TUESDAY SESSION

REPORT ON FERTILIZERS

By F. W. QUACKENBUSH (Purdue University, Agricultural Experiment Station, Lafayette, Ind.), *Referee*

Reports were received from Associate Referees on Manganese, Nitrogen, Ammoniacal Solutions and Liquid Fertilizers, Phosphorus, and Potassium. Their recommendations are approved.

It is recommended* that the work of all Associate Referees be continued.

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 21, 22 (1957).

REPORT ON MANGANESE IN FERTILIZERS*

By JOHN B. SMITH (Agricultural Experiment Station, Kingston, R.I.), *Associate Referee*

Acid solvents that were satisfactory when manganese sulfate was the sole carrier of manganese in fertilizers did not recover the manganese from a type of fritted material presented by Ferro Corporation in 1953 (*This Journal*, **37**, 331 (1954)). That frit proved too inactive for agronomic use and two new types were developed. It was suggested that hydrochloric acid be compared with the official nitric-sulfuric acid solvent.

Two fertilizers were used for the study: a 5–10–10 grade, factory-made to represent commercial practice, and a 4–8–8 sample dry mixed in the laboratory. Both fertilizers contained the frits; mixtures of the 4–8–8 also contained manganese sulfate, manganous oxide, manganese dioxide, and a manganese ore.

The results in Table 1 show no important differences in dissolving manganese by either hydrochloric-sulfuric acid or nitric-sulfuric acid mixtures. Manganese was recovered satisfactorily from all carriers except the 1953 frit. Slightly more manganese was found in the 1955 frits than was guaranteed, both in the frits alone and in the 5–10–10 fertilizers containing 10 per cent of the frits. Neither acid mixture recovered manganese from the inactive 1953 frit. The percentages of manganese contained in the fertilizers were low, but the solubility of the frits alone indicates that larger quantities could be recovered.

Hydrochloric acid is recognized to be a better solvent for certain man-

* Contribution No. 900 of this station.

TABLE 1.—*Comparison of acid solvents in methods for acid-soluble manganese*

MATERIAL	ACID-SOLUBLE Mn, PER CENT		
	HCl-H_2SO_4		HNO_3-H_2SO_4
	KIO_4	$NaBiO_3$	$NaBiO_3$
Frit, 1953, 2.34% Mn by H_2SO_4-HF	0.21	0.05	0.03
Frit, 501, 1955, high Fe	5.48	5.21	5.47
Frit, 502, 1955, high Mn	11.36	11.34	11.20
In 5–10–10 fertilizer[a]			
Frit, 501		0.51	0.54
Frit, 502		1.15	1.09
In 4–8–8 fertilizer[b] formulated to contain 1% Mn			
Frit, 1953			0.03
Frit, 501		0.98	0.95
Frit, 502		0.96	1.00
$MnSO_4$, technical grade			0.98
MnO, technical grade			0.99
MnO_2, technical grade			0.93
Manganese ore			1.11

[a] Ammonium sulfate, nitrogen solution, superphosphate, muriate of potash, tobacco stems, limestone, sand filler; trace of Mn.
[b] Sulfate of ammonia, nitrate of soda, superphosphate, muriate of potash, cocoa shell meal; 0.03% Mn.

ganese compounds than nitric acid is, but it is seldom used in analysis because all chlorides must be removed before oxidation to potassium permanganate. Most of the results were obtained by the volumetric bismuthate method, but the colorimetric method should be equally satisfactory since both methods use the same acids to dissolve the sample. From the results, the present official methods appear to be satisfactory.

It may be desirable to filter the silicate residue from the solutions of frits before taking an aliquot for oxidation with bismuthate; this step has not been proved essential, but it did facilitate the final filtration and gave better precision among replicate determinations.

It is recommended* that the study of magnesium and manganese in fertilizers be continued.

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 21, 22 (1957).

REPORT ON NITROGEN IN FERTILIZERS

By H. A. DAVIS (New Hampshire Agricultural Experiment Station, Durham, N.H.), *Associate Referee*, and S. R. MILES (Purdue University, Lafayette, Ind.)

As a result of the 1955 collaborative work on nitrogen in fertilizers and the reports presented at the 1955 A.O.A.C. meeting, the 1956 program was planned to consist of two parts:

Part I.—The report of the work done in 1955 listed definite specifications for heat output of burner, time of digestion, and amount of potassium sulfate used in the digestion of materials containing nitrogen not in the form of nitrate (*Official Methods of Analysis*, 8th Ed., 1955, sec. **2.23**). The 1956 program applied similar conditions to commercial fertilizers; the method used was the improved Kjeldahl method for nitrate-containing samples, **2.24**.

Part II.—The official method, **2.24**, was compared with a modified method for the determination of nitrogen in high chloride-high nitrate fertilizers, proposed by O. W. Ford (*This Journal*, **39**, 763 (1956)).

PART I

Digestion of Mixed Fertilizers Containing Nitrates—Variation of Conditions

The instructions to collaborators were the same as those for the 1955 work (*This Journal*, **39**, 550 (1955)) except to use sec. **2.24** instead of **2.23**.

Of 36 laboratories contacted, 27 agreed to participate in the program. Because of the amount of work involved, the collaborators were divided at random into two groups, A and B. The 13 collaborators of Group A were sent Sample A and 10 of them submitted reports. The 14 collaborators of Group B were sent Sample B; 10 submitted reports.

Sample A consisted of 8–6–4 commercial fertilizer containing considerable organic matter but no nitrate. Collaborators were instructed to analyze the sample according to **2.24**, since in routine work there may be no distinction between samples that do or do not contain nitrate. Sample B was 14–14–14 commercial fertilizer containing no organic matter; all the nitrogen present was in the form of nitrate.

Both samples were prepared for the collaborators in the same way. The material was ground in a Mikro-Sampl Mill, passed through a 1 mm sieve, mixed thoroughly, and packaged in glass bottles.

Six collaborators used gas as a heat source and 14 used electricity. A few collaborators reported that they had trouble in regulating the heat. They also commented that at higher temperatures, some acid was lost and the consequent drying in the digestion resulted in loss of nitrogen.

STATISTICAL ANALYSIS OF RESULTS[1]

Principal Results

Both Samples.—Each of the 20 collaborating laboratories used 24 sets of analytical conditions consisting of all possible combinations of two amounts of potassium sulfate, three heater speeds (temperatures), and four lengths of digestion time after clearing.

Heater speeds are expressed as "boiling time," which is the time required to bring 250 ml of water from 25°C. to a rolling boil. Each laboratory was to select a heater with a boiling time of about 4.5 minutes; this is called the medium-speed heater. In addition, the slowest and the fastest heaters were to be used. Among the 20 laboratories, the boiling time of the fastest heaters varied from 2 minutes 45 seconds to 4 minutes 30 seconds. The slowest heaters ranged from 4 minutes 17 seconds to 17 minutes 22 seconds.

The true analysis for either sample is not known. The highest analyses of each sample are considered the "best."

Sample A.—Table 1 gives the average of 10 laboratories for per cent nitrogen in Sample A (8–6–4 with considerable organic matter but no nitrate) under each of the 24 sets of conditions of analysis.

The average nitrogen analysis obtained with 15 grams of potassium sulfate (7.83 per cent N) is 0.02 per cent higher than with 10 grams (7.81 per cent N). This difference, though small, is highly significant statistically.

In 2 individual laboratories, the slow heaters, with boiling times of 8 minutes 5 seconds and 12 minutes, gave as high percentage of nitrogen as did the 4.5 minute heaters. However, in 2 other laboratories, heaters with boiling times of 7 minutes 35 seconds and 16 minutes gave 0.04 per cent and 0.05 per cent less nitrogen, respectively, than did the 4.5 minute heaters. Therefore it seems wise to avoid slow heaters.

Laboratory 9A tried a fourth heater with a boiling time of 2 minutes 50 seconds but used only 10 grams of potassium sulfate and only 30 and 60 minutes' digestion time; the determined per cent nitrogen was only 0.24. Apparently this heater was too hot.

Laboratory 6A made a complete set of 16 analyses with a fourth extra-hot heater that had a boiling time of 3 minutes. The analyses were extremely erratic: they varied from 0.02 to 7.83 per cent nitrogen. Digestion times of more than 30 minutes resulted in the lowest analyses but even 30 minutes produced low values. This 3 minute heater was too hot, also. However, in 3 other laboratories, the hottest heaters, with boiling times of 2 minutes 45 seconds, 3 minutes 27 seconds, and 3 minutes 30 seconds, did not appear to be too hot, since they did not seem to affect the analyses.

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[1] Prepared by S. R. Miles.

TABLE 1.—*Average % nitrogen in Sample A[a] obtained by 10 laboratories with each of the 24 combinations of 2 amounts of K_2SO_4, 3 temperatures, and 4 digestion times*

HEATER		PER CENT NITROGEN WITH DIGESTION TIME AFTER CLEARING:			
TEMPERATURE	RANGE IN BOILING TIME, MIN. AND SEC.	30 MIN.	60 MIN.	90 MIN.	120 MIN.
		10 g K_2SO_4			
Low	4′ 17″ to 17′ 22″	7.80	7.81	7.81	7.81
Medium	4′ 5″ to 5′ 5″	7.81	7.80	7.82	7.83
High	2′ 45″ to 4′ 30″	7.82	7.81	7.84	7.80
		15 g K_2SO_4			
Low	4′ 17″ to 17′ 22″	7.84	7.83	7.81	7.83
Medium	4′ 5″ to 5′ 5″	7.84	7.82	7.82	7.82
High	2′ 45″ to 4′ 30″	7.87	7.82	7.83	7.86

[a] 8–6–4 fertilizer containing organic matter but no nitrate.

With 15 grams of potassium sulfate and medium or fast heaters, any digestion time from 30 to 120 minutes appeared to give essentially the same per cent nitrogen.

Sample B.—Table 2 gives data for Sample B similar to the data for Sample A in Table 1. Sample B was 14–14–14 fertilizer containing no organic matter. Each average is for 9 laboratories. The data from laboratory 14 were omitted because its fastest heater gave very erratic analyses.

The average percentage of nitrogen obtained with 15 grams of potassium sulfate (14.31 per cent N) is 0.02 per cent higher than with 10 grams

TABLE 2.—*Average % nitrogen in Sample B[a] obtained by 9 laboratories with each of the 24 combinations of 2 amounts of K_2SO_4, 3 temperatures, and 4 digestion times[b]*

HEATER		PER CENT NITROGEN WITH DIGESTION TIME AFTER CLEARING			
TEMPERATURE	RANGE IN BOILING TIME, MIN. AND SEC.	30 MIN.	60 MIN.	90 MIN.	120 MIN.
		10 g K_2SO_4			
Low	4′ 40″ to 7′ 45″	14.23	14.28	14.32	14.32
Medium	4′ 30″ to 4′ 50″	14.23	14.30	14.31	14.32
High	3′ 20″ to 4′ 15″	14.29	14.26	14.32	14.32
		15 g K_2SO_4			
Low	4′ 40″ to 7′ 45″	14.29	14.34	14.29	14.30
Medium	4′ 30″ to 4′ 50″	14.32	14.34	14.33	14.32
High	3′ 20″ to 4′ 15″	14.31	14.32	14.31	14.28

[a] 14–14–14 fertilizer containing no organic matter.
[b] The data for laboratory 14B are omitted because of extremely erratic percentages of nitrogen obtained with the fastest burner.

of potassium sulfate (14.29 per cent N). This difference, though small, is highly significant statistically.

Study of the data from individual laboratories indicates that the slowest heaters (as slow as 7 minutes 45 seconds boiling time) gave as high nitrogen values as heaters with a boiling time of 4.5 minutes. In laboratory 14 the hottest heater, 3 minutes 30 seconds boiling time, caused extremely erratic analyses: the nitrogen varied from 0.10 to 14.26 per cent with little relation to the amount of potassium sulfate, to the temperature, or to the digestion time. However, in 4 other laboratories with fast burners varying from 3 minutes 20 seconds to 3 minutes 45 seconds, the high temperatures did not appear to affect the per cent nitrogen.

With 15 grams of potassium sulfate and medium or slow heaters, any digestion time from 30 to 120 minutes appeared to give essentially the same per cent nitrogen.

CONCLUSIONS

These conclusions are based on both the 1955 and the 1956 collaborative researches:

(1) Heaters should bring 250 ml of water from 25°C. to a rolling boil in 4–6 minutes. Faster or slower heaters may result in poor analyses.

(2) For nicotinic acid and other materials equally difficult to digest, 15 grams of potassium sulfate should be used, and digestion should continue at least 120 minutes after clearing.

(3) If 15 grams of potassium sulfate is used, for materials as easy to digest as 8–6–4 fertilizer containing considerable organic matter but no nitrate, 14–14–14 fertilizer containing no organic matter, or urea-formaldehyde compound, 30 minutes' digestion after clearing is enough but 120 minutes is not too much. However, if only 10 grams of potassium sulfate is used, digestion should continue at least 90 minutes after clearing, but 120 minutes is not too much.

Other Results

Variation within Laboratories.—Table 3 reports the variation in nitrogen analyses within laboratories. The standard deviation of duplicate analyses, columns B and E, show that laboratories varied considerably in the precision of their analyses. Although duplicate analyses were made a week or more apart, the values of these standard deviations apply to duplicate analyses made at one time.

On the basis of collaborative work by members of the Association of Official Agricultural Chemists, Miles and Quackenbush (*This Journal*, **38**, 108 (1955)) gave values of the standard deviation of chemical analysis for nitrogen within laboratories.

From their data, the standard deviation of duplicate analyses for material containing 8 per cent nitrogen is expected to be 0.064 per cent (last line of Table 3). This is slightly less than the 0.073 per cent average stand-

TABLE 3.—*Variation of % nitrogen within laboratories*

SAMPLE A: 8-6-4			SAMPLE B: 14-14-14		
LABORATORY (A)	STD DEV., DUPLICATE ANALYSES (B)	DIFFERENCE BETWEEN REPLICATE AVERAGES[a] (C)	LABORATORY (D)	STD DEV., DUPLICATE ANALYSES (E)	DIFFERENCE BETWEEN REPLICATE AVERAGES[a] (F)
2	0.067	0.02	1	0.095	0.00
3	0.062	0.11[c]	2	0.082	0.04
4	0.109	0.02	4	0.085	0.03
5	0.044	0.14[c]	5	0.054	0.01
6	0.059	0.01	7	0.082	0.00
7	0.062	0.01	9	0.136	0.15[c]
8	0.111	0.04	10	0.046	0.02[c]
9	0.056	0.02	11	0.097	0.02
10	0.039	0.01	13	0.057	0.01
12	0.081	0.02	14	0.033	0.01
Average	0.073	—	Average	0.082	—
Earlier work[b]	0.064	—	Earlier work[b]	0.075	—

[a] Difference between the average of a set of 24 analyses made "at one time" (within 1–3 days) and the average of the same set of 24 analyses made at least a week later.
[b] From Miles and Quackenbush, *This Journal*, **38**, 108 (1955).
[c] Highly significant difference.

ard deviation in the present research (next to last line of Table 3). For 14 per cent material, the earlier work indicates a standard deviation of 0.075 per cent; this also is slightly less than the average value, 0.082 per cent, from the present work.

Each laboratory made 24 analyses "at one time" (within 1–3 days) by each of the 24 sets of conditions. The 24 analyses are the first replicate. A second replicate was analyzed at least a week later. Columns C and F, Table 3, give the difference between the replicate averages for each laboratory (each of 24 analyses). Most of these differences are small, but for 3 laboratories the differences are from 0.11 to 0.15 per cent nitrogen and are highly significant statistically. These data illustrate the fact that analyses of the same material differ more from day to day than within one day. This is probably true for all laboratories.

Variation among Laboratories.—Table 4, columns B and D, show considerable variation in the average per cent nitrogen determined by the various laboratories. Each average is of 48 analyses. Column B shows averages from 7.75 to 7.95 per cent nitrogen, a range of 0.20 per cent. For Sample B, the averages vary from 13.99 to 14.50 per cent, a range of 0.51 per cent. For both samples, the variation among laboratories is very highly significant statistically.

The variation among laboratories is expressed on line 1 of Table 5 as a

TABLE 4.—*Average % nitrogen by laboratories*

SAMPLE A: 8-6-4		SAMPLE B: 14-14-14	
LABORATORY (A)	AV. % N (B)	LABORATORY (C)	AV. % N (D)
2	7.75	1	14.45
3	7.91	2	14.39
4	7.75	4	14.26
5	7.87	5	14.46
6	7.82	7	14.30
7	7.79	9	14.06
8	7.95	10	14.50
9	7.77	11	13.99
10	7.83	13	14.31
12	7.77	14	14.32
Average	7.82	Average	14.30

net standard deviation. This table includes the two 1955 samples, as well as two 1956 samples; data are given in order of the per cent nitrogen. (The net standard deviation among laboratories is less than the standard deviation commonly calculated directly from laboratory averages, because the net standard deviation is the remainder after the variation among laboratories due to analytical variation within laboratories has been removed. The net standard deviation is the square root of the component of variance among laboratories.)

The variation among laboratories is due, to a very small extent, to the real differences among the subsamples sent the various laboratories, but the major source of variation is a real difference among laboratories in the analyses they would obtain from identical subsamples. Note that the net standard deviation among laboratories increases with the per cent nitrogen in the material.

Least significant differences are given on lines 2 and 3 of Table 5. These differences are most easily explained by specific examples. Suppose (*a*)

TABLE 5.—*Variation among laboratories in determining % nitrogen*

LINE	ITEM (A)	1956: 8% N, 8-6-4 FERTILIZER (B)	1955: 11% N, NICOTINIC ACID (C)	1956: 14% N, 14-14-14 FERTILIZER (D)	1955: 38% N, UREA-FORMALDEHYDE COMPOUND (E)
1	Net Std Dev.	0.07	0.12	0.18	0.21
2	5% lsd[a]	0.24	0.35	0.53	0.60
3	1% lsd[a]	0.31	0.46	0.69	0.78

[a] lsd: least significant difference.

that a well-mixed sample of material containing 8 per cent nitrogen is divided into 2 subsamples; (*b*) that these subsamples are sent to 2 randomly chosen laboratories such as those that participated in the present collaborative project; (*c*) that each laboratory analyzes its subsample in duplicate; and (*d*) that the difference between the 2 subsample averages is calculated. Also suppose that this procedure is repeated many times with the same or different materials containing 8 per cent nitrogen and with many laboratories:

The 5 per cent least significant difference (lsd), column B, line 2, means that 5 per cent of the many differences between 2 laboratory averages would be expected to exceed 0.24 per cent nitrogen; also, 1 per cent of the many differences would be expected to exceed 0.31 per cent nitrogen (column B, line 3). The other least significant differences are interpreted similarly. Note that these values increase with the per cent nitrogen in the material analyzed.

PART II

At the 1955 meeting of this Association, O. W. Ford reported a comparison of results obtained by his proposed reduced-iron method with those by the official method for the determination of nitrogen in fertilizers having a high content of chloride in relation to nitrate (*This Journal*, **39**, 763 (1956)). Results for fertilizers containing no organic matter and a high chloride:nitrate ratio appear to be higher by the proposed method than by the official method, **2.24** (*Official Methods of Analysis*, 8th Ed., 1955).

A sample of 5–10–30 fertilizer was furnished by Professor Ford. It was prepared as follows: 360 grams reagent grade ammonium nitrate; 570 grams 20 per cent superphosphate; 320 grams triple superphosphate; and 1250 grams reagent grade potassium chloride. (Calculated nitrogen content, 5.04 per cent; chloride:nitrate ratio, about 2:1.) Professor Ford stated that there was a trace of nitrogen in the superphosphate used, and that a fair value for this sample is 5.3 to 5.4 per cent nitrogen.

A portion of this sample, labeled Sample C, was sent to each collaborator in both Group A and Group B. In addition, collaborators in Group B were asked to analyze Sample B by the Ford reduced-iron method if time permitted. Sample B, 14–14–14 commercial fertilizer, had a chloride:nitrate ratio of about 1:3 and contained no organic matter.

INSTRUCTIONS TO COLLABORATORS

(1) Determine total nitrogen by method **2.24**, *Official Methods of Analysis*, 8th Ed., 1955.

(2) Determine total nitrogen by Ford's reduced-iron method as follows:

Place 0.5–2.0 g sample in an 800 ml Kjeldahl flask and add 2–5 g reduced iron (depending on nitrate in sample; 5 g iron is sufficient for 0.185 g NO_3^-). Rinse the flask with 25 ml distilled H_2O, swirl to mix, and let stand 15–30 min. to insure solution of all salts. Rinse the flask with 25 ml 1:1 H_2SO_4, let stand until visible reaction ceases, add boiling chips, and boil 15–20 min. to complete the reduction, but

TABLE 6.—*Per cent nitrogen in Sample C*

COLLABORATOR	OFFICIAL METHOD, 2.24	RANGE	FORD METHOD	RANGE
2A	4.89	0.40	5.11	0.05
3A	5.32	0.12	5.17	0.04
5A	5.09	0.04	5.04	0.09
6A	4.59[a]	1.31	4.97[b]	0.54
7A	4.76	0.34	4.99	0.02
8A	4.76	0.32	5.19	0.10
9A	5.09	0.12	5.21	0.04
10A	4.92	0.07	4.94	0.09
12A	4.81	0.05	5.07	0.16
4B	4.80	0.32	4.84	0.26
5B	4.96	0.06	5.02	0.08
7B	4.82	0.15	5.27	0.06
9B	4.89	0.06	4.78	0.07
10B	4.83	0.03	5.25	0.02
11B	5.39	0.10	5.40	0.10
13B	5.07	0.14	5.20	0.18
Average (Mean)	4.94		5.09	
Std error[c]	0.0526		0.0414	

[a] Average of 15 determinations.
[b] Average of 9 determinations.
[c] Difference between the means is 0.15. Standard error of the difference between means, 0.0669; "*t*" value is 2.24.

do not take to dryness. If the sample contains organic nitrogen, use 50 ml cold 1:1 H_2SO_4 instead of 25 ml; then after boiling add 0.7 g HgO and digest 30 min. (on burner or heater adjusted for 4–5 min. boil test). Cool, dilute with 300–400 ml H_2O, add K_2S and alkali, and distill the NH_3 in the usual way. (Reduced iron, J. T. Baker No. 1-2228 or Fisher No. 1-62, has been found satisfactory.)

DISCUSSION

Sixteen of the 27 collaborators in Groups A and B reported an analysis of Sample C. Most collaborators reported results in triplicate as well as the average. Table 6 shows the average per cent nitrogen and the range (low to high) of the replicate results.

The average difference between results obtained by the two methods is significant at the 5 per cent level but not at the 1 per cent level.

Seven collaborators analyzed Sample B, a 14–14–14 fertilizer with a chloride:nitrate ratio of 1:3 (described in Part I), by the Ford method. The data appear in Table 7.

Not enough collaborators reported on Sample B by the Ford method to warrant drawing definite conclusions. However, from the results it might be predicted that the difference between the official method and the Ford method for Sample B would be less than that for Sample C, since the chloride:nitrate ratio is 2:1 in Sample C but only 1:3 in Sample B.

It was pointed out that when no organic matter is present in the ferti-

TABLE 7.—*Per cent nitrogen in Sample B (14–14–14)*

COLLABORATOR	OFFICIAL METHOD, 2.24	RANGE	FORD METHOD	RANGE
	per cent		*per cent*	
2B	——	——	14.31	0.06
4B	14.25	——	14.26	0.09
5B	14.39	0.06	13.81	0.18
7B	14.32 (1 g)	0.08	14.45	0.08
	13.90 (2 g)[a]	0.07	14.39[a]	0.07
9B	——	——	14.35	0.16
11B	14.31	0.05	14.53	0.08
13B	14.30	0.13	14.40	0.27
Average	14.31		14.33	

[a] 2 g result omitted from average. Other sample weights, 0.35 g to 1 g.

lizer mixture and the nitrogen is derived solely from nitrate or ammonium salts, it is not necessary to add the catalyst in the digestion. Adding sulfide in the distillation to remove mercury becomes unnecessary. One collaborator pointed out that when no organic nitrogen is present and only nitrate nitrogen is in the fertilizer, the Devarda method is satisfactory and simpler than the Ford method.

To use these various modifications properly, the analyst must know the form in which the nitrogen is present in the sample. Apparently, if an analyst obtains a low result for nitrogen by the usual method and he suspects a high chloride:nitrate ratio, he should use the Ford method; if no organic matter is present, he should use the Devarda method. These procedures are much easier to carry out than the Shuey method that has been studied collaboratively (*This Journal*, **34**, 653 (1951); **36**, 644 (1953)).

SUMMARY AND RECOMMENDATION

The results of the collaborative work reported in Part I show that the recommendations incorporated in the improved Kjeldahl method for nitrate-free samples, **2.23**, as a result of the 1955 study (*This Journal*, **39**, 63, 81 (1956)) should also be applied to the improved Kjeldahl method for nitrate-containing samples, **2.24**.

The Ford reduced iron method gives higher results when applied to fertilizers containing a high ratio of chloride to nitrate. The Associate Referee prefers to make no recommendation about the Ford method at this time. There is some question whether further collaborative work is needed, or whether the method should be recommended for use whenever the analyst decides it is applicable.

It has been observed that although the averages of replicate determinations of collaborators agree quite closely, the variation between replicates

often leaves much to be desired. Close attention by the analyst to critical details of the method should improve the agreement between replicate determinations, and the factors of error now "allowed" statistically should become much smaller.

It is recommended* that the following changes be incorporated in the improved method for nitrogen in nitrate-containing samples, **2.24**: (1) The use of 15 grams of potassium sulfate or anhydrous sodium sulfate; (2) adjustment of burners to bring 250 ml of water from 25°C. to a rolling boil in approximately 5 minutes; and (3) continuation of the digestion period for samples containing organic matter for 2 hours after clearing.

ACKNOWLEDGMENTS

The Associate Referee wishes to thank S. R. Miles for his interest in this project. His statistical studies and comments on the 1955 and 1956 programs have been very helpful.

The Consolidated Rendering Co., Boston, Mass., supplied the 8–6–4 fertilizer for Sample A; Associated Cooperatives, Sheffield, Ala., supplied the 14–14–14 fertilizer for Sample B; Professor O. W. Ford of Purdue University supplied the 5–10–30 fertilizer for Sample C.

The following collaborators took part in the program:

Joan Ryan and Harry R. Allen, University of Kentucky, Lexington, Ky.
Terry C. Woodis, Jr., Tennessee Valley Authority, Wilson Dam, Ala.
H. I. Macomber, Southern Testing Co., Wilson, N. C.
Paul D. Cretien, Texas Testing Laboratory, Inc., Dallas, Texas
E. L. Dunn, Department of Agriculture Laboratories, Reynoldsburg, Ohio
Charles W. Gehrke, University of Missouri, Columbia, Mo.
Edward R. Hahn, Hahn Laboratories, Columbia, S. C.
Dorothy M. Carroll, Fertilizer and Agricultural Lime Section, Soil and Water Conservation Research Branch, Agricultural Research Service, U.S.D.A., Beltsville, Md.
Vidabelle O. Cirino, Southern Regional Research Laboratory, New Orleans, La.
W. J. Ingram, Department of Agriculture, Salem, Ore.
Howard Hammond, State Laboratories, Bismarck, N. Dak.
G. Conner Henry, Law and Co., Atlanta, Ga.
P. A. Lang and Robert Reynolds, American Agricultural Chemical Co., Carteret, N. J.
Harry A. Miller, Department of Agriculture, Raleigh, N. C.
W. A. Morgan, Polychemicals Department, E. I. DuPont de Nemours and Co., Wilmington, Del.
James H. Cox and C. L. Orcutt, Nitrogen Div., Allied Chemical and Dye Corp., Hopewell, Va.
C. H. Perrin, Canada Packers Ltd., Toronto, Ontario, Canada
O. W. Ford, Department of Agricultural Chemistry, Purdue University, Lafayette, Ind.
P. F. Lineberry, Armour Fertilizer Works, Navassa, N. C.
M. V. Moore, Armour Fertilizer Works, P.O. Box 287, Chicago Heights, Ill.
C. L. Manning, Fort Worth Laboratories, Fort Worth, Texas.

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 21, 22 (1957).

REPORT ON SAMPLING OF LIQUID FERTILIZERS

By FRED J. ROTH (Bureau of Chemistry, State Department of Agriculture, Sacramento 14, Calif.), *Associate Referee*

The popularity and volume of sales of liquid fertilizers have gained tremendously during the past several years. They accounted for one-third of the total tonnage of fertilizers sold in California during the quarter April–May–June, 1956. One of the most popular forms of nitrogen fertilizers—an aqueous ammonia solution containing 20 per cent nitrogen—made up one-fourth of the total tonnage of commercial fertilizers reported in this period.

The methods for sampling liquid fertilizers specified in the California regulations are as follows:

LIQUID MATERIALS PACKAGED IN DRUMS OR BARRELS

(1) Use a sampling tube that will remove a cross section from top to bottom of the container. From each package entered place one sampler-full into sample container.

(2) Enter all containers if fewer than 3; at least 3 if fewer than 10; and 5 if 10 or more are present.

Inspectors, when sampling solutions in small drums or barrels, are instructed to lower the sampling tube completely to the bottom of the container and examine the withdrawn portion to detect sediment, crystallization, or other lack of uniformity of the material.

LIQUID MATERIALS IN LARGE CONTAINERS

To sample a liquid fertilizing material which is packaged in storage tanks, railway tank cars, or similar containers, a sample jar may be lowered into the material and allowed to fill. If this is not possible, the sample jar may be filled from faucet after sufficient material has flowed through faucet to clear connecting line.

Different types of liquid fertilizers require special sampling methods, some of which are discussed in the report by Fudge published in *This Journal*, **39**, 558 (1956). The Associate Referee has received additional ideas from others and would welcome still further thoughts on the subject. As a start, it was decided to investigate sampling methods and testing of aqua ammonia solutions. These solutions are usually made at "converter" plants, where anhydrous ammonia is absorbed in water, cooled to some extent, and stored in closed horizontal or vertical tanks with pressure release valves, for transfer to delivery trucks when needed. These converter plants are usually located in agricultural areas where the product will be used.

The quick turn-over of the product and losses in storage and handling made it advisable to devise equipment and plan a program of field testing.

DeWitt Bishop, District Inspector of the Bureau of Chemistry, California State Department of Agriculture, designed special equipment. Preliminary trials indicate that it will be quite effective, particularly for

screening tests to show whether a sample is deficient in its guarantee for ammonia nitrogen, and whether laboratory analyses are necessary.

The equipment used is shown in Fig. 1 and described in the accompanying legend. The cylinder is made of acrylic material, $\frac{1}{8}$ inch wall, 2.5 inches outside diameter, and 18.5 inches high from the bottom of the wooden base. The cylinder has a round acrylic plate cemented in the bottom with acrylic cement, and an acrylic tube cemented in the side near the bottom for an inlet or outlet tube. This acrylic material is quite resistant to the strong ammonia solutions, and does not become cloudy on repeated use. (A glass cylinder may be used for experiments, but it etches in time and breakage in field use is quite a problem.)

The sample of aqua ammonia is introduced into the cylinder by gravity or suction, and the time is noted. Approximately 4–5 minutes later, the temperature and specific gravity are read. The specific gravity reading should be taken by looking up at the hydrometer spindle from below the surface of the liquid. The time delay is needed to let the temperature of the hydrometer bulb come to equilibrium with that of the surrounding liquid.

The percentage of ammonia nitrogen can be found from a temperature-gravity correction chart (shown in Fig. 1). This type of physical analysis is possible because the specific gravity of pure aqueous ammonia solutions becomes increasingly lighter with increase in ammonia. This test cannot be used where additives, such as water modifiers, have been used in the solution. Such additives are seldom used in California and the label indicates when they are used.

Since turn-over of these solutions is fast, a quick field analysis is useful to show immediately if a user is getting what he pays for.

Table 1 shows results of field tests made in the spring of 1956 when temperatures were moderately high. The tests show a standard deviation of 0.13 per cent nitrogen between the hydrometer test and the actual chemical analysis of the same material. It will be noted that the deviations are all minus, and that the field test is consistently lower than the laboratory analysis.

As summer temperatures rose the following method was devised to cool the ammonia solution: a cooling coil inserted between the source of solution and the hydrometer well is put into ice water and the solution is run through it while the coil is moved up and down in the ice water to allow greater heat exchange.

To make the coil, a 7 foot length of $\frac{1}{2}$ inch aluminum tubing is curved around itself into a six-loop coil approximately 4 inches in diameter. Both ends of the tube are adjacent for easy attachment of Tygon or rubber connections (see Fig. 2). Aluminum tubing can be formed into a coil by hand without the sides collapsing by stoppering the end of the tube, filling it with sand with frequent tapping for solid packing, and stoppering the fill

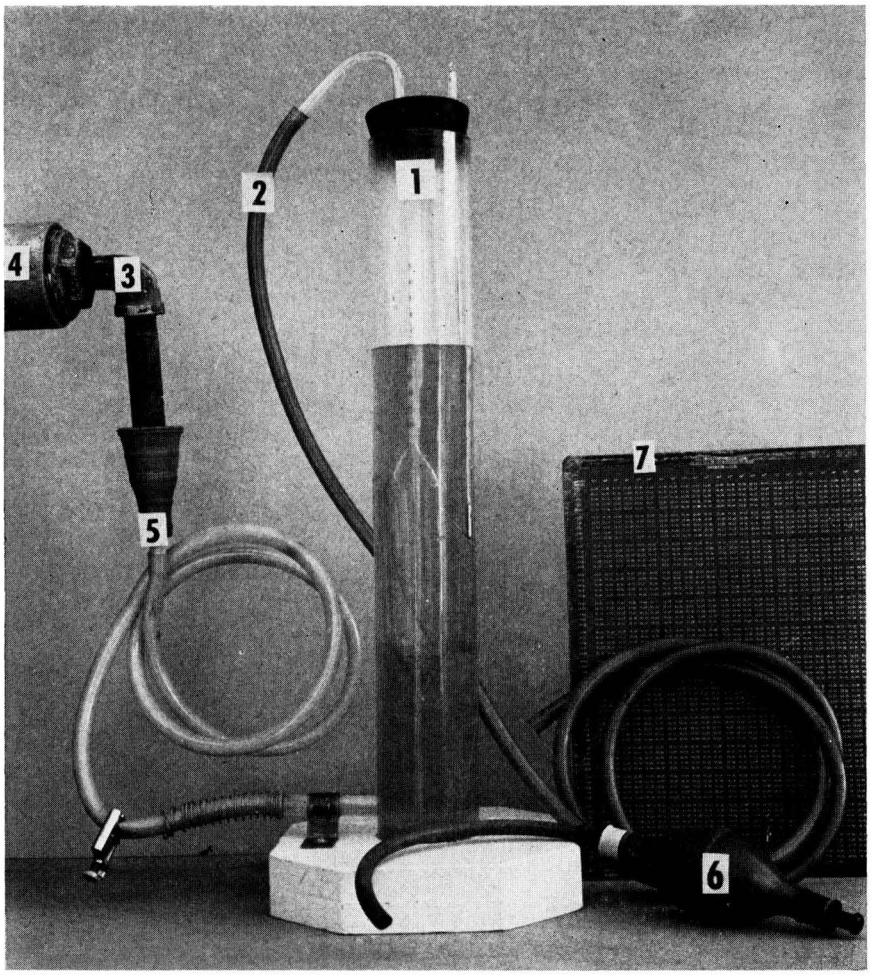

FIG. 1.—Equipment for determining aqua ammonia concentration by field inspection.

1: Upright cylinder containing sensitive hydrometer (Adolf Frese Corp., Los Angeles, Calif., No. 47557), marked in specific gravity (range 0.9000 to 0.9200) and thermometer in single degrees F. Cylinder made of "cast acrylic" is break-resistant for field handling. **2**: Transparent Tygon tubing used for any overflow of aqua ammonia while taking representative sample. Also used in place of **5** to siphon liquid from fill hole on tank tops. **3**: Various pipe fittings used to draw liquid from roadside field supply tanks. **4**: Field tank liquid take-off opening. **5**: Rubber adapter from shower hose. It fits any pipe to $1\frac{1}{4}$-inch maximum diameter. **6**. Suction bulb used with **2** to start siphon when drawing sample from top fill hole on tanks. **7**: Conversion chart. Per cent nitrogen is read from specific gravity and temperature readings of aqua ammonia sample enclosed in cylinder. In the interest of conservation of space, this chart is not included in this printed report. For those who may wish to try this method and do not have a chart, the author has a limited number of mimeographed copies that are available upon request.

TABLE 1.—*Comparison of hydrometer tests and chemical analyses of ammonia solutions*

SAMPLE NUMBER	TEMPERATURE OF SOLUTION, °F.	PER CENT AMMONIA NITROGEN BY HYDROMETER	DEVIATION FROM CHEMICAL ANALYSIS, PER CENT NITROGEN	PER CENT AMMONIA NITROGEN BY CHEMICAL ANALYSIS
1	73	20.46	−0.04	20.50
2	75	20.31	0.00	20.31
3	72.5	20.08	−0.06	20.14
4	73	20.06	−0.10	20.16
5	60	19.60	−0.12	19.72
6	58.5	19.11	−0.06	19.17
7	61.5	18.93	−0.23	19.16
8	72	20.56	−0.02	20.58
9	80	19.96	−0.17	20.13
10	81	19.97	−0.14	20.11
11	79.5	19.83	−0.13	19.96
12	77	19.77	−0.16	19.93
13	75	19.69	−0.15	19.84
14	75	19.74	−0.14	19.88
15	78	20.30	−0.16	20.46
16	77.5	20.16	−0.18	20.34
17	77.5	20.17	−0.12	20.29
18	75	19.89	−0.16	20.05
Standard Deviation from Chemical Analysis			0.13%	

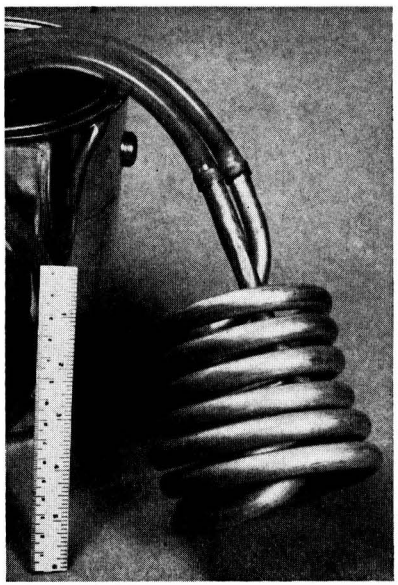

FIG. 2.—Cooling coil.

TABLE 2.—*Comparison of hydrometer tests of ammonia solutions at normal and cooled temperatures*

SAMPLE NUMBER	NORMAL TEMPERATURE OF SOLUTION, °F.	PER CENT AMMONIA NITROGEN BY HYDROMETER TEST AT NORMAL TEMPERATURE	DEVIATION FROM CHEMICAL ANALYSIS, PER CENT NITROGEN	TEMPERATURE OF COOLED SOLUTION, °F.	PER CENT AMMONIA NITROGEN BY HYDROMETER TEST OF COOLED SOLUTION	AVERAGE OF COOLED TESTS	DEVIATION FROM CHEMICAL ANALYSIS, PER CENT NITROGEN	PER CENT AMMONIA NITROGEN BY CHEMICAL ANALYSIS
1	80	19.79	−0.32	63	20.20	20.20	0.09	20.11
2	—	—	—	61	20.23			
				61.5	20.18	20.20	0.04	20.16
3	[a]	—	—	63	20.20			
				71	20.18			
				75	20.21	20.20	−0.12	20.32
4	81	18.65	−0.23	64	18.77			
				66	18.77	18.77	−0.11	18.88
5	80	17.47	−0.26	63	17.84	17.84	0.11	17.73
6	76	20.15	0.00	62	20.23	20.23	0.08	20.15
7	78	18.64	−0.11	61	18.76	18.76	0.01	18.75
8	86.5	19.48	−0.91[b]	55	20.37			
				56.5	20.35	20.36	−0.03	20.39
9	76	19.73	−0.12	59	19.88	19.88	0.03	19.85
10	72	19.50	0.00	62	19.63	19.63	0.13	19.50
Standard Deviation from Chemical Analysis			0.22				0.07	

[a] Atmospheric temperature, 101°F.
[b] Omitted in calculation of standard deviation.

end. When the coil is completed, the sand is removed by gentle tapping. After washing, the coil is ready for use.

Table 2 shows results of the tests on some ammonia solutions before and after cooling. Deviations in the uncooled samples shown in Table 2 were all minus, but those in the cooled samples were both plus and minus; apparently the consistent minus errors shown in the uncooled samples of Tables 1 and 2 had been overcome by the cooling. The cooled samples in Table 2 were colder than the samples of Table 1.

The standard deviation of the hydrometer analysis from the chemical analysis was 0.22 per cent before cooling and 0.07 per cent after cooling (about one-third as much). It is evident that the most accurate hydrometer tests are made between 55 and 65°F. This ties in with the fact that the hydrometer is calibrated at 60/60°F., at which point it would have its greatest accuracy in spite of correction charts for other temperatures.

As indicated by Fudge, it is impractical to make collaborative studies of ammonia solutions by the usual method of sending out collaborative samples. It is proposed in the coming year to ask collaborators to correlate field testing by the described equipment with chemical analyses done in their own laboratories.

In the meantime, this laboratory and field force will study suggested sampling methods for chemical analysis. The Associate Referee would welcome names of volunteers willing to assist in this work.

It is recommended* that this study be continued.

ACKNOWLEDGMENTS

The writer is deeply indebted to DeWitt Bishop, District Inspector of the Bureau of Chemistry staff, who designed the equipment and collaborated in the field testing; and to Brea Chemicals, Inc., who supplied the conversion chart and the original idea of an enclosed hydrometer well.

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 21, 22 (1957).

REPORT ON PHOSPHORUS IN FERTILIZERS

I. PREPARATION OF SOLUTION OF SAMPLE FOR TOTAL PHOSPHORUS DETERMINATION

By K. D. Jacob, *Associate Referee*, and W. M. Hoffman
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Last year's report (*This Journal*, **39**, 562 (1956)) showed that the several methods by which solutions of fertilizers are prepared for determina-

tion of total phosphorus should be studied further. Samples containing phosphorus in organic combinations present a particular problem. Accordingly, further collaborative study was made of the nitric-perchloric acid, magnesium nitrate-hydrochloric acid, and sulfuric-nitric acid methods of preparing solutions of organic materials.

Samples 1 and 2—Milorganite and cottonseed meal, respectively—were from the same batches of materials used in the previous study. Samples 3 and 3a were technical-grade calcium phytate containing about 39 per cent and 40 per cent total P_2O_5, respectively. All results with calcium phytate were obtained on Sample 3, except those by Collaborator 17. Samples 1 and 2 were ground to pass the U. S. No. 20 and No. 14 sieves, respectively. Samples 3 and 3a were used in the form in which they were received: a fine powder.

Although calcium phytate is of no interest as a fertilizer material, it was included in this and the previous study because of its very high content of organically combined phosphorus similar to the principal phosphorus compound (phytin) in cottonseed meal. Like phytin, calcium phytate requires severe treatment to completely convert the phosphorus into ionizable orthophosphate.

INSTRUCTIONS TO COLLABORATORS

Without further grinding, mix the sample thoroughly and analyze by the following methods. Make all determinations in triplicate, each on a separate portion of the sample, and report the individual results on the form enclosed with these instructions. If for any reason a determination is repeated the repetition should be made in triplicate, and the results reported should be those obtained in simultaneous replications.

PREPARATION OF SOLUTION

Method A. Nitric and Perchloric Acids.—Digest 2 g sample as in *Official Methods of Analysis*, 8th Ed., 1955, p. 379, sec. **22.51(a)**, with final dilution to 200 ml.

Method B. Magnesium Nitrate and Hydrochloric Acid.—Proceed as on p. 8, sec. **2.10(c)**.

Method C. Sulfuric and Nitric Acids.—Proceed as on p. 8, sec. **2.10(d)**.

TOTAL P_2O_5

Determine P_2O_5 in appropriate aliquots of the solutions by the volumetric method as on pp. 9–10, sec. **2.14(a)**.

COMMENTS OF COLLABORATORS

The procedure for the nitric-perchloric acid digestion used in the collaborative study in 1955 differed somewhat from the official procedure for preparation of solutions of feeds, which was used in the present work. Thus, a 250 ml Erlenmeyer or other suitable flask and boiling with nitric acid for 15 minutes were specified in the previous study, whereas the official procedure specifies a 500 or 800 ml Kjeldahl flask and boiling with nitric acid for 30–45 minutes. Like several of the previous collaborators, Collaborator 13 preferred digestion in Kjeldahl flasks—with the use of a solid glass bead in each flask to reduce bumping—while Collaborators 2, 4, and 16 favored 200 ml "phosphoric acid" flasks or 300 ml Erlenmeyer flasks. As the averages of closely agreeing determinations in triplicate, Collaborator 2 reported

2.73, 2.34, and 38.72 per cent P_2O_5 in Samples 1, 2, and 3, respectively, by the official procedure with Kjeldahl flasks, compared with 2.71, 2.35, and 38.60 per cent by the same procedure with "phosphoric acid" flasks.

Collaborators 2 and 13 noted that some phosphorus is lost when the nitric-perchloric acid digestion is carried too far. Thus Collaborator 2 found 34.97 per cent P_2O_5 in Sample 3 when the contents of the flask were evaporated nearly to dryness, as compared with 38.72 per cent when the digestion was stopped at the appearance of copious white fumes.

Collaborator 4 stated that too rapid digestion in the nitric-perchloric acid procedure may result in excessive volatilization of the acid and incomplete destruction of the organic matter.

Collaborators 7 and 17 had difficulty in completely precipitating the phosphorus from solutions of Sample 1 prepared with sulfuric-nitric acids. Collaborator 17 had similar difficulty with Sample 2. The results were improved by longer or more vigorous agitation during the precipitation of the ammonium phosphomolybdate.

Collaborator 11 reported that, with magnesium nitrate-hydrochloric acid treatment, 5 ml of magnesium nitrate solution did not completely wet the 2 g portions of Samples 1 and 2. Collaborator 13 stated that the danger of mechanical loss by spurting when hydrochloric acid is added to the ignited residue can be obviated by first wetting the residue with water. Collaborators 15 and 16 had trouble in completely dissolving Sample 3; Collaborator 15 corrected the difficulty by using 20 ml of magnesium nitrate solution.

To prepare solutions of organic materials, Collaborator 3 recommended igniting the sample with an equal weight of calcium lactate and treating the residue with hydrochloric acid.

No explanations were offered for the low results with the nitric-perchloric acid digestion by Collaborator 21 on Sample 1, by Collaborators 19 to 21 on Sample 2, and by Collaborators 18 to 21 on Sample 3, or with the sulfuric-nitric acid digestion by Collaborator 21 on Sample 3.

RESULTS

In this study, as in the previous one, the collaborators were instructed to precipitate the phosphomolybdate at 25–30°C., with continuous agitation, to reduce interference of the sulfate ion in the analysis of solutions prepared with sulfuric-nitric acids.

The results of some 200 analyses in triplicate were received from 22 collaborators. The reported data omit those by Collaborator 22, however, because they were received too late to be included.

The averages of the individual collaborator's results for total P_2O_5 in each of the samples by the three methods of solution preparation are given in Table 1. This table also shows the differences between the average results with nitric-perchloric acid digestion and those with each of the other methods, as well as the individual differences between the high and low results in triplicate determinations.

Excluding the results by certain collaborators (see footnotes to Tables 2 and 3) the average of all comparable analyses on each of the samples showed higher values with the sulfuric-nitric acid digestion (Method C) than with either of the other methods of solution preparation. The results by Method C were higher in 84 per cent of the 55 individual comparisons

TABLE 1.—*Percentages of total P_2O_5 determined by three methods of preparing the solutions of the samples*

COLLABORATOR	AVERAGE[a] BY METHOD:[b]			DIFFERENCE[c] BY METHOD:[b]			DIFFERENCE IN AVERAGE RESULTS BY METHOD A AND METHOD:	
	A	B	C	A	B	C	B[d]	C[d]
Sample 1: Milorganite								
1	2.91	2.84	2.79	0.08	0.08	0.08	−0.07	−0.12
2	2.73	2.72	2.82	0.05	0.04	0.05	−0.01	0.09
3	2.62	2.69	2.71	0.02	0.02	0.05	0.07	0.09
4	2.80	2.62	2.51	0.04	0.01	0.14	−0.18	−0.29
5	2.70	2.76	2.78	0.01	0.03	0.05	0.06	0.08
6	2.61	2.57	2.76	0.02	0.06	0.01	−0.04	0.15
7	2.75	2.73	2.68	0.01	0.01	0.09	−0.02	−0.07
8	2.66	2.74	2.65	0.04	0.10	0.06	0.08	−0.01
9	2.70	2.77	2.75	0.00	0.03	0.05	0.07	0.05
10	3.03	2.98	3.04	0.05	0.05	0.08	−0.05	0.01
11	2.70	2.70	2.77	0.02	0.02	0.01	0.00	0.07
12	2.85	2.78	2.90	0.10	0.05	0.10	−0.07	0.05
13	2.83	2.69	2.97	0.10	0.02	0.05	−0.14	0.14
14	2.79	2.78	2.94	0.02	0.15	0.08	−0.01	0.15
15	2.70	2.65	2.83	0.10	0.10	0.05	−0.05	0.13
16	2.92	2.95	2.99	0.15	0.11	0.03	0.03	0.07
17	2.75	2.66	2.55	0.06	0.04	0.19	−0.09	−0.20
18	2.48	2.68	2.51	0.05	0.07	0.04	0.20	0.03
19	2.50	2.75	2.63	0.03	0.02	0.12	0.25	0.13
20	2.74	2.96	3.09	0.05	0.09	0.06	0.22	0.35
21	1.95	2.90	3.01	0.10	0.11	0.05	0.95	1.06
Sample 2: Cottonseed Meal								
1	2.49	2.49	2.57	0.12	0.08	0.04	0.00	0.08
2	2.34	2.33	2.37	0.01	0.01	0.02	−0.01	0.03
3	2.26	2.30	2.41	0.02	0.04	0.01	0.04	0.15
4	2.34	2.35	2.35	0.00	0.02	0.05	0.01	0.01
5	2.60	2.36	2.40	0.01	0.03	0.04	−0.24	−0.20
6	2.26	2.16	2.32	0.04	0.06	0.02	−0.10	0.06
7	2.31	2.26	2.37	0.01	0.02	0.02	−0.05	0.06
8	2.28	2.46	2.35	0.12	0.16	0.01	0.18	0.07
9	2.44	2.43	2.48	0.02	0.00	0.05	−0.01	0.04
10	2.46	2.58	2.73	0.05	0.05	0.05	0.12	0.27
11	2.33	2.35	2.40	0.02	0.01	0.03	0.02	0.07
12	2.33	2.35	2.42	0.13	0.10	0.05	0.02	0.09
13	2.38	2.48	2.56	0.05	0.05	0.07	0.10	0.18
14	2.42	2.39	2.52	0.03	0.10	0.05	−0.03	0.10
15	2.28	2.33	2.45	0.05	0.06	0.10	0.05	0.17
16	2.72	2.59	2.58	0.05	0.05	0.11	−0.13	−0.14
17	2.34	2.38	2.39	0.03	0.02	0.01	0.04	0.05
18	2.23	2.22	2.31	0.05	0.04	0.11	−0.01	0.08
19	1.37	2.41	2.39	0.05	0.04	0.02	1.04	1.02
20	1.84	2.58	2.70	0.04	0.03	0.02	0.74	0.86
21	0.55	2.40	2.36	0.10	0.12	0.16	1.85	1.81

TABLE 1—(*continued*)

COLLABORATOR	AVERAGE[a] BY METHOD:[b]			DIFFERENCE[c] BY METHOD:[b]			DIFFERENCE IN AVERAGE RESULTS BY METHOD A AND METHOD:	
	A	B	C	A	B	C	B[d]	C[d]
Sample 3: Calcium Phytate								
1	38.73	38.55	39.04	0.12	0.24	0.20	−0.18	0.31
2	38.72	38.66	39.21	0.14	0.16	0.14	−0.06	0.49
3	39.03	37.38	40.20	0.16	1.46	0.20	−1.65	1.17
4	38.89	37.44	39.62	0.25	0.36	0.30	−1.45	0.73
5	38.80	38.47	39.33	0.25	0.15	0.10	−0.33	0.53
6	38.51	37.95	38.99	0.04	0.06	0.01	−0.56	0.48
7	38.70	38.70	38.80	0.05	0.05	0.05	0.00	0.10
8	38.05	38.51	37.97	0.18	0.13	0.17	0.46	−0.08
9	39.33	39.27	39.40	0.10	0.10	0.20	−0.06	0.07
10	38.37	38.42	39.63	0.16	0.14	0.10	0.05	1.26
11	38.37	38.77	39.42	0.10	0.09	0.12	0.40	1.05
12	38.55	38.60	40.33	0.10	0.20	0.05	0.05	1.78
13	38.73	38.77	39.97	0.05	0.30	0.10	0.04	1.24
14	38.77	39.00	41.83	0.40	0.40	0.10	0.23	3.06
15	38.04	38.40	38.42	0.37	0.16	0.20	0.36	0.38
16	39.13	38.88	39.00	0.20	0.20	0.20	−0.25	−0.13
17[e]	39.75	39.84	39.91	0.00	0.06	0.26	0.09	0.16
18	33.33	38.42	39.50	7.25	0.25	0.00	5.09	6.17
19	22.72	38.08	39.91	1.43	1.60	0.15	15.36	17.19
20	18.71	37.15	40.84	3.87	0.68	0.07	18.44	22.13
21	36.07	39.12	13.51	0.46	0.48	5.36	3.05	−22.56

[a] Average of triplicate determinations.
[b] A, HNO_3-$HClO_4$ method; B, $MgNO_3$-HCl method; C, H_2SO_4-HNO_3 method.
[c] Difference in high and low results of triplicate determinations.
[d] The minus sign denotes that the result by this method is lower than that by Method A.
[e] Sample 3a.

with Method A (nitric-perchloric acid digestion) and in 76 per cent of those with Method B (magnesium nitrate-hydrochloric acid treatment). The differences were smallest for the low-phosphorus materials (Samples 1 and 2). These data provide further evidence of the tendency toward high results with the sulfuric-nitric acid digestion.

With nitric-perchloric acid digestion the average results were very close to those with magnesium nitrate-hydrochloric acid treatment. The values by the first method were higher in 51 per cent of the individual comparisons. The agreement between the results by the two methods was generally better than in the previous study.

The average differences between all the comparisons of nitric-perchloric acid digestion with magnesium nitrate-hydrochloric acid treatment were not statistically significant in the case of Samples 1 and 2 (Table 3). For Sample 3 the difference was significant at the 5 per cent level but not at

TABLE 2.—*Summary of percentages of total P_2O_5*

SAMPLE	RANGE[a] BY METHOD:			AVERAGE BY METHOD:		
	A	B	C	A	B	C
1[b]	2.48– 3.03	2.57– 2.96	2.51– 3.09	2.74	2.75	2.78
2[c]	2.23– 2.72	2.16– 2.59	2.31– 2.73	2.38	2.38	2.44
3[d]	38.04–39.33	37.38–39.27	37.97–41.83	38.67	38.49	39.45

[a] Range of average of triplicate determinations.
[b] 20 collaborators—excludes Collaborator 21.
[c] 18 collaborators—excludes Collaborators 19 to 21.
[d] 16 collaborators—excludes Collaborators 17 to 21.

the 1 per cent level. In the comparisons of digestions with nitric-perchloric and sulfuric-nitric acids the average difference was not significant for Sample 1. It was significant at the 5 per cent level but not at the 1 per cent level for Sample 2, and was significant at both levels for Sample 3.

The results of other comparisons of nitric-perchloric acid digestion with official methods of preparing solutions of fertilizers for determination of total phosphorus are summarized in Table 5 of Part II (Photometric Determination of Phosphorus) of this report. In 22 comparisons, each on a triple superphosphate and a 4–12–4 mixed fertilizer, the average differences between the results for total phosphorus in solutions prepared with nitric-perchloric and nitric-hydrochloric acids, respectively, were not statistically significant. For a mixture of organic materials, the average difference on solutions prepared by nitric-perchloric acid digestion and magnesium nitrate-hydrochloric acid treatment, respectively, was significant at the 5 per cent level but not at the 1 per cent level.

In this study, as in previous collaborative investigations, there was a

TABLE 3.—*Summary of differences between percentages of total P_2O_5*

SAMPLE	RANGE,[a,b] METHOD A VS. METHOD:		AVERAGE[c,b] METHOD A VS. METHOD:	
	B	C	B	C
1[d]	−0.18, 0.25	−0.29, 0.35	0.01[e]	0.04[e]
2[f]	−0.24, 0.18	−0.20, 0.27	0.00[e]	0.07[g]
3[h]	−1.65, 0.46	−0.13, 3.06	−0.17[g]	0.74[i]
1 to 3	−1.65, 0.46	−0.29, 3.06	−0.05	0.27

[a] Range of the difference in average triplicate results of individual comparisons.
[b] The minus sign denotes that Method A gave the higher result.
[c] Average of the difference in all comparisons.
[d] 20 comparisons—excludes Collaborator 21.
[e] Not statistically significant at either 5% or 1% level.
[f] 18 comparisons—excludes Collaborators 19 to 21.
[g] Statistically significant at 5% level but not at 1% level.
[h] 17 comparisons—excludes Collaborators 18 to 21.
[i] Statistically significant at 1% level.

wide range in the average values for total P_2O_5 obtained by the individual collaborators on the same material with the same method of solution preparation. In many instances the discrepancies appear to result from factors other than those involved in the solution procedure itself.

The differences between the high and low results in triplicate determinations of P_2O_5 with the three methods of solution preparation are summarized in Table 4. For the low-phosphorus materials (Samples 1 and 2)

TABLE 4.—*Summary of differences between high and low percentages of total P_2O_5 in triplicate determinations*[a]

SAMPLE	RANGE BY METHOD:			AVERAGE BY METHOD:		
	A	B	C	A	B	C
1	0.00–0.15	0.01–0.15	0.01–0.19	0.05	0.06	0.07
2	0.00–0.13	0.00–0.16	0.01–0.16	0.05	0.05	0.05
3	0.00–7.25	0.05–1.60	0.00–5.36	0.17[b]	0.22[c]	0.14[d]
1 to 3	0.00–7.25	0.00–1.60	0.00–5.36	0.09	0.11	0.08

[a] 21 triplicated determinations on each sample by each method.
[b] Excluding Collaborators 18(7.25%), 19(1.43%), and 20(3.87%).
[c] Excluding Collaborators 3(1.46%) and 19(1.60%).
[d] Excluding Collaborator 21(5.36%).

the differences exceeded 0.10 per cent in 10 per cent of all the analyses with Method A (nitric-perchloric acid digestion), 12 per cent with Method B (magnesium nitrate-hydrochloric acid treatment), and 14 per cent with Method C (sulfuric-nitric acid digestion). For the high-phosphorus material (sample 3) the differences exceeded 0.25 per cent in 29 per cent of the analyses with Method A, 33 per cent with Method B, and 14 per cent with Method C. The data indicate, in general, that for individual materials the precision of the results among the replicates of an analysis was not influenced greatly by the method of preparing the solution.

Several collaborators in this and the previous study reported very low and erratic results on solutions of cottonseed meal and calcium phytate prepared with nitric-perchloric acids, and it has been suggested that the difficulty resides in making the perchloric acid digestion at a too low temperature or for a too short time. Also, the opinion has been expressed that for the digestion of such refractory materials the volume of perchloric acid should be greater than 10 ml. The influence of these factors on the percentages of P_2O_5 found in cottonseed meal (Sample 2) was investigated by O. W. Ford, Purdue University Agricultural Experiment Station, and his data are summarized in Table 5. In experiments 4–9 the digestion was discontinued when the maximum temperature was reached. The results indicate that with cottonseed meal the temperature of the perchloric acid digestion should be raised to at least 170°C. over a period of

60 minutes or longer if more than 10 ml of the acid is used. It was noted that the appearance of copious white fumes in the bulb of the flask coincides with a temperature of about 170°C., but white fumes may evolve from the mouth of the flask at lower temperature.

DISCUSSION

The results of this and other investigations appear to justify the recommendation that digestion with nitric-perchloric acids be adopted as an official procedure for the preparation of solutions of fertilizers for determination of total phosphorus. The severe treatment specified in the procedure for preparing solutions of feeds (*Official Methods of Analysis*, 8th Ed., 1955, p. 379, sec. **22.51(a)**) is necessary with oilseed meals and other fertilizers containing organically combined phosphorus; there is evidence that with such materials more than 10 ml of perchloric acid is preferable. With inorganic fertilizers, however, the time of the nitric acid digestion and the time and temperature of the perchloric acid digestion usually can be decreased materially. With some inorganic materials—calcium metaphosphate, for example—the use of more than 10 ml of perchloric acid may be desirable.

TABLE 5.—*Influence of some factors in nitric-perchloric acid digestion of cottonseed meal*[a]

EXPERIMENT NO.	VOLUME OF $HClO_4$	DIGESTION PERIOD[b]	MAXIMUM TEMPERATURE	P_2O_5
	ml	*minutes*	*°C*	*per cent*
1	10[c]	120	130–140	0.80, 0.90
2	10[c]	180	150–160	2.14
3	10[c]	240	160–180	2.37
4	10	60	178	2.38, 2.38
5	10	150	178	2.35, 2.45
6	10	165	167	2.35, 2.42
7	30	60	170	2.40, 2.40
8	30	60	205	2.45, 2.47
9	30	150	197	2.43, 2.45
10	30	205[d]	192	2.40, 2.45

[a] Data from O. W. Ford, Purdue University Agricultural Experiment Station; 2 g samples digested with 72% $HClO_4$ in Kjeldahl flasks.
[b] With $HClO_4$, in addition to digestion for 45 minutes with 40 ml HNO_3.
[c] 1 g cottonseed meal.
[d] Including digestion for 10 minutes at 192° C.

Kjeldahl flasks are preferred for the nitric-perchloric acid digestion of organic materials, but inorganic fertilizers usually can be digested readily in other kinds of flasks. Boiling of the partially diluted digestate, as specified in the method for feeds, insures rapid and complete dissolution of the ionizable orthophosphate, but total expulsion of nitrogen dioxide fumes from the solution appears to be unnecessary.

Since nitric-perchloric acid digestion introduces no ions that interfere in the volumetric determination of phosphorus, it is preferable to sulfuric-nitric acid digestion in the analysis of organic materials. On the other hand, the nitric-perchloric acid procedure appears in general to have little or no advantage over nitric-hydrochloric acid digestion in the preparation of solutions of inorganic fertilizers.

Since the use of perchloric acid in preparing solutions of samples is more than ordinarily hazardous and is being extended to an increasingly wide variety of materials, *Official Methods of Analysis* should contain a section outlining in some detail the precautions to be observed in the handling and application of this acid as a laboratory reagent.

This investigation gives additional evidence of the tendency of the sulfuric-nitric acid digestion to yield high results for phosphorus by the volumetric method. Further study should be made of the factors influencing the interference of the sulfate ion.

The magnesium nitrate-hydrochloric acid method of preparing solutions of organic materials should be given further study, with the objectives of reducing the time requirements and of lessening the danger of loss of sample during the ignition with magnesium nitrate.

SUMMARY

The preparation of solutions of organic materials for determination of total phosphorus was further studied; 22 collaborators compared nitric-perchloric acid digestion to sulfuric-nitric acid digestion and to magnesium nitrate-hydrochloric acid treatment in more than 200 triplicated analyses of Milorganite, cottonseed meal, and calcium phytate. Phosphorus was determined by the volumetric method; the phosphomolybdate was precipitated at 25–30°C. under continuous agitation.

In agreement with the results of previous investigations, the phosphorus values reported by the individual collaborators usually were higher with sulfuric-nitric acid digestion than with either of the other methods of solution preparation. The differences, smallest with the low-phosphorus materials (Milorganite and cottonseed meal), reflect chiefly the interference of the sulfate ion in the volumetric determination of phosphorus.

With nitric-perchloric acid digestion the collaborators' individual results were usually close to those with magnesium nitrate-hydrochloric acid treatment. The agreement between the values by the two methods was generally better than in the previous study.

Investigation of the factors influencing the decomposition of organic materials by nitric-perchloric acid treatment indicated that with refractory substances, such as cottonseed meal, the temperature of the perchloric acid digestion should be raised to at least 170°C. over a period of 60 minutes or longer.

The results reported here, together with those of other investigations,

point to nitric-perchloric acid digestion as a suitable procedure for the preparation of solutions of either organic or inorganic fertilizers for the determination of phosphorus.

The official sulfuric-nitric acid and magnesium nitrate-hydrochloric acid procedures should be studied further to improve their utility and define their application more clearly.

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RECOMMENDATIONS

It is recommended*—

(1) That further study be made of:

(*a*) The official sulfuric-nitric acid and magnesium nitrate-hydrochloric acid methods of preparing solutions of fertilizers for phosphorus determination.

(*b*) Methods for direct determination of available phosphorus.

(2) That *Official Methods of Analysis* include a section on precautions in the handling and use of perchloric acid as a laboratory reagent.

(3) That the following method of preparing phosphate fertilizer solutions by digestion with nitric and perchloric acids be adopted as first action and that collaborative study of the method be discontinued.

2.10

(e) (Suitable for all fertilizers.) Boil gently 30–45 min. with 20–30 ml HNO_3 in suitable flask (preferably Kjeldahl for samples contg large quantities of org. matter) to oxidize all easily oxidizable matter. Cool and add 10–20 ml 70–72% $HClO_4$. Boil very gently until soln is colorless or nearly so and dense white fumes appear in flask. Do not boil to dryness at any time (Danger!). (With samples contg large quantities of org. matter, temp. should be raised to fuming point, ca 170°, over period of at least 1 hr.) Cool slightly, add 50 ml H_2O, and boil few min.

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 21, 22 (1957).

REPORT ON PHOSPHORUS IN FERTILIZERS

II. PHOTOMETRIC DETERMINATION OF PHOSPHORUS

By E. A. Epps, Jr.,* and K. D. Jacob,† *Associate Referee*

In accordance with the recommendation in the previous report,[1] further collaborative study was made of the photometric vanadomolybdate method for total phosphorus in fertilizers. The work was extended to include determinations on solutions prepared by nitric-perchloric acid digestion of the samples, as well as by official procedures, and determinations of phosphorus in the citrate-insoluble residues.

The fertilizers used in this investigation were commercial triple superphosphate (Sample 1), commercial 4–12–4 mixed fertilizer (Sample 2),

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[1] Epps, E. A., Jr., Hoffman, W. M., and Jacob, K. D., *This Journal*, 39, 579 (1956).

and a laboratory-prepared mixture of organic materials (Sample 3) consisting of cottonseed meal, bone meal, and other substances. Each of the samples was ground to pass the 60-mesh sieve and thoroughly mixed.

INSTRUCTIONS TO COLLABORATORS

Make all determinations in duplicate on the same solution of each sample, and report the individual results on the form enclosed with these instructions.

TOTAL P_2O_5

Preparation of solutions.—(a) With the following modifications, prepare solutions of Samples 1 and 2 as in *Official Methods of Analysis*, 8th Ed., 1955, p. 8, sec. **2.10(a)**, and of Sample 3 as in sec. **2.10(c)**: Use 0.5 g of Sample 1 and 1 g of Samples 2 and 3. Dilute to 250 ml instead of 200 ml. (b) Using the same weight of sample as before, boil 20 min. with 20 ml HNO_3 in Kjeldahl flask, cool, add 10 ml 70% $HClO_4$, and boil until solution is colorless or nearly so and dense white fumes appear. Cool, add 50 ml H_2O, and boil. Cool, filter into 250 ml volumetric flask, and dilute to volume.

Volumetric method.—Determine P_2O_5 in appropriate aliquots of the solutions as on pp. 9–10, sec. **2.14(a)**.

Photometric method.—Prepare vanadomolybdate reagent as follows: Dissolve 40 g molybdic acid (85%) in 400 ml H_2O, with heating if necessary to dissolve completely. Dissolve 1 g NH_4 vanadate in 300 ml H_2O and add 200 ml HNO_3. Cool the solutions and mix by pouring the molybdate solution into the vanadate solution. Dilute to 1 liter.

Prepare standard phosphate solution by dissolving 1.9168 g KH_2PO_4 in H_2O and diluting to 1 liter. This solution contains 1 mg P_2O_5 per ml.

Transfer 5 ml of the solution of the sample to a 100 ml volumetric flask. Add 25 ml H_2O and 25 ml vanadomolybdate reagent, dilute to 100 ml, mix thoroughly, and let stand 10 min. for development of color. In the same way, prepare concurrently from the KH_2PO_4 solution a series of color standards containing 2.0, 2.5, 3.0, 3.5, 4.0, and 4.5 mg P_2O_5 per 100 ml. Using a photometer, determine the absorbance of the solutions of the sample and the standards at a wavelength of 400 mμ with the 2.0 mg standard set at zero absorbance. Estimate the P_2O_5 in the sample by interpolation of the photometric reading on a graph of the readings obtained with the standard solutions.

CITRATE-INSOLUBLE P_2O_5

Preparation of solutions.—Proceed as on p. 11, sec. **2.18(a)(ii)**.

Volumetric method.—Determine P_2O_5 in 50 ml aliquots of the solutions as on pp. 9–10, sec. **2.14(a)**.

Photometric method.—Determine P_2O_5 in 20 ml aliquots of the solutions as for total P_2O_5, using standard KH_2PO_4 solutions containing appropriate concentrations of P_2O_5.

COMMENTS OF COLLABORATORS

Collaborator 7 pointed out that the differential spectrophotometric method of Gee and Deitz[2] calls for adjustment of the unknown and standard solutions to approximately the same acidity, a requirement not specified in the procedure used in the present study. He supplied data, shown later, which indicate that this refinement leads to better agreement between the results by the photometric and the volumetric methods.

[2] GEE, A., and DEITZ, V. R., *Anal. Chem.*, **25**, 1320 (1953).

Collaborator 3 indicated that the Coleman Universal (No. 14) photometer does not respond well to high intensities of color.

Collaborator 5 stated that the phosphorus concentration in the unknown should be such that the absorbance falls on the straight middle portion of the standard curve.

For the photometric determination of citrate-insoluble phosphorus, Collaborator 4 used standards containing 0.5, 1.0, and 1.5 mg P_2O_5 per 100 ml. Collaborator 11 noted that solutions of the citrate-insoluble residues, prepared with nitric-hydrochloric acids, had a slight greenish tinge.

Collaborator 1 reported difficulty in dissolving the ring of residue deposited in the Kjeldahl flask during the nitric-perchloric acid digestion of the triple superphosphate. Collaborators 2 and 10 stated that Kjeldahl flasks are not necessary for this digestion, especially in the case of samples containing little or no organic matter. According to Collaborator 5, addition of a small amount of sulfuric acid lessens the hazard of the digestion.

RESULTS

Collaborators 2, 4, 7, and 9 used the Beckman Model DU spectrophotometer in all the photometric determinations. Collaborators 6, 8, and 11 used the Beckman Model B instrument; Collaborators 5 and 10, the Bausch and Lomb Spectronic 20; Collaborator 3, the Coleman Universal (No. 14); and Collaborator 1, the Klett-Summerson photoelectric colorimeter. Determinations were also made by Collaborator 5 with the Beckman DU and by Collaborator 4 with the Bausch and Lomb Spectronic 20.

The results of 217 analyses in duplicate were received from 11 collaborators. The requested analyses were made by all collaborators except No. 2 who omitted the determination of citrate-insoluble phosphorus in Sample 3.

The averages of the individual collaborator's results for total P_2O_5 in solutions prepared by official procedures and by nitric-perchloric acid digestion, respectively, are given in Tables 1 and 2. These tables also show the differences between the average results by the volumetric and photometric methods, as well as the individual differences between the high and low results in duplicate determinations.

As in the previous study, the dominant trend of the results was toward lower values by the photometric method—88 per cent of the 66 comparisons. The sample averages by the photometric method were in all instances significantly lower (1 per cent level) than those by the volumetric method (Tables 3 and 4).

With the volumetric method, preparation of the solution by nitric-perchloric acid digestion gave sample averages that were not statistically different from those obtained on solutions prepared by official procedures (Table 5). With the photometric method the differences were not significant on the triple superphosphate; they were significant at the 5 per cent level on the mixed fertilizer and organic material.

Differences between duplicate determinations of total P_2O_5 by the volumetric and photometric methods with the several procedures for prepar-

TABLE 1.—*Percentages of total P_2O_5 found by volumetric and photometric methods in solutions prepared by official procedures*

COLLABORATOR[a]	AVERAGE[b]		DIFFERENCE[c]		DIFFERENCE IN AVERAGE RESULTS BY VOLUMETRIC AND PHOTOMETRIC METHODS[d]
	VOLUMETRIC	PHOTOMETRIC	VOLUMETRIC	PHOTOMETRIC	
Sample 1: Triple Superphosphate					
1	45.67	45.00	0.15	0.00	−0.67
2	46.25	45.40	0.30	0.40	−0.85
3	45.60	44.25	0.00	0.50	−1.35
4	46.30	46.10	0.12	0.20	−0.20
5	45.90	45.30	0.10	0.20	−0.60
6	45.64	45.70	0.13	0.20	0.06
7	46.02	45.65	0.05	0.00	−0.37
8	43.76	43.88	0.39	0.12	0.12
9	46.51	46.22	0.03	0.05	−0.29
10	46.50	45.40	0.60	0.00	−1.10
11	46.68	46.55	0.56	0.30	−0.13
Sample 2: Mixed Fertilizer (4–12–4)					
1	14.15	14.12	0.10	0.05	−0.03
2	14.12	13.87	0.05	0.05	−0.25
3	13.72	13.40	0.05	0.00	−0.32
4	13.99	14.00	0.06	0.04	0.01
5	14.13	14.06	0.10	0.12	−0.07
6	14.10	14.03	0.11	0.06	−0.07
7	13.98	13.83	0.03	0.00	−0.15
8	13.92	13.43	0.04	0.03	−0.49
9	14.21	14.11	0.06	0.02	−0.10
10	14.05	13.85	0.10	0.00	−0.20
11	14.30	14.38	0.04	0.05	0.08
Sample 3: Organic Material					
1	12.69	12.60	0.09	0.00	−0.09
2	12.85	12.52	0.20	0.05	−0.32
3	12.87	12.62	0.25	0.05	−0.25
4	12.57	12.56	0.06	0.03	−0.01
5	12.33	12.58	0.03	0.04	0.25
6	12.63	12.62	0.08	0.00	−0.01
7	12.81	12.70	0.00	0.02	−0.11
8	12.39	12.07	0.03	0.03	−0.32
9	12.14	12.31	0.06	0.03	0.17
10	13.00	12.42	0.00	0.05	−0.58
11	12.94	12.75	0.19	0.00	−0.19

[a] Photometric instruments: Beckman DU—Collaborators 2, 4, 7, and 9; Beckman B—Collaborators 6, 8, and 11; Bausch and Lomb Spectronic 20—Collaborators 5 and 10; Coleman Universal (No. 14)—Collaborator 3; Klett-Summerson—Collaborator 1.
[b] Average of duplicate determinations.
[c] Difference in high and low results of duplicate determinations.
[d] The minus sign denotes that the photometric method gave the lower result.

ing the solution of the sample are summarized in Table 6. The range of the differences was 0.00 to 0.60 per cent of P_2O_5 (average, 0.10 per cent) with the volumetric method and 0.00 to 0.50 per cent (average, 0.08 per cent) with the photometric method. The precision was better with the volumetric method in 33 per cent of the comparisons and with the photometric method in 58 per cent.

TABLE 2.—*Percentages of total P_2O_5 found by volumetric and photometric methods in solutions prepared with nitric-perchloric acids*

COLLABORATOR[a]	AVERAGE[b]		DIFFERENCE[c]		DIFFERENCE IN AVERAGE RESULTS BY VOLUMETRIC AND PHOTOMETRIC METHODS[d]
	VOLUMETRIC	PHOTOMETRIC	VOLUMETRIC	PHOTOMETRIC	
Sample 1: Triple Superphosphate					
1	46.25	46.20	0.10	0.10	−0.05
2	46.25	45.57	0.10	0.05	−0.68
3	45.55	44.75	0.10	0.00	−0.80
4	46.20	46.16	0.08	0.12	−0.04
5	45.90	45.25	0.00	0.50	−0.65
6	45.13	44.95	0.12	0.10	−0.18
7	45.97	45.30	0.05	0.00	−0.67
8	43.80	43.53	0.08	0.00	−0.27
9	46.40	46.27	0.12	0.05	−0.13
10	47.30	45.40	0.20	0.00	−1.90
11	46.56	46.45	0.32	0.10	−0.11
Sample 2: Mixed Fertilizer (4–12–4)					
1	14.15	14.10	0.10	0.10	−0.05
2	14.05	13.75	0.20	0.10	−0.30
3	13.75	13.50	0.00	0.10	−0.25
4	13.97	13.99	0.02	0.14	0.02
5	13.70	13.92	0.05	0.35	0.22
6	14.00	13.83	0.01	0.06	−0.17
7	13.99	13.81	0.02	0.00	−0.18
8	13.84	13.34	0.00	0.04	−0.50
9	14.29	14.07	0.07	0.06	−0.22
10	14.50	13.80	0.20	0.05	−0.70
11	14.60	14.36	0.16	0.00	−0.24
Sample 3: Organic Material					
1	12.47	12.37	0.15	0.05	−0.10
2	12.75	12.45	0.00	0.10	−0.30
3	12.50	12.42	0.00	0.05	−0.08
4	12.67	12.65	0.06	0.05	−0.02
5	12.46	12.37	0.03	0.05	−0.09
6	12.34	12.18	0.04	0.04	−0.16
7	12.77	12.65	0.03	0.00	−0.12
8	11.90	11.63	0.04	0.00	−0.27
9	12.53	12.20	0.01	0.10	−0.33
10	13.25	12.55	0.10	0.00	−0.70
11	12.69	12.55	0.19	0.00	−0.14

[a] Photometric instruments: Beckman DU—Collaborators 2, 4, 7, and 9; Beckman B—Collaborators 6, 8, and 11; Bausch and Lomb Spectronic 20—Collaborators 5 and 10; Coleman Universal (No. 14)—Collaborator 3; Klett-Summerson—Collaborator 1.
[b] Average of duplicate determinations.
[c] Difference in high and low results of duplicate determinations.
[d] The minus sign denotes that the photometric method gave the lower result.

The results obtained with the different photometers are compared with the results by the volumetric method (same collaborators) in Table 7. The performance of the Beckman DU, Beckman B, and Klett-Summerson instruments was about equal and was superior to that of the Bausch and Lomb and the Coleman instruments. The performance of the two Beck-

TABLE 3.—*Summary of percentages of total P_2O_5 by volumetric and photometric methods*

SAMPLE	SOLUTION METHOD[a]	RANGE[b]		DIFFERENCE		AVERAGE	
		VOLUMETRIC	PHOTOMETRIC	VOLUMETRIC	PHOTOMETRIC	VOLUMETRIC	PHOTOMETRIC
1	A	43.76–46.68	43.88–46.55	2.92	2.67	45.89	45.40
	B	43.80–47.30	43.53–46.45	3.50	2.92	45.94	45.44
	A, B	43.76–47.30	43.53–46.55	3.54	3.02	45.92	45.42
2	A	13.72–14.30	13.40–14.38	0.58	0.98	14.06	13.92
	B	13.70–14.60	13.34–14.36	0.90	1.02	14.08	13.86
	A, B	13.70–14.60	13.34–14.38	0.90	1.04	14.07	13.89
3	A	12.14–13.00	12.07–12.75	0.86	0.68	12.66	12.52
	B	11.90–13.25	11.63–12.65	1.35	1.02	12.58	12.37
	A, B	11.90–13.25	11.63–12.75	1.35	1.12	12.62	12.44

[a] A, official methods; B, HNO_3-$HClO_4$ digestion.
[b] Range of averages reported by individual collaborators.

TABLE 4.—*Summary of differences between percentages of total P_2O_5 by volumetric and photometric methods*

SAMPLE	SOLUTION METHOD[a]	VOLUMETRIC VS. PHOTOMETRIC[b]	
		RANGE[c]	AVERAGE[d]
1	A	−1.35, 0.12	−0.49[e]
	B	−1.90, −0.04	−0.50[f]
	A, B	−1.90, 0.12	−0.49[e]
2	A	−0.49, 0.08	−0.14[f]
	B	−0.70, 0.22	−0.22[f]
	A, B	−0.70, 0.22	−0.18[e]
3	A	−0.58, 0.25	−0.13[g]
	B	−0.70, −0.02	−0.21[e]
	A, B	−0.70, 0.25	−0.17[e]
1 to 3	A	−1.35, 0.25	−0.26
	B	−1.90, 0.22	−0.31
	A, B	−1.90, 0.25	−0.28

[a] A, official methods; B, HNO_3-$HClO_4$ digestion.
[b] The minus sign denotes that the photometric method gave the lower result.
[c] Range of the difference between average duplicate results of individual comparisons.
[d] Average of the differences in all comparisons.
[e] Statistically significant at 1% level.
[f] Statistically significant at 5% level but not at 1% level.
[g] Not statistically significant at either 5% or 1% level.

TABLE 5.—*Summary of differences between percentages of total P_2O_5 with different methods of preparing solution of sample*

SAMPLE	P_2O_5 METHOD[a]	OFFICIAL VS. HNO_3–$HClO_4$ METHOD OF SOLUTION PREPARATION[b]	
		RANGE[c]	AVERAGE[d]
1	I	−0.51, 0.80	0.04[e]
	II	−0.75, 1.20	0.03[e]
	I, II	−0.75, 1.20	0.04[e]
2	I	−0.43, 0.45	0.02[e]
	II	−0.20, 0.10	−0.06[f]
	I, II	−0.43, 0.45	−0.04[e]
3	I	−0.49, 0.39	−0.08[e]
	II	−0.44, 0.13	−0.16[f]
	I, II	−0.49, 0.39	−0.12[f]
1 to 3	I	−0.51, 0.80	−0.01
	II	−0.75, 1.20	−0.06
	I ,II	−0.75, 1.20	−0.03

[a] I, volumetric; II, photometric.
[b] The minus sign denotes that the HNO_3-$HClO_4$ method gave the lower result.
[c] Range of the difference between average results of individual comparisons.
[d] Average of the differences in all comparisons.
[e] Not statistically significant at either 5% or 1% level.
[f] Statistically significant at 5% level but not at 1% level.

TABLE 6.—*Summary of differences between high and low percentages of total P_2O_5 in duplicate determinations by volumetric and photometric methods*

SAMPLE	SOLUTION METHOD[a]	RANGE		AVERAGE	
		VOLUMETRIC	PHOTOMETRIC	VOLUMETRIC	PHOTOMETRIC
1	A	0.00–0.60	0.00–0.50	0.22	0.18
	B	0.00–0.32	0.00–0.50	0.12	0.09
	A, B	0.00–0.60	0.00–0.50	0.17	0.14
2	A	0.03–0.11	0.00–0.12	0.07	0.04
	B	0.00–0.20	0.00–0.35	0.08	0.09
	A, B	0.00–0.20	0.00–0.35	0.07	0.06
3	A	0.00–0.25	0.00–0.05	0.09	0.03
	B	0.00–0.19	0.00–0.10	0.06	0.04
	A, B	0.00–0.25	0.00–0.10	0.07	0.03
1 to 3	A	0.00–0.60	0.00–0.50	0.13	0.08
	B	0.00–0.32	0.00–0.50	0.08	0.07
	A, B	0.00–0.60	0.00–0.50	0.10	0.08

[a] A, official methods; B, HNO_3-$HClO_4$ digestion.

TABLE 7.—*Summary of results for total P_2O_5 in samples 1 to 3 with different types of photometers*

PHOTOMETER	COLLABORATOR	NO. OF COMPARISONS[a]	DIFFERENCE IN AVERAGE RESULTS FOR TOTAL P_2O_5 BY VOLUMETRIC AND PHOTOMETRIC METHODS[b]	
			RANGE[c]	AVERAGE[d]
			per cent	*per cent*
Beckman DU	2, 4, 5, 7, 9	30	−0.85, 0.47	−0.16
Beckman B	6, 8, 11	18	−0.50, 0.12	−0.17
Bausch and Lomb Spectronic 20	4, 5, 10	18	−1.90, 0.25	−0.37
Coleman Universal (No. 14)	3	6	−1.35, −0.08	−0.50
Klett-Summerson	1	6	−0.67, −0.03	−0.16
		78	−1.90, 0.47	−0.24[e]

[a] The individual comparisons are between the average results of duplicate determinations on solutions prepared, respectively, by official methods and by HNO_3-$HClO_4$ digestion.
[b] The minus sign denotes that the photometric method gave the lower result.
[c] Range of the difference between results of individual comparisons.
[d] Average of the difference in all comparisons.
[e] Weighted average; unweighted average is 0.27 per cent.

man photometers was somewhat better than in the previous collaborative investigation; the reverse was true of the Bausch and Lomb instrument. It should be noted, however, that Collaborator 4 obtained results with the Bausch and Lomb instrument very close to those obtained with the Beckman DU (Table 8). As pointed out by Collaborator 3 and supported by data in the previous study, the Coleman Universal (No. 14) photometer does not perform well at the color intensities resulting from the relatively high concentrations of P_2O_5 specified in the instructions to the collaborators.

The data of Table 9, reported by Collaborator 7, indicate that better agreement in the results by the volumetric and photometric methods is obtained when the solutions of the sample and the photometric standard are adjusted to approximately the same acidity, as recommended by Gee and Deitz (see footnote 2). The Beckman DU instrument was used in these determinations. The data of Table 10, reported by Collaborator 9, however, indicate that the photometric method as outlined in the instructions to collaborators is capable of giving results which agree well with those by both the volumetric and the gravimetric methods.

The results for citrate-insoluble P_2O_5 in the samples, as determined by the volumetric and photometric methods, are summarized in Table 11. Collaborator averages did not show statistically significant differences between the two procedures for either of the samples. As with total P_2O_5 (Table 3), however, there was a wide range in the average values obtained

TABLE 8.—*Percentages of total P_2O_5 found by two collaborators with Beckman DU and Bausch and Lomb Spectronic 20 instruments*

COLLABORATOR	AVERAGE[a]		
	VOLUMETRIC	PHOTOMETRIC	
		BECKMAN	B. AND L.
	Sample 1		
4	46.25	46.13	46.01
5	45.90	45.82	45.27
	Sample 2		
4	13.98	13.99	13.97
5	13.92	14.10	13.99
	Sample 3		
4	12.62	12.61	12.60
5	12.40	12.66	12.48

[a] Average of determinations made in duplicate on solutions prepared by official methods and by HNO_3-$HClO_4$ digestion, respectively.

by the individual collaborators on the same material with the same method of analysis.

DISCUSSION

In 2 years of collaborative study the results for total P_2O_5 with the photometric method generally have been lower, often considerably lower, than those with the volumetric method. With the photometric method in the present work, for example, the average values for each of the three samples were lower than those with the volumetric method, and the differences were statistically significant at the 1 per cent level. In both years,

TABLE 9.—*Percentages of total P_2O_5 determined by volumetric method and two photometric methods*

SAMPLE	AVERAGE[a]		
	VOLUMETRIC	PHOTOMETRIC	
		A[b]	B[c]
1	45.97	45.30	45.82
2	13.99	13.81	13.90
3	12.77	12.65	12.71

[a] Average of duplicate determinations by Collaborator 7. Solutions of samples were prepared with nitric-perchloric acids.
[b] Method outlined in instructions to collaborators.
[c] Method of Gee and Deitz.

TABLE 10.—*Percentages of total P_2O_5 determined by volumetric, gravimetric, and photometric methods*

SAMPLE	AVERAGE[a]		
	VOLUMETRIC	GRAVIMETRIC	PHOTOMETRIC[b]
1	46.51	46.33	46.22
2	14.21	14.18	14.11
3	12.14	12.29	12.31

[a] Average of duplicate determinations by Collaborator 9. Solutions of samples were prepared by official methods.
[b] Beckman DU spectrophotometer.

however, some of the collaborators obtained good agreement between the two methods.

What are the factors responsible for these variations? Do they result from inherent deficiencies in either or both of the methods, their inadequate delineation in the *Official Methods of Analysis* or in the instructions to the collaborators, failure of the analyst to adhere to the methods as outlined, inexperience of the analyst with the methods, poor techniques, or other causes?

The differences do not appear to be associated consistently with the use of a specific type or make of photometer nor with the method of preparing the solution of the sample for analysis. As previously indicated, better agreement between the two methods may be obtained by adjusting the solutions of the sample and the photometric standard to approximately the same level of acidity, an operation which was not specified in the instructions to the collaborators. The poor results that some of the analysts obtained with the photometric method may have been due, at least in part, to inexperience with the method and to failure to adjust the conditions of the determination to permit optimum performance of the photometer used. Some of the discrepancies may have resulted from variations in the purity of the photometric standard, monopotassium phosphate.

Some factors affecting the differences between the values by the two

TABLE 11.—*Summary of percentages of citrate-insoluble P_2O_5 by volumetric and photometric methods*

SAMPLE	RANGE[a]		DIFFERENCE		AVERAGE	
	VOLUMETRIC	PHOTOMETRIC	VOLUMETRIC	PHOTOMETRIC	VOLUMETRIC	PHOTOMETRIC
1	0.42–1.16	0.35–1.17	0.74	0.82	0.84	0.82
2	0.75–1.72	0.72–1.79	0.97	1.07	1.11	1.10
3[b]	0.70–1.79	0.65–1.78	1.09	1.13	1.28	1.28

[a] Range of averages reported by individual collaborators.
[b] Not analyzed by Collaborator 2.

methods may reside in the volumetric determination itself. For example, it appears to be a rather common experience that, even in the absence of sulfate interference, the volumetric method tends to give high results.

The data of these investigations, together with the foregoing considerations, show the need for further study of the conditions requisite for better agreement between the results by the volumetric and photometric methods.

SUMMARY

The photometric vanadomolybdate method was compared with the official volumetric method by 11 collaborators in 217 duplicated determinations of total and citrate-insoluble phosphorus in one sample each of triple superphosphate, mixed fertilizer, and organic material. Solutions of the samples were prepared by official procedures and by nitric-perchloric acid digestion.

In agreement with the results of the previous study, the values reported by the individual collaborators were usually lower with the photometric method than with the volumetric method. For all the samples, the values for total phosphorus by the photometric method were lower in 88 per cent of the 66 individual comparisons comprising the main portion of the study. The photometric method gave lower values for citrate-insoluble phosphorus in 63 per cent of the comparisons.

For all comparisons, the average values for total phosphorus by the photometric method were lower than those by the volumetric method on each of the samples, and the differences were statistically significant at the 1 per cent level. On the other hand, the differences between the average values for citrate-insoluble phosphorus were not significant with any of the samples.

Precision among the duplicate determinations by the individual collaborators—generally good with both methods—was better with the photometric method, but both methods showed wide ranges in the average results among the collaborators.

With proper conditions for the determination, the type and make of the photometer do not appear to be important considerations in the photometric method.

Digestion with nitric-perchloric acids is a suitable procedure for preparing solutions of fertilizers for analysis by the photometric method.

It is recommended* that further study be made of the factors that may contribute to the differences between the results by the photometric and volumetric methods.

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* For report of Subcommittee A and action of the Association, see *This Journal*, **40**, 21, 22 (1957).

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REPORT ON PHOSPHORUS IN FERTILIZERS

III. MECHANICAL ANALYSIS OF PHOSPHATE ROCK

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The report[1] on this subject presented at the 1955 meeting of the Association showed that in the determination of the fractions of two samples of ground Florida pebble phosphate rock passing 100-mesh and 200-mesh sieves, respectively, the results obtained by a combination of wet and dry sieving were generally much more accurate and precise than those by dry sieving alone. In further study, the results of which are given in this report, the investigation was extended to other types of raw mineral phosphates marketed for direct application to the soil.

The four samples used in the work were from commercial materials sold for direct application. Except for thorough mixing, they were not subjected to further manipulation.

[1] HAVEN, H. A., and JACOB, K. D., *This Journal*, 39, 589 (1956).

Sample 1: Florida pebble phosphate rock containing 34.76 per cent total P_2O_5 and 0.56 per cent moisture. This sample was from the material designated as No. 31 in the previous report.

Sample 2: Florida soft phosphate with colloidal clay,[2] containing 20.64 per cent total P_2O_5 and 6.33 per cent moisture. This material, which is marketed under several trade names, consists essentially of comparatively coarse aggregates of loosely cohering particles of finely divided phosphates and clay.[3]

Sample 3: Tennessee brown-rock phosphate containing 29.98 per cent total P_2O_5 and 0.48 per cent moisture.

Sample 4: Wyoming phosphate rock containing 31.01 per cent total P_2O_5 and 0.33 per cent moisture. Material from "hanging-wall" bed ("A" bed) of deposit at Leefe.

INSTRUCTIONS TO COLLABORATORS

Mix the sample thoroughly before weighing the portions for analysis. Make all determinations in triplicate, and report the individual results on the form enclosed with these instructions. If for any reason a determination is repeated, the repetition should be made in triplicate, and the three results reported should be those obtained in simultaneous replications. Make all weighings to the nearest 0.1 g. Use 100-mesh sieves (0.147 mm opening) and 200-mesh sieves (0.074 mm opening) of the Tyler Standard series, matched and checked against Tyler or National Bureau of Standards certified sieves, where practicable. If such comparisons are made, please note the fact in your report. Use the same sieves in all the determinations. The sieves may be either half-height (1″ depth to sieve cloth) or full-height (2″ depth to sieve cloth). Make all dry sievings with 8″ diameter sieves. Sieves of this diameter are suggested, but not required, for the wet sievings.

Make all dry-sieving determinations with a Tyler Ro-Tap Testing Sieve Shaker or other mechanical shaking device. To provide information on the performance of different types of shakers, it is requested that determinations be made with more than one kind of machine if available. For the triplicated determinations on each sample and by each method, place the set of 100-mesh, and 200-mesh sieves in the machine in the extreme top, the middle, and the extreme bottom positions, respectively, with intervening fillers if necessary. Thus, a determination in triplicate will consist of only one test in each of the three positions. Identify the position of each set of sieves with the corresponding analytical results in your report, in this order: top, middle, bottom. There is evidence that the efficiency of the dry sieving operation may be influenced by the position of the sieve in the shaker. The use of half-height sieves in the Ro-Tap machine permits the triplicate determinations to be made simultaneously, but this is not possible with full-height sieves.

Do not supplement the action of the shaker by brushing the sieve, or in other ways.

METHOD A—DRY SIEVING

Transfer 100 g sample to 100-mesh sieve in series with 200-mesh sieve of the same height and diameter. Shake the set of sieves in a mechanical shaker for 20 min. Estimate the percentage of the sample passing the 100-mesh sieve by subtracting from 100 the weight of material retained on the sieve. Likewise, estimate the per-

[2] *Assoc. Am. Fertilizer Control Officials, Official Pub.*, 9, 16 (1955).
[3] HILL, W. L., JACOB, K. D., ALEXANDER, L. T., and MARSHALL, H. L., *Ind. Eng. Chem.*, 22, 1392 (1930).

centage of the sample passing the 200-mesh sieve by subtracting from 100 the sum of the weights of material retained on this sieve and on the 100-mesh sieve.

METHOD B—WET AND DRY SIEVING

For washing the sample with water, assemble an apparatus, comprising pipe, valve, pressure gauge, and water aerator (such as Fisher aerator No. 14-551), of the kind illustrated in the W. S. Tyler Catalog 53, 1952 and 1955 editions, page 47.

Samples 1, 3, and 4: Transfer 100 g to a 200-mesh sieve having a depth of at least 2″ to the sieve cloth. Wash with a moderate stream of water at a maximum pressure of 4 lb (gauge pressure) until the water passing through the sieve is clear, with care to avoid loss of the sample by splashing. Dry the material remaining on the sieve at 105° C and transfer to a 100-mesh sieve in series with a 200-mesh sieve of the same height and diameter. Shake the set of sieves by means of a mechanical shaker for 8 min. Estimate the percentages of the sample passing the 100-mesh and 200-mesh sieves, respectively, in the same manner as in Method A.

Sample 2: Prepare a solution containing 36 g sodium hexametaphosphate and 7.9 g sodium carbonate per liter. Transfer 50 ml of this solution and 450 ml water to a 1 liter beaker. Slowly add 100 g of the sample to the rapidly stirred solution with care to avoid contact of the unwetted material with the shaft of the stirrer and the side of the beaker, and continue stirring for 5 min. after all of the sample has been added. Transfer the slurry to a 200-mesh sieve and proceed as previously described.

NOTES ON THE PROCEDURES

All the dry sievings were made with sieves 8 inches in diameter. Collaborators 1, 6, 8, and 10 used sieves 2 inches in depth to the sieve cloth, Collaborator 9 used both 1-inch and 2-inch sieves, and the others used 1-inch sieves.

Sieves 8 inches in diameter were used for all the wet sievings except the 4-inch sieves used by Collaborator 2. Collaborators 2 and 3 used sieves 4 inches in depth to the sieve cloth, Collaborator 7 used 1-inch sieves, and the others used 2-inch sieves. Stainless steel 250-mesh sieves were used for the wet sievings by Collaborator 2 to insure against the possibility of 200-mesh particles being forced through the sieves by the water pressure. This collaborator pointed out that sieve cloth made of stainless steel is much more durable than that made of bronze.

For dry sieving, the 200-mesh sieves used by Collaborator 4 and the 100-mesh and 200-mesh sieves used by Collaborator 5 were checked against National Bureau of Standards certified sieves. All the determinations by Collaborator 9 were made with matched sieves, and those by Collaborator 10 were made with calibrated sieves.

Dry sievings were made by all the collaborators with the aid of the Ro-Tap Testing Sieve Shaker manufactured by the W. S. Tyler Company, Cleveland, Ohio. Collaborators 1 and 10 also used the Syntron Electric Vibrator manufactured by the Syntron Company, Homer City, Pa.

Wet sieving of the soft phosphate with colloidal clay (Sample 2) was facilitated greatly by prior dispersion of the material with a solution of sodium hexametaphosphate and sodium carbonate, as in the mechanical analysis of soils.[4] Without the dispersing agent the results were low and erratic.

All the results by dry sieving alone were obtained with a shaking period of 20 minutes. For dry sieving after wet sieving, the shaking period was 8 minutes in all cases.

RESULTS

The averages of the individual collaborator's results on each of the four

[4] KILMER, V. J., and ALEXANDER, L. T., *Soil Sci.*, 68, 15 (1949).

samples by the two methods of mechanical analysis are shown in Table 1; also given are the differences in the average results by dry sieving alone (Method A) and those by combination of wet and dry sieving (Method B), as well as the individual differences between the high and low results by each collaborator. The results are summarized in Table 2.

With Method B the average values reported by the individual collaborators generally were considerably higher than those with Method A. Agreement in the high and low results of replicated determinations was much poorer with Method A. In both of these respects, the differences in the results by the two methods were generally greater for the finer fraction (−200 mesh) of each sample. The data, like those of the previous study, provide further evidence of the superiority of a combination of wet and dry sieving over dry sieving alone in both the precision and the accuracy of the results.

With Method A the soft phosphate with colloidal clay (Sample 2) showed relatively little tendency to clog the sieves, and the precision of the results was generally very much better than for the other samples. Also, the differences in the average results by Method A and Method B were generally much higher and more uniform than those for Samples 1, 3, and 4. These data reflect the stability of the coarse aggregates of fine particles in Sample 2 under the conditions of Method A, as well as the effectiveness of the dispersing agent in breaking down the aggregates with Method B.

Another sample of commercial Florida soft phosphate with colloidal clay, from an unknown producer, gave results close to those obtained on Sample 2 with the respective methods of mechanical analysis. As with Sample 2, the wet sieving operation in Method B was facilitated greatly by the dispersing agent.

Collaborators 1 and 10 compared the performance of the Ro-Tap and Syntron machines under the conditions of Method A as outlined in the instructions to collaborators. Collaborator 1 obtained better precision in his results of replicate determinations with the Ro-Tap than with the Syntron machine, whereas Collaborator 10 usually obtained better precision with the Syntron machine (Table 3). In most of the comparisons by both collaborators the average results by Method A with the Syntron machine were closer to, but still considerably below, the averages by Method B than were those with the Ro-Tap. The agreement of Collaborator 10's results for the fraction passing the 100-mesh sieve was especially good. In additional determinations by Collaborator 10, not shown in Table 3, comparison of the two machines in the dry sieving operation in Method B indicated that the Syntron shaker may yield somewhat higher values for the fraction passing the 200-mesh sieve.

The previous study indicated that the position of the sieve in the Ro-Tap machine may influence markedly the results obtained by Method A (dry sieving alone). The highest values usually were obtained when the sieve was placed in the extreme top of the machine, whereas the trend was

TABLE 1.—*Percentage fractions of phosphate rock as determined by two methods of mechanical analysis*[a]

COLLABORATOR	THROUGH 100 MESH				THROUGH 200 MESH				DIFFERENCE IN AVERAGE RESULTS BY METHOD A AND METHOD B	
	AVERAGE[b] BY METHOD:		DIFFERENCE[c] BY METHOD:		AVERAGE[b] BY METHOD:		DIFFERENCE[c] BY METHOD:			
	A	B	A	B	A	B	A	B	100 MESH	200 MESH
Sample 1: Florida Pebble Phosphate										
1	97.5	99.0	1.2	0.3	80.7	88.2	3.6	0.7	1.5	7.5
2	86.5	98.5	24.9	0.2	63.5	88.1	25.5	1.3	12.0	24.6
3	90.3	98.5	10.8	0.3	61.0	87.0	23.8	0.3	8.2	26.0
4	88.6	98.6	11.9	0.1	55.6	87.0	20.8	0.5	10.0	31.4
5	94.2	98.2	3.3	0.1	74.1	87.9	19.4	0.9	4.0	13.8
6	95.4	98.6	3.7	0.1	65.7	85.4	5.8	0.5	3.2	19.7
7	97.2	98.5	0.7	0.4	77.8	88.4	3.8	0.4	1.3	10.6
8	86.7	98.0	28.1	0.2	70.2	88.0	19.4	0.2	11.3	17.8
9	94.5	98.0	4.6	0.0	74.8	86.5	2.7	0.2	3.5	11.7
10[d]	95.1	98.1	6.2	0.2	54.4	84.0	21.7	2.0	3.0	29.6
Sample 2: Florida Soft Phosphate with Colloidal Clay										
1	40.5	97.1	1.3	0.4	26.1	94.3	0.8	0.2	56.6	68.2
2	39.4	96.7	2.0	0.4	25.4	94.2	1.3	0.0	57.3	68.8
3	38.4	96.2	0.7	0.2	24.0	93.4	1.1	0.4	57.8	69.4
4	42.5	96.5	1.1	0.1	27.1	93.9	1.4	0.1	54.0	66.8
5	40.7	95.5	4.4	0.9	25.7	93.0	3.2	0.7	54.8	67.3
6	42.4	96.4	0.8	0.1	27.3	93.6	0.3	0.1	54.0	66.3
7	39.6	96.8	1.0	0.2	26.4	94.8	0.8	0.0	57.2	68.4
8	39.4	95.1	0.8	0.1	26.8	91.6	0.7	0.3	55.7	64.8
9	43.2	96.6	1.0	0.2	29.5	93.6	1.0	0.2	53.4	64.1
10[d]	40.9	95.5	0.2	1.0	25.7	92.7	1.0	1.5	54.6	67.0
Sample 3: Tennessee Brown-Rock Phosphate										
1	87.1	99.7	7.7	0.1	65.9	92.8	8.2	1.2	12.6	26.9
2	60.4	99.5	63.2	0.0	35.2	93.8	48.4	2.1	39.1	58.6
3	78.5	99.5	32.1	0.1	40.8	92.5	26.3	0.1	21.0	51.7
4	41.7	99.6	22.1	0.2	9.6	92.8	2.1	0.5	57.9	83.2
5	92.8	99.4	10.3	0.1	73.9	93.0	9.6	0.4	6.6	19.1
6	60.7	99.6	48.4	0.1	46.6	90.6	28.7	0.1	38.9	44.0
7	86.1	99.6	22.3	0.2	55.5	93.3	51.6	0.5	13.5	37.8
8	85.7	99.2	29.6	0.0	40.3	92.8	10.4	0.2	13.5	52.5
9	81.2	99.5	28.5	0.0	62.5	91.8	22.5	0.5	18.3	29.3
10[d]	95.9	99.5	3.5	0.0	44.9	89.5	2.7	4.0	3.6	44.6
Sample 4: Wyoming Phosphate										
1	84.9	96.4	6.0	0.5	59.4	77.6	7.1	0.5	11.5	18.2
2	78.5	95.9	29.4	0.3	47.5	80.2	25.1	3.2	17.4	32.7
3	67.5	95.6	30.2	0.3	48.3	78.1	27.2	0.4	28.1	29.8
4	78.6	95.5	32.7	0.1	35.0	78.9	44.4	0.4	16.9	43.9
5	83.8	95.5	8.4	0.2	61.8	79.6	14.9	1.5	11.7	17.8
6	86.6	96.6	12.9	0.2	40.8	79.1	21.3	0.4	10.0	38.3
7	92.2	95.3	4.0	0.5	64.3	85.4	4.2	0.3	3.1	21.1
8	63.5	94.4	35.8	0.3	48.8	78.6	7.4	0.8	30.9	29.8
9	82.2	95.0	19.5	0.5	58.5	78.9	21.4	0.4	12.8	20.4
10[d]	91.9	96.2	4.6	0.6	51.2	74.2	0.8	1.3	4.3	23.0

[a] Method A, dry sieving in Ro-Tap machine. Method B, combination of wet and dry sieving. 100-gram samples.
[b] Average of triplicate determinations, except as indicated otherwise.
[c] Difference in high and low results of triplicate determinations, except as indicated otherwise.
[d] Duplicate determinations with sieves in top and bottom positions in the machine.

TABLE 2.—*Summary of results for percentage fractions of phosphate rock as determined by two methods of mechanical analysis*[a]

SAMPLE	FRACTION, MESH	AVERAGE RESULTS				DIFFERENCE IN AVERAGE RESULTS BY METHOD A AND METHOD B		DIFFERENCE IN HIGH AND LOW RESULTS OF TRIPLICATE DETERMINATIONS			
		RANGE[b] BY METHOD:		AVERAGE BY METHOD:				RANGE BY METHOD:		AVERAGE BY METHOD:	
		A	B	A	B	RANGE[b]	AVERAGE	A	B	A	B
1	−100	86.5–97.5	98.0–99.0	92.3	98.4	1.3–12.0	6.1	0.7–28.1	0.0–0.4	9.9	0.2
	−200	55.6–80.7	85.4–88.4	69.3	87.4	7.5–31.4	18.1	2.7–25.5	0.2–1.3	13.9	0.6
2	−100	38.4–43.2	95.1–97.1	40.7	96.3	53.4–57.8	55.6	0.7– 4.4	0.1–0.9	1.5	0.3
	−200	24.0–29.5	91.6–94.8	26.5	93.6	64.1–69.4	67.1	0.3– 3.2	0.0–0.7	1.2	0.2
3	−100	41.7–92.8	99.2–99.7	74.9	99.5	6.6–57.9	24.6	7.7–63.2	0.0–0.2	29.4	0.1
	−200	9.6–73.9	90.6–93.8	47.8	92.6	19.1–83.2	44.8	2.1–51.6	0.1–2.1	23.1	0.6
4	−100	63.5–92.2	94.4–96.6	79.8	95.6	3.1–30.9	15.8	4.0–35.8	0.1–0.5	19.9	0.3
	−200	35.0–64.3	77.6–85.4	51.6	79.6	17.8–43.9	28.0	4.2–44.4	0.3–3.2	19.2	0.9

[a] Excluding determinations by Collaborator 10, which were made in duplicate. Method A, dry sieving in Ro-Tap machine. Method B, combination of wet and dry sieving. 100-gram samples.

[b] Range of averages of triplicate determinations.

TABLE 3.—*Percentage fractions of phosphate rock as determined with Ro-Tap and Syntron machines by dry sieving alone*

FRACTION, MESH	COLLABORATOR[a]	METHOD A[b]				METHOD B,[c] AVERAGE
		DIFFERENCE[d]		AVERAGE[e]		
		RO-TAP	SYNTRON	RO-TAP	SYNTRON	
Sample 1						
−100	1	1.2	1.7	97.5	97.7	99.0
	10	6.2	0.2	95.1	98.1	98.1
−200	1	3.6	8.4	80.7	78.5	88.2
	10	21.7	0.3	54.4	80.4	84.0
Sample 3						
−100	1	11.1	24.0	55.1	78.5	99.7
	10	3.5	0.1	95.9	99.1	99.5
−200	1	7.4	16.0	32.5	53.9	92.8
	10	2.7	0.5	44.9	80.8	89.5
Sample 4						
−100	1	6.1	13.6	81.9	74.3	96.4
	10	4.6	0.1	91.9	95.1	96.2
−200	1	7.7	12.0	41.8	46.2	77.6
	10	0.9	3.0	51.2	69.0	74.2

[a] Triplicate determinations by Collaborator 1 with sieves placed respectively in top, middle, and bottom positions in the machines; the determinations on Sample 1 with the Ro-Tap machine were made about 15 weeks earlier than the other determinations by Collaborator 1 reported in this table. Duplicate determinations by Collaborator 10 with sieves in top and bottom positions in the machines.
[b] Dry sieving only. 100-gram samples.
[c] Combination of wet and dry sieving, with dry sieving in Ro-Tap machine. 100-gram samples. Results from Table 1.
[d] Difference in high and low results of replicate determinations.
[e] Average of replicate determinations.

to the lowest values with the sieve in the extreme bottom position. Further evidence of the influence of sieve position is given in Table 4. Thus, the highest result was obtained in 98 per cent of the triplicate determinations on Samples 1, 3, and 4 when the sieve was in the top position. Likewise, the lowest result was obtained in 70 per cent of the determinations with the sieve in the bottom position. Furthermore, the average percentages of material passing either the 100-mesh or 200-mesh sieve were much higher with the top than with the bottom sieves.

In agreement with the previous study, the results obtained with the Ro-Tap machine in Method B were not influenced by the position of the sieve in the shaker. As reported by Collaborators 1 and 10, there was no

TABLE 4.—*Effect of position of sieve in Ro-Tap machine on results obtained in analysis of samples 1, 3, and 4 by dry sieving alone*[a]

POSITION OF SIEVE IN MACHINE	DETERMINATIONS[b] RANKING:			PORTION THROUGH:		
	HIGH	MEDIUM	LOW	100 MESH[c]	200 MESH[c]	AVERAGE[d]
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Top	98	0	2	92.5	65.8	79.2
Middle	0	72	28	79.4	53.6	66.5
Bottom	2	28	70	75.1	49.2	62.2

[a] Based on the data of Table 1. Excluding determinations by Collaborator 10, which were made in duplicate with sieves in top and bottom positions only.
[b] 54 determinations in each position, 27 each with 100-mesh and 200-mesh sieves.
[c] Average of 27 determinations in each position.
[d] Average of results with 100-mesh and 200-mesh sieves.

relation between the position of the sieve in the Syntron shaker and the results by either Method A or Method B.

The data of Table 5 indicate that closer agreement between the results by the two procedures can be obtained by reducing the weight of the sample used with the Ro-Tap machine in Method A (dry sieving alone). Even with a 25 gram sample, however, the values by the latter method usually were substantially lower than those by Method B (combination of wet and dry sieving), especially in the case of the 200-mesh fraction. All the determinations were made with sieves 8 inches in diameter and 2 inches in depth, and the shaking periods in Methods A and B were 20 minutes and 8 minutes, respectively. Similar tests were not made with the Syntron machine.

TABLE 5.—*Effect of sample weight on percentage fractions of phosphate rock as determined with Ro-Tap machine by dry sieving alone*

SAMPLE	FRACTION, MESH	METHOD A[a]						METHOD B,[b] AVERAGE
		DIFFERENCE[c] WITH SAMPLE WEIGHT OF:			AVERAGE[d] WITH SAMPLE WEIGHT OF:			
		25 G	50 G	100 G	25 G	50 G	100 G	
1	−100	0.7	0.2	1.2	97.8	98.1	97.5	99.0
	−200	1.5	1.0	3.6	83.2	83.6	80.7	88.2
3	−100	7.0	13.4	7.7	95.0	90.8	87.1	99.7
	−200	11.9	9.2	8.2	78.4	70.3	65.9	92.8
4	−100	2.6	2.5	6.0	94.1	94.5	84.9	96.4
	−200	6.6	8.2	7.1	68.4	64.2	59.4	77.6

[a] Dry sieving only.
[b] Combination of wet and dry sieving, with dry sieving in Ro-Tap machine. Triplicate determinations. 100-gram samples.
[c] Difference in high and low results of triplicate determinations.
[d] Average of triplicate determinations.

TABLE 6.—*Percentage fractions of Florida pebble phosphate rock No. 30 as determined by two methods of mechanical analysis*[a]

FRACTION, MESH	DIFFERENCE[b] WITH METHOD:		AVERAGE[c] WITH METHOD:		DIFFERENCE IN AVERAGE RESULTS BY METHODS A AND B
	A	B	A	B	
−60	0.1	0.1	96.1	96.4	0.3
−80	2.2	0.2	85.2	87.4	2.2
−100	3.6	0.3	76.6	79.9	3.3
−200	2.6	0.3	49.6	54.0	4.4

[a] Method A, dry sieving in Ro-Tap machine. Method B, combination of wet and dry sieving. 100-gram samples.
[b] Difference in high and low results of triplicate determinations.
[c] Average of triplicate determinations.

To obtain information on the performance of the methods on sieve fractions coarser than 100 mesh, determinations were made on Florida pebble phosphate rock No. 30. This coarsely ground material was one of the two samples used in the previous study. The diameter and depth of the sieves and the duration of the shaking were those stated in the preceding paragraph. The results by the two methods (Table 6) were in progressively better agreement as the sieve aperture increased, and they were substantially equal for the fraction passing the 60-mesh sieve. It appears, therefore, that Method A gives accurate values for the 60-mesh and coarser fractions of ground phosphate rock.

As previously pointed out, wet sieving of such materials as soft phosphate with colloidal clay is greatly facilitated and the precision and accuracy of the results are considerably improved by the use of a dispersing agent (sodium hexametaphosphate and sodium carbonate) in the manner outlined in Method B. It was found, however, that with this method the dispersing agent is of little or no benefit in the wet sieving of ground phosphate rock. Thus, the dispersing agent had practically no effect on the values for the 100-mesh and 200-mesh fractions in Samples 1, 3, and 4 (Table 7).

DISCUSSION

The data presented in this report confirm those of the previous study in showing the generally much greater precision and accuracy of a combination of wet and dry sieving over dry sieving alone for the mechanical analysis of raw mineral phosphate marketed for direct application to the soil. Since in these studies the action of the mechanical shaker was not supplemented by brushing the sieve or in other ways, the poor results with dry sieving can be attributed to the tendency of the finely divided particles to clog the sieve and to coalesce into aggregates that resist the parting action of the machine.

With the dry sieving method it is a common practice to supplement the

TABLE 7.—*Effect of dispersing agent on percentage fractions of phosphate rock as determined by combination of wet and dry sieving*[a]

SAMPLE	FRACTION, MESH	DIFFERENCE[b] WITH DISPERSING AGENT:		AVERAGE[c] WITH DISPERSING AGENT:	
		ABSENT	PRESENT	ABSENT	PRESENT
1	−100	0.3	0.1	99.0	99.0
	−200	0.7	0.1	88.2	88.3
2	−100	1.1	0.4	89.5	97.1
	−200	0.8	0.2	82.9	94.3
3	−100	0.1	0.1	99.7	99.7
	−200	1.2	1.1	92.8	92.5
4	−100	0.5	0.2	96.4	96.2
	−200	0.5	0.8	77.6	77.6

[a] Dry sieving with Ro-Tap machine. Dispersing agent, sodium hexametaphosphate and sodium carbonate. 100-gram samples.
[b] Difference in high and low results of triplicate determinations.
[c] Average of triplicate determinations.

action of the shaking machine by brushing the sieve to break down the aggregates and clear the apertures. The conditions requisite to complete separation of the sample into the desired particle-size fractions cannot be defined precisely, however, and the technique and efficiency of the brushing operation are highly subject to variation among analysts and among determinations by the same analyst. The pitfalls are avoided by first wet sieving the sample to remove most of the fine material and then dry sieving the moisture-free residue.

SUMMARY

Further study was made of methods for the mechanical analysis of phosphate rock, in which dry sieving alone was compared with combination of wet and dry sieving by 10 collaborators in 160 replicated analyses (chiefly in triplicate) of 1 sample each of ground phosphate rock from Florida, Tennessee, and Wyoming and a sample of Florida soft phosphate with colloidal clay. The dry sievings were done with mechanical shakers.

In agreement with the results of the previous study, the percentages of material passing the 100-mesh and 200-mesh sieves with the combination of wet and dry sieving usually were considerably higher than those with dry sieving alone, and the precision among the replicates of a determination was generally much poorer with the latter method.

Wet sieving of the Florida soft phosphate with colloidal clay was facilitated and the precision and accuracy of the results were improved by the use of a dispersing agent consisting of sodium hexametaphosphate and sodium carbonate, but the dispersing agent was of practically no benefit in the wet sieving of ground phosphate rock.

In comparisons by two collaborators, the trend in the results by dry sieving alone was toward higher percentages of material passing the 100-mesh and 200-mesh sieves with the Syntron Electric Vibrator than with the Ro-Tap Testing Sieve Shaker.

As in the previous study, the position of the sieve in the Ro-Tap machine markedly influenced the results by dry sieving alone, but not those by the combination of wet and dry sieving. There appeared to be no definite relation between the position of the sieves in the shaker and the results obtained with the Syntron machine.

In tests with the Ro-Tap machine, the results by the dry sieving method were improved somewhat by decreasing the weight of the sample from 100 grams to 25 grams but not enough to bring them to the level of those by the combination wet and dry sieving method.

The results of this and the previous study point to the combination wet and dry sieving method as a suitable procedure for the mechanical analysis of raw mineral phosphates marketed for direct application to the soil.

ACKNOWLEDGMENT

Grateful acknowledgment is made to the International Minerals and Chemical Corp., Chicago, Ill., the San Francisco Chemical Co., Montpelier, Ida., and the Soil Builders, Inc., Dunnellon, Fla., for the samples of Tennessee and Wyoming phosphate rocks and Florida soft phosphate with colloidal clay, respectively; to the Association of Florida Phosphate Mining Chemists and the Association's secretary, H. A. Haven, for the sample of Florida pebble phosphate rock and for continued interest in this study; and to the following collaborators and their respective organizations for their fine cooperation (the alphabetical listing of the collaborators does not correspond with the numbered designations in this report):

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C. D. McDowall and G. D. French, Davison Chemical Co., Division of W. R. Grace and Co., Bartow, Fla.

J. H. Nunez, Virginia-Carolina Chemical Corp., Nichols, Fla.

E. M. Powell, American Cyanamid Co., Brewster, Fla.

Dick Read, Illinois Farm Supply Co., East St. Louis, Ill.

W. N. Simpson and J. F. Kilpatrick, The American Agricultural Chemical Co., Pierce, Fla.

B. F. Turner, R. S. Hedden, and H. C. Auman, International Minerals & Chemical Corp., Bartow, Fla.

D. B. Underhill and S. E. Shearin, Coronet Phosphate Co., Plant City, Fla.

RECOMMENDATION

It is recommended* that the following method for the mechanical anal-

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 21, 22 (1957).

ysis of phosphate rock be adopted as first action and that collaborative study of the subject be discontinued:

MECHANICAL ANALYSIS OF PHOSPHATE ROCK

APPARATUS

(a) *Water pressure control.*—*See* Fig. 1. Connect valve, *A*, std pressure gauge, *B*, and aerator, *C*, with 3/8″ diam. pipe.

(b) *Sieves.*—Nos. 100 and 200, bronze or stainless steel cloth, calibrated against certified sieves. Sieves 8″ diam. and 2″ in depth to sieve cloth are recommended for both wet and dry sieving, but other sizes may be used if detd to be suitable under conditions of method. (Other sieves in U. S. series may be used, with precaution to insure complete sepn of sample into desired fractions.)

(c) *Sieve shaker.*—Ro-Tap, Syntron, or other suitable machine.

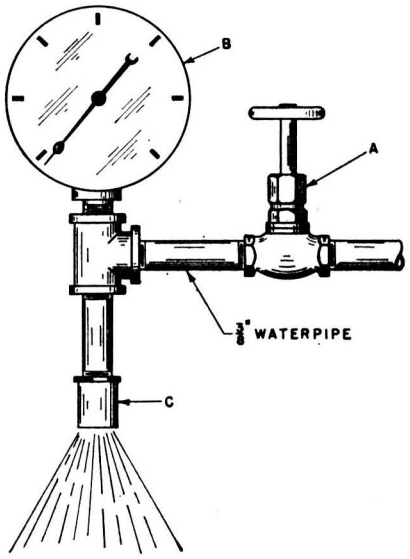

FIG. 1.—Apparatus for control of water pressure

REAGENT

Dispersing agent.—Dissolve 36 g Na hexametaphosphate and 8 g Na_2CO_3 in H_2O and dil. to 1 l.

DETERMINATION

(a) *Ground phosphate rock.*—Place 100 g sample on No. 200 sieve and wash with moderate stream of tap H_2O at max. gauge pressure of 4 lb until H_2O passing sieve is clear, with care to avoid loss of sample by splashing. Dry material remaining on sieve at 105° and transfer to No. 100 sieve in series with No. 200 sieve of same diam. and depth. Shake 8 min. in mechanical shaker. Det. % sample passing No. 100 sieve by subtracting wt of material retained on that sieve from 100. Det. % sample passing No. 200 sieve by subtracting sum of wts of material retained on that sieve and on No. 100 sieve from 100.

(b) *Soft phosphate with colloidal clay.*—Add 100 g sample to rapidly stirred soln of 50 ml dispersing agent and 450 ml tap H_2O, with care to avoid contact of unwetted material with shaft of stirrer and side of beaker. Stir 5 min. after addn of sample is completed. Transfer slurry to No. 200 sieve and proceed as in (a).

REPORT ON POTASH IN FERTILIZERS

By O. W. FORD (Purdue Agricultural Experiment Station, Lafayette, Ind.), *Associate Referee*

The flame photometric procedure for potash in fertilizers developed by Gehrke, *et al.* (*J. Agr. and Food Chem.*, **3**, 48 (1955)) was selected for the 1956 collaborative study. In this procedure, interfering anions are re-

moved from the sample solution by means of an exchange resin. The procedure appeared to offer the following distinct advantages over the flame photometric methods studied collaboratively in the past:

(1) Anion interference is eliminated.

(2) Because the total salt content of the atomized solution is much less than where "radiation buffers" are used, atomizer performance is more efficient.

(3) The slightly acid condition of the aspirated solution (pH 5) helps keep the atomizer clean.

(4) Magnesium interference is eliminated because magnesium ions are completely removed during preparation of the sample solution.

Eight laboratories agreed to participate in the study. These laboratories were sent samples representative of the several types normally encountered in fertilizer laboratories: the potash content ranged from 5 to 63 per cent.

Shortly after the study began, the manufacturers of the exchange resin specified in the procedure announced that they were discontinuing production of the resin. A number of the collaborators had already secured the resin or could obtain enough from remaining stocks to complete the work this year. However, since the resin is no longer available, the flame photometric procedure studied this year cannot be considered for adoption as an official method. Therefore the details of the method have not been included in this report.

The work of the collaborators was still valuable, however. The ion exchange treatment had been criticized because of the extra time required; such criticism would not be valid if this step increased the precision of the determination. Therefore, the data were analyzed statistically to determine whether removing the interfering anions improved the precision. The coefficient of variation was calculated and compared with values obtained by other flame photometric methods and by the official gravimetric method.

In a 1954 collaborative study of flame photometric methods, anion interferences were handled by adding known amounts of these ions to both the standards and samples to bring the concentration up to the "plateau level." By chance, the compositions of the collaborative samples in 1954 and 1956 were quite similar, and results could be compared. The data (Table 1) indicate quite clearly that removing the interfering anions increases the precision to the point where it equals the precision of the gravimetric method, as the following shows:

(1) In the 1954 study, the coefficient of variation for 6 out of 7 samples was larger by the flame procedure than by the gravimetric method.

(2) In the 1956 study, the coefficient of variation for 5 out of 8 samples was smaller by the flame procedure than by the gravimetric method.

(3) The average coefficient of variation for the 1956 flame method was

TABLE 1.—*Comparison of 1954 and 1956 A.O.A.C. collaborative results*

APPROX. LEVEL OF POTASH	COEFFICIENT OF VARIATION			
	1954		1956	
	GRAVIMETRIC	FLAME	GRAVIMETRIC	FLAME
per cent				
6	2.48	2.33	1.72	3.20
10	2.19	2.38	2.03	1.38
16	1.70	1.88	1.83	1.52
17	1.67	2.56	3.02	2.36
30	1.24	1.41	1.67	1.12
63	0.95	1.30	0.95	1.24
12	1.47	2.09	—	—
20	—	—	1.72	1.66
23	—	—	1.38	1.47
Average	1.67	1.99	1.79	1.74

very close to that obtained for the gravimetric method in both 1954 and 1956; it was lower than the 1954 flame value (the average values are based upon approximately 500 individual determinations in 1956 and approximately 700 in 1954).

The question then arose as to what average coefficient of variation could be expected if the gravimetric method was used in a number of laboratories over a period of several years. In addition to the two years already reported, collaborative data were also available from 1955. Another source of data was the monthly Magruder check sample results. The information was calculated for these samples from December 1953 to July 1954 because the check samples of that period were similar to those of the collaborative studies: they covered the range up to 30 per cent K_2O.

The results are summarized in Table 2 and indicate that the normal average coefficient of variation in the gravimetric analysis of all types of samples is about 1.7–1.8. It is of interest that the value obtained by the flame photometric method fell within this range for the first time this year. Apparently the additional step required to remove interfering anions by an exchange resin is justified by the increased precision. In the future, however, a method should include several choices of resins.

RECOMMENDATIONS

It is recommended*—

(1) That collaborative study be continued on the flame photometric procedure of determining potash in fertilizers, employing anion exchange resins.

* For report of Subcommittee A and action of the Association, see *This Journal*, **40**, 21, 22 (1957).

TABLE 2.—*Average coefficient of variation by years*

DATE AND SOURCE OF DATA	AVERAGE COEFFICIENT OF VARIATION	
	GRAVIMETRIC METHOD	FLAME METHOD
Magruder 1953–54	1.79	2.10
A.O.A.C. collaborative samples:		
1954	1.67	1.99
1955	1.78	—
1956	1.79	1.74

(2) That the volumetric determination of potash in fertilizers by the sodium tetraphenylboron procedure be studied collaboratively.

ACKNOWLEDGMENT

The Associate Referee wishes to express his thanks to the many collaborators for their fine cooperation and to F. W. Quackenbush and E. D. Schall for their suggestions and criticisms in the development of this report.

No reports were given on acid- and base-forming quality, boron, copper and zinc, free water, inert materials, or sampling and preparation of sample of fertilizers.

REPORT ON SOIL CONDITIONERS

By LYLE T. ALEXANDER (Soil Survey Laboratory, Soil Conservation Service, U.S. Department of Agriculture, Plant Industry Station, Beltsville, Md.), *Referee*

Associate Referee Toth has reported on Performance of Soil Conditioners and Associate Referee Roth on Chemical Analysis of Soil Conditioners. It has been impossible to carry out an active program of work on this subject because there is very little sale of soil conditioners at the present time, and consequently there is little interest in control methods.

It is recommended* that the entire topic of soil conditioners be discontinued. If the question should again become active, a new Referee and Associate Referees could be appointed. However, if the Association feels that the topic should be kept on a standby basis, the Referee has no objection.

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* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 24 (1957).

REPORT ON METHODS FOR EVALUATING THE EFFECTIVENESS OF SOIL CONDITIONER CHEMICALS

By S. J. Toth (New Jersey Agricultural Experiment Station, Rutgers University, New Brunswick, N.J.), *Associate Referee*

Methods for determining the effectiveness of soil conditioner chemicals have been under study for the past three years. In 1953 excellent response was obtained from a number of collaborators in evaluating methods. Since this time, however, the study has been completely inactive for the following reasons:

(1) Too few investigators have responded to requests for a collaborative study, and the Associate Referee feels that any additional evaluation would not reflect a true cross-section of the opinions of the workers in this field.

(2) The literature dealing with the effectiveness of various soil conditioning chemicals is sufficient to warrant the conclusion that little difference exists between the effectiveness of the various products now being marketed.

(3) The present high cost of soil conditioning chemicals, plus their small volume of sale during the past two years, precludes any large-scale agricultural use of these products.

It is therefore recommended* that evaluation of the effectiveness of soil conditioning chemicals be discontinued as of 1956 and not be renewed until these products reach large-scale agricultural use.

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 24 (1957).

REPORT ON CHEMICAL ANALYSIS OF SOIL CONDITIONERS

By Fred J. Roth (Bureau of Chemistry, State Department of Agriculture, Sacramento 14, Calif.), *Associate Referee*

It has not been possible to carry out any work on the chemical analysis of soil conditioners during the past year. Apparently interest in synthetic soil conditioners has dropped from the high level of several years ago, although there is still interest in various materials for conditioning soils. These materials all require individual techniques for evaluation. In general, chemical analysis has not been successful for this evaluation. It is suggested that any future studies on this subject could well be reported in contributed papers.

It is recommended* that the study of chemical analysis of soil conditioners be discontinued.

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 24 (1957).

REPORT ON ZINC AND COPPER IN LIMESTONE

By JOHN G. A. FISKELL (Department of Soils, University of Florida, Gainesville, Fla.), *Associate Referee*

Methods were studied for the determination of zinc and copper in limestone and dolomite. The dithizone-carbamate method for zinc, developed by Holland and Ritchie (1) and modified by Cowling and Miller (2) for plant material, was selected for its sensitivity and specificity. Carbamate-EDTA and 2,2′-biquinoline, found by Cheng and Bray to be specific for copper in soil samples (3), were used as reagents.

The copper and zinc were separated from the calcium, magnesium, aluminum, and iron in the limestone samples by the anion exchanger Dowex-1 in a column at appropriate molarity of hydrochloric acid (4, 5). The main advantage of such a separation was to reduce the concentration of metal, other than copper or zinc, to be complexed by the reagents. Actually, adding more of the complexing citrate or EDTA reagent kept the sample in solution during the extraction into carbon tetrachloride.

Zinc and copper were determined colorimetrically in these samples with 2-carboxy-2′-hydroxy-5′-sulfoformazylbenzene (zincon) (5), but the determination was not successful, either with or without the anion exchange step; the interfering element was not identified, however.

Wet-digestion of the limestone samples with concentrated hydrochloric acid was found to be adequate. The zinc or copper values did not increase further when peroxide, aqua regia, or 1:1 nitric-perchloric acid or EDTA was added to the sample before it was evaporated to dryness. A correction was made for any zinc or copper in these reagents.

METHOD

REAGENTS

(a) *Hydrochloric acid.*—Prepare 3*N*, 1*N*, and 0.01*N* solutions of HCl, using glass-distilled H_2O for the dilutions. (Reagent grade concentrated HCl, Baker and Adamson, was used in this work and gave very low blanks for Cu and Zn.)

(b) *Ammonium hydroxide.*—Reagent grade NH_4OH usually contains less than 1 mmg Cu or Zn per 10 ml and is satisfactory without redistillation.

(c) *Dithizone.*—Dissolve 0.20 g diphenylthiocarbazone in 2 l CCl_4. Store in brown bottle in refrigerator. Purification of some reagent lots may be necessary.

(d) *Ammonium citrate.*—Dissolve 113 g NH_4 citrate in 1 l redistilled H_2O. Add concentrated NH_4OH to pH 8.7 and dilute to 2 l. Purify if necessary by shaking with dithizone, (c), and discarding CCl_4 phase.

(e) *Citrate-carbamate reagent.*—Prepare as in (d) but add 0.5 g Na diethyldithiocarbamate before diluting to 2 l.

(f) *Citrate-EDTA reagent.*—Weigh out 50 g ethylenediaminetetraacetic acid into a 1000 ml Pyrex beaker. Add 200 g NH_4 citrate and 500 ml H_2O distilled from glass. Add concentrated NH_4OH until completely dissolved and adjust pH to 8.5. Dilute to 1 l. Use acid form of EDTA, rather than sodium salt, for lower copper blanks.

(g) *Carbamate reagent.*—Dissolve 1 g disodium diethyldithiocarbamate in 100 ml H_2O.

(h) *Carbon tetrachloride.*—Reagent grade or redistilled.

(i) *Biquinoline reagent.*—Dissolve 0.2 g 2,2′-biquinoline in 800 ml hot iso-amyl alcohol, cool, and dilute to 1 l with the iso-amyl alcohol.

(j) *Sodium acetate solution.*—2*N*.

(k) *Anion exchange columns.*—Prepare Pyrex glass tubes about 30 cm long, tapered at bottom and flanged at top. Place 1 cm glass wool in bottom of tube and pour in slurry of 50–100 mesh Dowex-1 to 10 cm depth. Wash well with 3*N* HCl; then rinse with six 10 ml portions 0.01*N* HCl, and twice more with 3*N* HCl before adding sample solution.

(l) *Zinc standard solution.*—Prepare solution containing 1 mmg Zn/ml in 3*N* HCl by diluting stock solution containing 1 g pure Zn metal/100 ml 3*N* HCl.

(m) *Copper standard solution.*—Prepare solution containing 2 mmg Cu/ml by diluting stock solution containing 1 g pure Cu metal/100 ml 3*N* HCl.

PREPARATION OF SAMPLE

Grind sample of limestone in a mortar and pestle free of zinc or copper. Transfer to porcelain crucible and dry at 110°. Weigh 5, 3, and 1 g portions of the sample into 250 ml Erlenmeyer flasks. Add 5 ml concentrated HCl per g sample, heat slowly, and evaporate to dryness. Repeat. Add 75 ml 3*N* HCl; when completely dissolved, dilute to 100 ml in a volumetric flask, and shake well. Filter, using Whatman No. 32 paper and discard precipitate.

DETERMINATION OF ZINC

Pipet 25 ml aliquot of prepared sample solution into a 250 ml separatory funnel, add 40 ml reagent (d), and adjust pH to 8.5–9.0 with NH_4OH, using suitable external indicator such as pHydrion paper. Add 10 ml dithizone reagent, shake 1 min., and drain CCl_4 layer into second separatory funnel. Shake sample with few ml CCl_4 and transfer to second funnel. Repeat until CCl_4 layer is green, discarding aqueous phase. Add 50 ml 0.02*N* HCl to second funnel, shake 2 min., and discard CCl_4 layer. Shake twice with 10 ml CCl_4, discarding CCl_4 layer. Add 50 ml reagent (e) and adjust pH to 8.5–9.0 with NH_4OH. Add 10 ml dithizone reagent, shake 1 min., and transfer CCl_4 layer to a 25 ml volumetric flask. Shake with 5 ml CCl_4 and transfer CCl_4 layer to flask. Repeat until CCl_4 layer is green only. Dilute to volume with CCl_4, shake, and read absorbance at 535 mμ on spectrophotometer, such as Beckman Model DU. Prepare standard curve representing concentrations of 0–20 mmg Zn, using this procedure.

DETERMINATION OF COPPER BY CARBAMATE-EDTA

Pipet 25 ml aliquot of the sample into a separatory funnel, add 25 ml reagent (f), and adjust pH to 8.5–10.0, using concentrated NH_4OH and external pH indicator. Add 3 ml reagent (g) and 10 ml CCl_4, shake 1 min., and transfer to 15 ml centrifuge tube. Add 5 ml CCl_4 to separatory funnel, shake 1 min., and add CCl_4 layer to tube. Read absorbance at 500 mμ on spectrophotometer. Construct standard curve representing concentrations of 0–35 mmg Cu, using this procedure. (If aqueous phase is yellow at pH 9, extract with CCl_4 in absence of carbamate reagent to provide the blank.)

DETERMINATION OF COPPER BY BIQUINOLINE

Pipet 25 ml aliquot of the sample into a separatory funnel containing no citrate or EDTA. Add 0.5 g hydroxylamine hydrochloride, 7.5 ml 2*N* Na acetate, and 6 ml concentrated NH_4OH, adjusting pH to 4.5–5.5, using external pH indicator or

paper. Add 10 ml reagent (i), shake 2 min., and discard aqueous phase. Drain the iso-amyl alcohol into a 15 ml centrifuge tube and centrifuge 1 min. to eliminate any H_2O remaining. Read absorbance at 540 mμ in the spectrophotometer. Prepare standard curve representing concentrations of 0–50 mmg Cu, using this procedure.

SEPARATION OF COPPER AND ZINC BY ANION EXCHANGE

Pass 25 ml sample solution through the anion resin column. (Metal chloride anions of Fe, Al, Cu, and Zn are retained; Ca and Mg are not.) To complete separation, elute column with 30 ml 3*N* HCl. Put clean 125 ml flask under the column and elute with 50 ml 1*N* HCl to remove copper. Proceed with copper analysis by either carbamate-EDTA or biquinoline method. Elute the column with 0.01*N* HCl to recover the zinc and determine by dithizone method.

RESULTS AND DISCUSSION

The results obtained for the analysis of zinc and copper in seven samples are shown in Table 1. The data show that the size of sample used—2, 3, or 5 g—did not cause results to vary greatly; this indicates that other metals did not interfere in the determination. Considerable black precipitate was found in the samples of dolomite N.B.S. 88 and argillaceous limestone N.B.S. 1a, regardless of the wet-ashing reagents used. The filtrates from the argillaceous limestone were yellow. All samples contained appreciable quantities of iron, which, however, could be complexed by the citrate and EDTA. In the biquinoline procedure a large excess of hydroxylamine was added to prevent ferric hydroxide from precipitating.

TABLE 1.—*Zinc and copper assay of limestone and dolomite samples*[a]

SAMPLE	ZINC CONTENT	COPPER CONTENT		
		BIQUINOLINE METHOD		CARBAMATE-EDTA METHOD
		DIRECT	RESIN	
	ppm	*ppm*	*ppm*	*ppm*
Dolomite N.B.S. 88	57.5 ±0.50	6.75 ±0.30	6.65 ±0.72	6.80 ±0.60
Dolomite, Birmingham, Ala.	3.40 ±0.20	1.00 ±0.10	0.86 ±0.15	0.96 ±0.21
Marble Limestone, Tate, Ga.	2.65 ±0.21	2.55 ±0.33	2.50 ±0.41	2.60 ±0.25
Agricultural Limestone, Ocala, Fla.				
Lot A	6.53 ±0.13	0.90 ±0.06	0.70 ±0.10	1.15 ±0.11
Lot B	6.29 ±0.37	0.85 ±0.02	0.86 ±0.15	0.99 ±0.11
Lot C	6.53 ±0.13	1.00 ±0.12	1.25 ±0.23	0.95 ±0.20
Argillaceous Limestone, N.B.S. 1a	39.6 ±1.6	7.25 ±0.30	6.93 ±1.0	7.00 ±0.26

[a] Variation shown is that obtained between 5 g, 3 g, and 2 g samples.

Reagents used without purification gave a very low blank for both copper and zinc.

Copper was separated successfully by anion exchange resin, as shown in Table 1. However, recovery of zinc by this method was somewhat erratic and the data have not been reported. The zinc was completely recovered from several samples but not from others; apparently one or more elements in the sample reduced the release of zinc from the column.

It is recommended* that the study of the determination of zinc and copper in liming materials be continued.

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No reports were given on exchangeable calcium and magnesium, exchangeable hydrogen, exchangeable potassium, fluorine, molybdenum, or phosphorus.

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 24 (1957).

REPORT ON PESTICIDES

By THOMAS H. HARRIS (Pesticide Regulation Section, Plant Pest Control Branch, Agricultural Research Service, U. S. Department of Agriculture, Washington 25, D. C.), *Referee*

Reports were received from eight Associate Referees and a brief summary of each follows:

Allethrin.—A joint industry-government collaborative study of several proposed methods for the determination of allethrin in samples of technical allethrin was begun several years ago. The ethylenediamine method for allethrin was subsequently adopted as one of the official methods of the Chemical Specialties Manufacturers' Association. As the Associate Referee pointed out in his report this year, this method as adopted offered the option of reaction with ethylenediamine at either 25–30°C. for two hours or 98° ±2°C. for 0.5 hours in heat-resistant pressure bottles.

The Associate Referee has reported this year on a study to determine whether a statistically significant difference can be noted when the determination of allethrin is carried out under these two different reaction conditions. No significant difference was found, and the industry Associa-

tion chose to standardize the reaction temperature at 25° ±2°C. for two hours.

Benzene Hexachloride.—The Associate Referee's report this year is concerned with the new radioactive isotope dilution procedure for the determination of gamma benzene hexachloride. It contains results of a collaborative evaluation of this method and a recommendation that it be adopted as first action. The data suggest that this is likely to be the most precise and accurate method available for the determination of gamma BHC.

Amine Salts of 2,4-D Herbicides.—As the Associate Referee has pointed out, the objective of the year's program was to adapt the Parr bomb procedure to the analysis of aqueous solutions of 2,4-D and 2,4,5-T herbicides. Five collaborators compared the A.O.A.C. extraction method with the proposed modification. Work is to be continued next year.

Parathion.—The Associate Referee reports that some analysts prefer the "spot test" titration of O'Keefe and Averill as it appeared in method **5.166** originally adopted as first action. If this titration step is found to yield acceptable results, the Referee favors its inclusion in the official method.

Physical Properties of Pesticides.—The Associate Referee continued his studies on granulated insecticides and outlined procedures for grain breakdown and dustiness. These are important properties of granulated insecticides and more information on such properties is needed for both manufacturing and regulatory reasons. An interim government specification for a granulated dieldrin formulation is under consideration. There is also a government purchase description covering a granular heptachlor formulation. Both of these include specifications pertaining to physical properties.

Pyrethrins.—Last year the Associate Referee outlined a collaborative study to evaluate a chromatographic cleanup procedure in the determination of pyrethrins by the official method. In this year's report he points out some of the difficulties encountered and the corrective measures to be taken.

Rotenone.—The Associate Referee has found that results are more reproducible when the rotenone for the standard curve is purified. He recommends that a collaborative study be conducted. The Referee recommends that this study be continued.

Volatility of Ester Forms of Hormone Type Herbicides.—The Associate Referee has outlined details of three methods for the qualitative response of plants to esters of 2,4-dichlorophenoxyacetic acid and related compounds. A collaborative study of the Baskin and Walker method (*Agr. Chemicals*, **8**, 46 (1953)), as well as other methods, is planned for the coming year.

RECOMMENDATIONS

It is recommended*—

(1) That the method for the analysis of technical allethrin by reaction with ethylenediamine, as published by the Insecticide Chemical Analysis Committee of the Chemical Specialties Manufacturers' Association and as modified in this collaborative study, be adopted as first action.

(2) That the method of Craig, Tryon, and Brown (*Anal. Chem.*, **25**, 1661 (1953)) for the determination of gamma BHC be adopted as first action.

(3) That the method for herbicides, **5.135**, as modified to include aqueous solutions of 2,4-D and 2,4,5-T herbicides, be studied further.

(4) That the first action method for parathion, **5.166**, be adopted as official with the inclusion of an alternate method of titration in which the "spot test" end point of O'Keefe and Averill (*This Journal*, **35**, 385 (1952)) is used.

(5) That further study be made of physical properties of insecticide formulations with particular emphasis on suspensibility, granulation, and particle size determination.

(6) That the proposed chromatographic cleanup procedure for pyrethrins be again studied collaboratively.

(7) That work on the determination of rotenone in insecticide formulations be continued.

(8) That a collaborative study be made of the various methods for ascertaining plant response to 2,4-D type herbicides.

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 23, 24 (1957).

REPORT ON ALLETHRIN

By DAVID KELSEY (Pesticide Regulation Section, Plant Pest Control Branch, Agricultural Research Service, U. S. Department of Agriculture, Washington 25, D. C.), *Associate Referee*

This Association joined the Insecticide Chemical Analysis Committee of the Chemical Specialties Manufacturers' Association in a collaborative program on the analysis of commercial allethrin. A progress report was given at the 1955 meeting (*This Journal*, **39**, 379 (1956)). The purpose of this work was to determine the optimum reaction time of allethrin with ethylenediamine in the first step of the standard method of analysis for allethrin as published by the Committee, and to determine whether the method should be adopted by this Association. Details of the method are as follows:

METHOD FOR ALLETHRIN

PRINCIPLES

Allethrin reacts quantitatively with ethylenediamine to form chrysanthemum monocarboxylic acid which is detd by titrn with std NaOMe in pyridine. Chrysanthemum monocarboxylic acid, anhydride, and acid chloride interfere quantitatively and are detd independently.

REAGENTS

(a) *Absolute alcohol.*—Special Denatured Formula No. 2-B is satisfactory.

(b) *Standard methanolic hydrochloric acid.*—0.1*N*. Dil. 17 ml HCl (1+1) to 1 l with anhyd. MeOH. Stze against std 0.1*N* NaOH, using phenolphthalein. If used at temp., T, different from that at which stdzed, T_0, calcd corrected normality $= N[1-0.001(T-T_0)]$.

(c) *Standard sodium methylate.*—0.1*N* in pyridine. Transfer 50 ml 2*N* NaOMe to 1 l bottle contg 75 ml anhyd. MeOH and dil. to 1 l with redistd pyridine. Stdze against National Bureau of Standards benzoic acid, using pyridine as solvent and thymolphthalein, (i), as indicator. Dispense from 50 ml automatic buret with vents connected to Ascarite tubes. Stdze daily against std methanolic HCl, (b).

(d) *Standard methanolic potassium hydroxide.*—0.02*N*.

(e) *Morpholine soln.*—Transfer 8.7 ml redistd morpholine to liter bottle and dil. to 1 l with anhyd. MeOH. Fit bottle with 2 hole rubber stopper; thru 1 hole insert 20 ml pipet so that tip extends below surface of liquid, and thru other hole insert short piece of glass tubing to which is attached aspirator bulb.

(f) *Ethylenediamine.*—Redistd commercial grade contg less than 3% H_2O. Dispense from automatic buret with vents connected to Ascarite tubes.

(g) *Dimethyl yellow-methylene blue mixed indicator.*—Dissolve 1 g dimethyl yellow (*p*-dimethylaminoazobenzene) and 0.1 g methylene blue in 125 ml anhyd. MeOH.

(h) *α-Naphtholbenzein indicator.*—1% alc. soln.

(i) *Thymolphthalein indicator.*—1% pyridine soln.

DETERMINATION OF CHRYSANTHEMUM MONOCARBOXYLIC ACID CHLORIDE

Add 8–10 drops of the mixed indicator, (g), to ca 150 ml anhyd. MeOH and add 0.1*N* HCl, (b), dropwise until soln appears reddish-brown by transmitted light. Add 0.02*N* KOH, (d), dropwise until appearance of first green color. Transfer 25 ml of this soln to each of three 125 ml glass-stoppered erlenmeyers, reserving 1 flask as reference color for end point. Into each of other flasks add 1.5–2.5 g sample from weighing pipet, swirling flask during addn of sample. Within 5 min., titr. with 0.02*N* KOH, (d), to first green end point, using blank as reference color. Calc. milliequiv. chrysanthemum monocarboxylic acid chloride/g sample: $C = A \times N$/g sample, where A = ml N normal KOH required; $C \times 18.67 =$ % chrysanthemum monocarboxylic acid chloride.

DETERMINATION OF CHRYSANTHEMUM MONOCARBOXYLIC ACID

Transfer 25 ml anhyd. EtOH to each of two 125 ml glass-stoppered erlenmeyers, add 8–9 drops α-naphtholbenzein indicator, and cool to 0° in ice bath. Neutralize by dropwise addn of 0.02*N* NaOH, **41.32**, to bright green end point. To each flask add 1.5–2.5 g sample from weighing pipet. Immediately titr. with 0.02*N* NaOH, **41.32**, to first bright green end point. Calc. milliequiv. chrysanthemum monocarboxylic acid and acid chloride/g sample: $D = A \times N$/g sample, where A = ml N normal NaOH required; $(D-C) \times 16.82 =$ % chrysanthemum monocarboxylic acid.

DETERMINATION OF CHRYSANTHEMUM MONOCARBOXYLIC ANHYDRIDE

Pipet 20 ml morpholine soln, (e), into each of four 250 ml erlenmeyers, using same pipet. Fill pipet by exerting pressure in bottle with aspirator bulb. Reserve 2 flasks for blanks; into each of other flasks add 1.5–2.5 g sample from weighing pipet. Swirl flasks and allow samples and blanks to stand at room temp. 5 min. Add 4–5 drops mixed indicator, (g), to each flask and titr. with 0.1N HCl, (b), until color changes from green to faint red when viewed by transmitted light. Calc. milliequiv. chrysanthemum monocarboxylic anhydride/g sample: $E=(B-A)\times N/g$ sample, where A = ml N normal HCl required for sample, and B = ml normal HCl required for blank; $(E-2C)\times 31.84 = \%$ chrysanthemum monocarboxylic anhydride.

DETERMINATION OF ALLETHRIN

Add sample contg 0.8–1.1 g allethrin to each of two 250 ml erlenmeyers from weighing pipet. To each of 2 flasks as blanks and to samples add 25 ml ethylenediamine, (f), with swirling. Allow samples and blanks to stand 2 hr at 25 ± 2°. Wash down sides of flasks with 50 ml redistd pyridine. To each flask add 6–10 drops thymolphthalein indicator, (i), and titr. with 0.1N NaOMe, (c), to first permanent blue-green end point. (With colorless samples, first blue end point may be used.) Calc. milliequiv. allethrin/g sample: $F=(A-B)\times N/g$ sample, where $A=$ ml N normal NaOMe required for sample, and B = av. ml N normal NaOMe required for blank; $(F+C-D-E)\times 30.24 = \%$ allethrin.

COLLABORATIVE STUDY

As originally published by Hogsett, *et al.* (*Anal. Chem.*, **25**, 1207 (1953)), this method offered the option of reaction with ethylenediamine either at 25–30°C. for 2 hours, or at 98° ± 2°C. for 0.5 hour, in heat-resistant pressure bottles. Samples of the commercial product were analyzed by the standard method with these different reaction conditions; results by both options for the same samples were compared to determine if a statistically significant difference could be noted in the allethrin content. If no difference could be observed, the Committee was to decide which reaction temperature should be specified in the standard method; the collaborative program would then be continued to determine the optimum reaction time at that temperature.

Four samples of refined and commercial allethrin were analyzed at both 25° and 98°. No significant difference could be observed in the allethrin content of any single sample, although results for the four different samples obtained from the basic manufacturers of the chemical varied considerably.

RESULTS AND RECOMMENDATION

As a result of this work, the Insecticide Chemical Analysis Committee standardized the reaction temperature at 25° ± 2°C. Equivalent samples of refined and commercial allethrin were then analyzed again at this temperature, with reaction times of 2, 4, 6, 16, and 24 hours. The results obtained by the five collaborators for each sample are shown in Table 1.

These results have been corrected for chrysanthemum monocarboxylic acid, acid anhydride, and acid chloride. These sets of data were statisti-

TABLE 1.—*Collaborative results for allethrin by ethylenediamine method*

SAMPLE	REACTION TIME, HOURS	ALLETHRIN, PER CENT									
		COLL. 1		COLL. 2		COLL. 3		COLL. 4		COLL. 5	
A	2	94.1	94.2	91.0	90.9	93.2	93.0	94.7	94.9	93.6	93.4
	4	94.2	94.3	92.6	91.0	93.6	94.4	95.0	94.8	93.7	93.6
	6	94.3	94.7	88.9	90.4	93.7	93.6	95.3	95.2	93.7	93.6
	16	94.3	94.3	90.8	92.0	94.1	93.2	95.5	95.4	93.7	93.5
	24	94.5	94.4	91.8	91.9	93.6	93.8	95.5	95.5	93.8	93.7
B	2	92.9	93.0	90.0	90.1	92.0	92.1	93.6	93.4	92.4	92.2
	4	93.1	93.1	90.7	91.0	92.5	92.8	93.3	93.2	92.5	92.3
	6	93.2	93.2	89.9	89.7	92.8	92.5	93.4	93.4	92.5	92.3
	16	93.1	93.1	90.7	89.3	92.8	92.4	93.6	93.5	92.7	92.4
	24	93.0	93.0	89.8	90.5	92.7	92.5	93.4	93.4	92.7	92.7
C	2	87.8	87.8	85.6	85.2	87.8	87.7	87.9	87.4	86.7	86.4
	4	87.8	88.0	86.3	86.8	88.4	88.3	89.4	89.5	88.0	87.9
	6	88.1	88.1	85.9	84.4	88.8	88.3	89.1	88.8	88.0	87.9
	16	87.9	87.9	86.6	85.4	88.2	87.9	89.3	89.2	88.0	87.9
	24	87.9	88.0	86.0	83.7	88.2	88.0	89.1	89.2	88.6	87.8
D	2	90.0	89.6	87.6	86.3	94.1	93.7	90.9	91.0	89.7	89.6
	4	89.9	90.0	86.5	87.5	94.3	94.1	90.5	90.6	89.8	89.7
	6	90.2	89.9	87.0	88.1	94.4	94.3	90.5	90.6	90.0	89.7
	16	89.9	89.8	83.7	84.8	94.1	94.1	90.7	90.8	89.9	89.6
	24	90.0	90.0	85.4	86.2	94.2	94.4	91.0	90.8	90.1	89.8

cally analyzed for variance; the findings are shown in Table 2. The significance of the different causes of variations in test results is denoted under column F by the number in parentheses.

TABLE 2.—*Analysis of variance in collaborative results for allethrin*

SOURCE OF ESTIMATE	SUM OF SQUARES	D.F.	F
Between reaction times	4.396	4	1.099 (NS)[a]
Between laboratories	390.094	4	97.524 (0.01)
Between Laboratories 1, 3, 4, 5, and 2	348.348	1	348.348 (0.001)
Between Laboratories 1, 3, 4, and 5	25.715	1	25.715 (NS)
Between Laboratories 3, 4, and 1	15.965	1	15.965 (NS)
Between Laboratories 3 and 4	0.066	1	0.066 (NS)
Between samples	1011.002	3	337.001 (0.001)
Between reaction times: Laboratories	7.896	16	0.494 (NS)
Between reaction times: Samples	7.286	12	0.607 (NS)
Between laboratories: Samples	143.010	12	11.918 (0.001)
Between reaction times: Laboratories: Samples	17.922	43	0.373 (NS)
Within replicates (error)	49.140	100	0.491

[a] Not significant.

Since no attempt was made to control the quality of the samples, the considerable variation between allethrin values was relatively unimportant to the statistical study.

The mean square for the reaction times (1.099) indicated that no further reaction took place after 2 hours; the mean square for the reaction time-sample interaction (0.607) indicated that 2 hours at 25° ±2° is adequate for all samples of refined and commercial allethrin, regardless of purity differences. The deliberate use of different samples was an integral part of this collaborative program, to determine whether the proposed method would be suitable for any sample of technical allethrin that might be encountered in a practical situation. The differences in the values reported for any one sample by the participating laboratories, however, were found to be statistically significant; therefore the details of the analytical procedure must be followed closely to obtain accurate and reproducible results.

It is recommended* that the method for the determination of technical allethrin by reaction with ethylenediamine, as published by the Insecticide Chemical Analysis Committee of the Chemical Specialties Manufacturers' Association and modified as a result of this collaborative study, be adopted as first action.

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 23, 24 (1957).

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Charles V. Marshall, Canadian Department of Agriculture, Ottawa, Ontario, Canada
M. S. Schechter, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Md.

REPORT ON BENZENE HEXACHLORIDE

By IRWIN HORNSTEIN (U. S. Department of Agriculture, Agricultural Research Service, Entomology Research Division, Beltsville, Md.), *Associate Referee*

The partition chromatographic method and the radioactive-isotope dilution method for determining the gamma isomer content of technical benzene hexachloride have been studied by two industry-government committees; the committees' conclusions regarding the partition chromatographic procedure have been reported (*This Journal*, **39**, 373 (1956)).

The present report summarizes the work of the committee headed by John T. Craig, Commercial Solvents Corporation, who developed the radioactive-isotope dilution procedure.[1] In this procedure, pure gamma isomer labeled with radioactive Cl^{36} is added to a sample of benzene hexachloride containing an unknown amount of gamma. The determination of the decrease in radioactivity, from the standard level to the diluted level, on a pure gamma fraction recovered from the mixture is a measure of the gamma isomer content of the sample.

This method is inherently an absolute one. The primary requirement is that a weighable sample of pure gamma isomer be isolated from the mixture of standard plus unknown. The isolation need not be quantitative.

The collaborative study included the analyses of samples of two technical benzene hexachlorides containing 13 and 15 per cent of the gamma isomer, as well as a 35 per cent gamma concentrate, and gamma-reinforced samples prepared by adding known amounts of pure gamma to technical grade samples of previously established gamma content. The method is as follows:

[1] CRAIG, J. T., TRYON, P. F., and BROWN, W. G., *Anal. Chem.*, **25**, 1661 (1953).

RADIOACTIVE-ISOTOPE DILUTION METHOD FOR GAMMA BENZENE HEXACHLORIDE

PRINCIPLES

Method is based on addn of pure gamma isomer labeled with radioactive Cl^{36} to sample of benzene hexachloride contg unknown amount of gamma isomer. Detn of decrease in radioactivity from std level to dild level, on pure weighable gamma fraction recovered from mixt., is measure of gamma isomer content of sample. The isolation of pure gamma material need not be quantitative.

(This analytical technique can be applied to benzene hexachloride samples having wide range of gamma content. Wt of unknown sample to be analyzed should be increased or decreased according to its estimated gamma content, so that ratio of labeled gamma added to ordinary gamma in sample will approximate ratio used in this method.)

PREPARATION OF LABELED GAMMA ISOMER STANDARD

(Radioactive chlorination of benzene is based on rapid establishment of exchange equilibrium between Cl and Cl-ion in aq. soln. Inactive Cl bubbled thru radioactive HCl becomes active by exchange; near-quant. transfer of radioactive Cl into Cl phase is achieved.)

Place 15 ml benzene in benzene reaction tube (2, Fig. 1). By means of 4 ml pipet to which is attached hypodermic syringe, place 4 ml aq. radioactive 0.2*N* HCl contg 12 microcuries Cl^{36} in Cl-exchange tube (1, Fig. 1).

Pass ordinary Cl from cylinder (3, Fig. 1) 30 min. thru system up to tube contg radioactive HCl in order to displace all air from system. Open stopcock above radioactive HCl tube and bubble ordinary Cl into the HCl 6 min. at rate of 0.1 g/min. Radioactive Cl, along with excess ordinary Cl, passes into benzene reaction tube and dissolves in the benzene.

After all Cl has passed into benzene, place 150 watt lamp 3″ from center of benzene and allow it to remain there until yellow-green color disappears, when reaction is complete. Introduce stream of air thru bubbler in benzene tube, apply heat to coil, and distill benzene. Air stream will flush vapors thru condenser. Dry cake

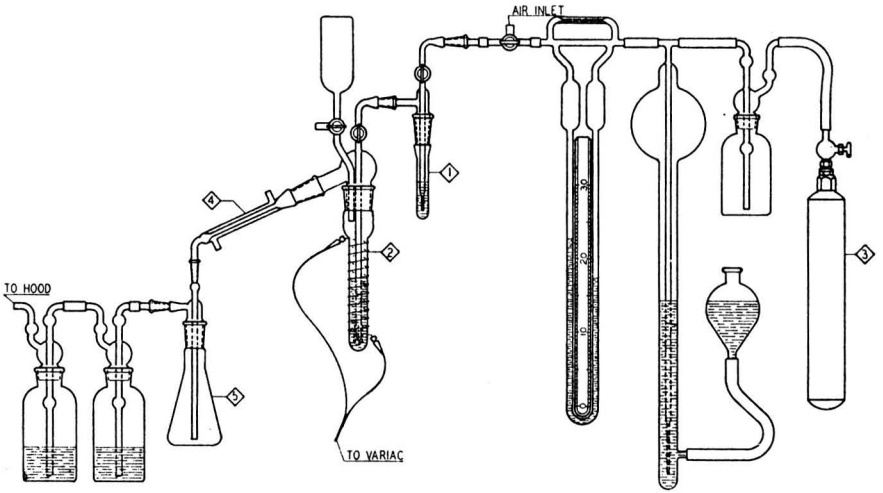

FIG. 1.—Chlorination apparatus.

of ca 1 g crude radioactive benzene hexachloride in 75° vacuum oven to remove traces of benzene.

Ext. gamma isomer from cake with 2 ml *n*-hexane satd with nitromethane by heating to b.p. and stirring vigorously. Decant ext. into 50 ml beaker and repeat extn. Wash spent cake with 2 ml cold *n*-hexane and combine with previous exts. Evap. solvent, using heat lamp placed ca 6″ above beaker, to obtain gamma oil.

Add 3.5 g pure unlabeled gamma isomer to gamma oil. Dissolve mixt. in 16 ml alcohol (Special Denatured Formula No. 3A is satisfactory) by heating and stirring. Cool to 20° with constant stirring. Allow slurry to stand in bath 15 min. after crystals appear. Filter, and wash with alcohol. M.p. of dried crystals is 112.0–112.8°.

Repeat recrystn at least twice to remove all traces of isomers other than gamma. Dry final crystals 3 hr at 70° in vacuum oven to obtain labeled std.

ISOLATION OF GAMMA ISOMER

Weigh 120 ± 5 mg (to 0.1 mg) of labeled std into tared 15 × 50 mm shell vial and add 1000 ± 5 mg (to 0.1 mg) of tech. grade benzene hexachloride sample.

Add 1.2 ml perchloroethylene, place cap (can be made from 5 ml beaker cut in half) on vial, and insert vial into well of 115° perchloroethylene heating bath (1, Fig. 2). Allow vial to remain in bath 15 min., stirring occasionally. (Not all of crude samples will necessarily dissolve.) Cool mixt. in 20° H_2O bath (3, Fig. 2) 30 min. with occasional stirring to allow crystn of isomers other than gamma. Leave cap on during crystn.

Tare 15 × 50 mm shell vial and place in Niederl-Niederl sulfur filtration app. (2, Fig. 2). Filter supernatant liquid from crystals thru filter stick into tared vial. Wash crystals by adding 0.3 ml perchloroethylene and cooling to 20° while stirring. Filter wash into vial contg original filtrate.

Place vial contg filtrate and wash in evapn tube inserted in top of heating bath and evap. solvent, using air stream directed at surface of soln and adjusted to avoid splashing. Most of perchloroethylene will evap. in 30 min. Weigh residue, which is gamma oil. Usually yield is 300–450 mg.

Crystallize gamma oil by dissolving in 0.8 ml of 1,4-dioxane and *n*-butyl alcohol (1 + 1)/g of mixt. while heating and stirring until homogeneous soln is obtained. Cool 15 min. at 20° in H_2O bath; then scratch walls of vial to induce crystn. After crystals appear, allow slurry to stand in bath 10 more min.; then filter in Niederl-Niederl filtration app. Wash crystals with ca 5 drops cooled *n*-butyl alcohol. M.p. of crystals is 100–112°, after drying 30 min. in 75° vacuum oven.

Recrystze material from *n*-butyl alcohol, using ca 4 ml solvent/g crystals, by dissolving and then cooling to 20° as before. Filter, wash with ca 3 drops *n*-butyl alcohol, and dry 30 min. in 75° vacuum oven. Repeat recrystn, using 3 ml alcohol/g (Special Denatured Formula No. 3A alcohol is satisfactory). Dry crystals in 75° vacuum oven 2 hr. Yield is usually ca 50 mg; m.p. 112.0–112.8°. If m.p. is not in this range, repeat recrystn.

COUNTING

Use thin-wall, glass, liquid-jacketed counting tube (25 mg/sq. cm., 10 ml capacity) (Fig. 3) for soln counting.

Take background count before counting sample, filling counter tube with same solvent used to dissolve sample. Subtract this value from count of both sample and std.

Weigh, to 0.1 mg, isolated pure gamma sample into 12 ml snap cap vial, add 10.0 ml acetone, and shake until sample dissolves. Transfer soln slowly into counter tube with hypodermic syringe (take care not to subject thin glass wall to too sudden pressure changes or tube will break) and count ca 15,000 total counts, noting time.

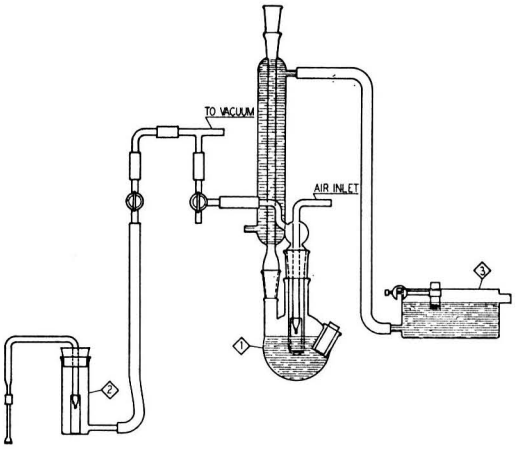

FIG. 2.—Sample isolation apparatus.

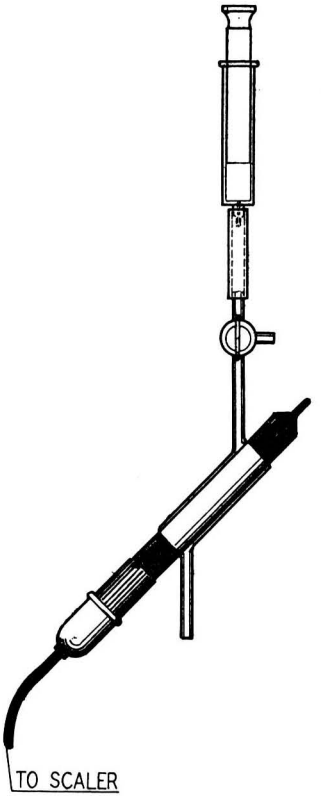

FIG. 3—Counting apparatus. Available as Model J.T., N. Wood Counter Lab., 5491 Blackstone Ave., Chicago 15, Ill.

Wash out tube 3 times with fresh acetone, and siphon dry. Check background again for 10 min. to make sure tube is decontaminated. Similarly prep. soln of 50 mg std and count to same approx. total count as sample, noting time.

Calc. mg gamma benzene hexachloride in sample: $X = A(B-C)/C$, where A = mg radioactive std added to sample; B = specific activity of std, *i.e.*, counts per min. of std/mg std counted; and C = specific activity of isolated sample, *i.e.*, counts per min. of isolated sample/mg sample counted.

NOTE.—Before radioactive compounds are made or purchased, authorization for their use must be obtained from Atomic Energy Commission, to ensure that proper precaution will be observed in handling these materials.

RESULTS

The results of the study of the 13 and 15 per cent benzene hexachloride samples are shown in Tables 1 and 2. Agreement among the laboratories that analyzed these samples was good.

To evaluate this method further, known amounts of pure unlabeled gamma were added to sample DSI-1519 (the 13 per cent gamma sample). Three laboratories added approximately 5, 10, or 20 per cent of gamma, based on the weight of the total sample, and the fourth laboratory added 5 and 10 per cent gamma, based on the gamma content of the sample. The

TABLE 1.—*Per cent of gamma isomer in technical benzene hexachloride sample DSI-1519*

RUN NO.	LAB. A	LAB. B	LAB. C	LAB. D	LAB. E
1	12.9	12.9	12.7	13.0	12.8
2	12.9	12.9	13.3	13.0	13.3
3	12.9	12.8	12.8	—	13.3
4	12.8	12.7	13.1	—	12.9
5	13.0	12.8	13.1	—	12.8
6	12.8	12.8	13.0	—	—
7	—	—	13.2	—	—
Mean	12.9	12.8	13.0	13.0	13.0
Range	0.2	0.2	0.6	0.0	0.5
Standard deviation (±)	0.08	0.08	0.2	0.0	0.26

TABLE 2.—*Per cent of gamma isomer in technical benzene hexachloride sample DSI-1489*[a]

RUN NO.	LAB. A	LAB. B	LAB. C	LAB. D
1	15.5	16.0	15.2	15.2
2	15.6	16.1	15.5	15.2
3	15.2	15.9	15.5	14.9
4	15.2	15.9	15.7	15.0
5	15.6	16.0	15.4	14.8
6	15.7	15.8	15.7	15.3
7	—	—	15.4	—
8	—	—	15.5	—
9	—	—	15.5	—
Mean	15.5	15.9	15.5	15.1
Range	0.5	0.3	0.5	0.5
Standard deviation (±)	0.22	0.12	0.2	0.2

[a] Lab. C prepared its own Cl^{36}-labeled gamma. The other laboratories used a radioactive gamma sample prepared and supplied by J. T. Craig.

total gamma content was then determined by the radioactive-isotope dilution method. The results are all in good agreement with theory, as shown in Table 3.

Analyses of a 35 per cent gamma concentrate (sample No. 35) by three laboratories are reported in Table 4. It was difficult to obtain a representative sample for analysis. The removal of a molten aliquot was not feasible, since the sample did not melt completely until the temperature was high enough to cause the gamma fraction to sublime. This difficulty would be common to any method used.

A statistical analysis of all the data received from the six laboratories,

TABLE 3.—*Per cent of gamma isomer in DSI-1519 enriched with different amounts of added gamma*

LAB. A		LAB. B		LAB. C		LAB. D	
THEORY	FOUND	THEORY	FOUND	THEORY	FOUND	THEORY	FOUND
18.2	18.0	19.4	19.4	18.0	18.2	13.5	13.1
18.2	18.0	19.5	19.5	18.0	18.0	13.5	13.7
23.5	23.7	17.6	17.3	18.0	17.8	13.5	13.5
23.6	23.6	20.6	21.1	18.0	18.3	14.0	14.1
34.3	34.2	24.0	24.3	18.0	18.3	14.0	14.6
34.5	34.8	22.2	22.0	33.0	33.3	14.0	14.6
				33.0	33.1		
				33.0	32.7		
				33.0	32.6		
				33.0	33.2		

TABLE 4.—*Per cent of gamma isomer found in sample 55, benzene hexachloride concentrate containing approximately 35% gamma isomer*

RUN NO.	LAB. A	LAB. B	LAB. C
1	32.3	35.6	32.0
2	31.8	35.7	—
3	33.2	35.8	—
4	31.5	35.8	—
5	31.9	36.0	—
6	32.2	36.2	—
Mean	32.2	35.8	32.0

covering all samples except the 35 per cent gamma concentrate, revealed an over-all relative precision of ±4.2 per cent. This value was based on a 95 per cent confidence level, i.e., if one laboratory reported 12.0 per cent of gamma, for example, it could be assumed that another laboratory would find between 11.5 and 12.5 per cent gamma. If the original laboratory reanalyzed the sample, it could be assumed with the same confidence that a value between 11.7 and 12.3 per cent would be found, since within-laboratory variation is ±2.6 per cent.

Two additional studies were made to check the adequacy of the suggested total count of 15,000 per sample and to compare the isotope dilution method with other procedures. A. H. Sutton compared the values obtained with 15,000 counts to values obtained with a much larger total count. The data in Table 5 indicate that 15,000 is adequate. C. H. Schmieges submitted data comparing polarographic, chromatographic, and infrared analyses with results obtained by isotope dilution. The results for samples DSI-1519 and DSI-1489 are shown in Table 6.

A survey of the data indicates that the accuracy and precision of the

TABLE 5.—*Effect of total count on gamma values for technical benzene hexachloride sample DSI-1489*

RUN NO.	TOTAL COUNT	FIRST 15,000 COUNTS (APPROX. 30 MINUTES) GAMMA	GAMMA
	number	*per cent*	*per cent*
1	63,000	15.2	15.4
2	52,000	15.5	15.3
3	53,000	15.5	15.6
4	66,000	15.7	15.7
5	170,000	15.4	15.8
6	40,000	15.7	15.7
7	15,000	15.4	15.4
8	552,000	15.5	15.2
9	523,000	15.5	15.5
Mean	——	15.5	15.5
Range	——	0.5	0.6

TABLE 6.—*Per cent of gamma isomer in benzene hexachloride when determined by various methods (number of runs indicated in parentheses)*

SAMPLE	POLAROGRAPHY	CHROMATOGRAPHY	INFRARED	ISOTOPE DILUTION
DSI-1519	13.25 (2)	13.4 (1)	13.1 (3)	13.0 (7)
DSI-1489	15.0 (2)	14.7 (1)	15.0 (2)	15.1 (6)

method are excellent. It is recommended* that the method described in this report, essentially that of Craig, *et al.*, be adopted as first action.

ACKNOWLEDGMENTS

The following investigators took part in the collaborative study:

J. T. Craig, *Chairman*, Commercial Solvents Corporation, Terre Haute, Ind.

M. E. Griffing, Ethyl Corporation, Ferndale, Mich.

A. D. Turissini and R. Suffis, General Chemical Division, Allied Chemical and Dye Corporation, Long Island City, N. Y.

A. H. Sutton, General Chemical Division, Allied Chemical and Dye Corporation, Marcus Hook, Pa.

J. W. Churchill, J. A. Curtis, and C. H. Schmieges, Mathieson Chemical Company, Niagara Falls, N. Y.

R. L. Tracy, John Powell Laboratories, Port Jefferson, N. Y.

I. Hornstein, U. S. Department of Agriculture, Agricultural Research Service, Entomology Research Branch, Beltsville, Md.

J. T. Craig prepared the radioactive gamma benzene hexachloride used in this study. Fifteen companies contributed $250 each for preparing the labeled gamma isomer.

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 23, 24 (1957).

REPORT ON HERBICIDES

By HOWARD HAMMOND (State Laboratories Department, Bismarck, N. Dak.), *Associate Referee*

The objective of this year's collaborative study on herbicides was to broaden the use of the Parr bomb to include herbicides in water solution. A commercial amine salt of 2,4-dichlorophenoxyacetic acid was sent to collaborators with the request that analysis be made by the A.O.A.C. extraction method, **5.133**, and by the following method:

TOTAL CHLORINE IN AMINE SALTS OF 2,4-DICHLOROPHENOXYACETIC ACID (2,4-D) AND 2,4,5-TRICHLOROPHENOXYACETIC ACID (2,4,5-T) IN LIQUID HERBICIDES

Weigh 0.5 g sample (or sample containing ca 0.07 g Cl) from a weighing bottle or weighing buret into the cup of a 42 ml Parr electric ignition bomb (22 ml flame-ignited bomb may also be used). Add 6–10 drops of 1:1 NaOH, avoiding excess (excessive NaOH makes the residue difficult to break up), to decompose the amine radical and yield the Na salt. Place in air oven at 100° and dry 30–60 min. Break up the dried residue with a glass rod and mix with 0.5 g powdered sugar. Mix well with 15 g calorimetric grade Na_2O_2 (one standard measuring dipper). Proceed as in **5.135**, beginning "Withdraw rod . . ."

RESULTS AND DISCUSSION

Results from 5 collaborators are shown in Table 1. Collaborators obtained good agreement with the different methods; some of the variation may be due to differences in the silver chloride titration. The two collaborators who obtained the higher results by the Parr bomb used A.O.A.C. method **5.153**(a) which includes digestion and filtration of the precipitated

TABLE 1.—*Collaborative results for 2,4-dichlorophenoxyacetic acid*

METHOD	2,4-DICHLOROPHENOXYACETIC ACID, PER CENT				
	COLL. 1	COLL. 2	COLL. 3	COLL. 4	COLL. 5
A.O.A.C. extraction method, **5.133**	41.68	41.35		42.01	42.47
Same, expressed as 2,4-D acid	41.69	41.34		41.90 41.91 41.63	42.27
Proposed Parr Bomb method	42.86 42.95	41.58 41.49	41.39 41.68 41.01	42.45 42.89 42.98 42.68	41.51 41.68 41.93
Dow Method 21410[a]	42.80 42.79				

[a] A non-aqueous titration method.

silver chloride. Another collaborator used a Fisher Senior Model Titrimeter.

One collaborator expressed preference for the extraction method over the proposed method because he had difficulty in breaking up the sodium salt of 2,4-D and mixing the charge. Another collaborator, however, preferred the proposed method because it is less time consuming and requires fewer manipulations.

An investigation was begun of a partition chromatographic procedure for the determination of mixtures of 2,4-D and 2,4,5-T (*Anal. Chem.*, **24**, 1968 (1952)) but time did not permit collaborative study. A titrimetric assay of trichloroacetate (*Anal. Chem.*, **27**, 1774 (1955)) was investigated and thought very promising. It is hoped that both these studies can be continued.

It is recommended* that studies on herbicides be continued.

COLLABORATORS

The Associate Referee wishes to express his appreciation to the following collaborators for their part in this study:

L. E. Streeter, The Dow Chemical Co., Midland, Mich.

M. E. Mandrell, The Dow Chemical Co., Midland, Mich.

H. A. Thompson, Naugatuck Chemicals, Elmira, Ontario, Canada

C. H. Hall, Pesticide Regulation Section, Plant Pest Control Branch, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Md.

Lloyd G. Keirstead, Connecticut Agricultural Experiment Station, New Haven, Conn.

* For report of Subcommittee A and action of the Association, see *This Journal*, **40**, 23, 24 (1957)

REPORT ON PHYSICAL PROPERTIES OF PESTICIDES

By Ernest L. Gooden (U. S. Department of Agriculture, Agricultural Research Service, Entomology Research Branch, Beltsville, Md.), *Associate Referee*

Developments in insecticide testing since the last report (*This Journal*, **39**, 386 (1956)) mainly concern granulated insecticides. Grain breakdown and dustiness still give the most trouble.

Grain Breakdown.—The latest form of the grain breakdown test seems to be adequately simple and reproducible. Details are as follows:

TEST FOR GRAIN BREAKDOWN

Transfer a 10 g sample to a 250 ml glass-stoppered graduated cylinder and fill to 250 ml with distilled water at room temperature (ca 25°C.). Rotate the cylinder end over end by machine at 30 rpm for 2 hr; then remove, let stand undisturbed for 30 min., and draw off, by suction, any oil that has risen to the top. Support the

cylinder upright over a U.S. No. 60 sieve, and transfer the sample to the sieve, using a vigorous stream of tap water introduced through a hose extending nearly to the bottom of the cylinder. (Use 8″ diameter sieve and support cylinder so that its base is within the sieve frame and ca 1 cm above the sieve cloth.) Rinse out the residue from the sieve onto a filter, using a gentle stream of water. Dry the residue to constant weight at not higher than 55°C., and calculate the percentage of sample passing through the sieve.

The amount that has been specified to pass the sieve in this test is 80 per cent (minimum). Normal discrepancy in results between successive runs is about 1 per cent.

The only unsolved problem is whether a breakdown test is necessary. Various tentative specifications and purchase descriptions now under consideration or in actual use omit the breakdown requirement; yet the test is still often requested to be run on current samples. Even when the test is not part of the specification, it sometimes gives the buyer useful evidence of whether the formulation was made in the prescribed manner. The importance of breakdown *per se* may depend on the composition of the granules and the purpose for which the insecticide is being used.

Dustiness.—Dustiness of granulated insecticides is commonly measured by the relative weight of the fraction passing a U. S. No. 60 sieve by dry test. Such a measurement seems appropriate, since this sieve is approximately the upper limit in the most lenient specifications for dusting powders. The object of the test is not an absolute separation at the designated particle size. Any dust particles that cling persistently to the granules are, for practical purposes, part of the granules. The mobile dust particles, on the other hand, constitute the objectionable fraction; regardless of its chemical composition, which is unpredictable, in a mechanical sense this fraction is an impurity.

The dustiness of a given sample is often unstable. Thus it is difficult to establish the reproducibility of a test method, especially in comparisons between laboratories. Even in the same laboratory and on the same day, reproducibility between successive runs requires extreme precautions in handling; since the dust does not occupy all the space between granules, even after it is thoroughly dispersed throughout the sample it continually tends to segregate. The sample must be frequently reblended to ensure representative subsamples and to preserve the original particle-size distribution in the remainder of the stock sample.

The test method for grain-size distribution developed in the Entomology Research Branch is as follows (the paragraphs have been numbered to conform to the Federal Specifications system):

TEST FOR DUSTINESS AND OVERSIZE

4.3.2. *Grain-Size Distribution*

4.3.2.1. *Sieves.* Sieves shall conform to the requirements of Federal Specification RR-S-366b.

4.3.2.2. *Sample Preparation.* Each time any portion is removed from the stock

sample, the entire sample must first be blended to ensure uniform distribution of the dust among the granules. For this purpose, tumble the sample end-over-end at approximately 30 rpm for 1 minute in a jar or drum whose volume is about twice the apparent volume of the sample.

4.3.2.3. *Procedure.* Screen a 20 g sample through a nest of 8 inch U.S. Standard sieves of the designated mesh sizes, using a single-eccentric type of mechanical shaker that imparts to the sieve a rotary motion and tapping action at a uniform speed of approximately 300 gyrations and approximately 150 taps per minute. Continue the screening for 15 minutes. Weigh the residues and calculate the percentage passing through each sieve. In the weighing and transferring of the sieve sample and its size fractions, any dust clinging to the sieve cloth, sieve wall, scale pan, and miscellaneous surfaces, or clogging the sieve-cloth apertures, is to be regarded as part of the finest fraction (sieve-pan fraction). Neither this dust nor the material actually entering the sieve pan need be recovered for weighing; the granules lying on the respective sieve cloths are to be recovered and weighed, and the pan fraction calculated by difference.

It is recommended* that the study of physical properties of pesticides be continued.

* For report of Subcommittee A and action of the Association, see *This Journal*, **40**, 23, 24 (1957).

REPORT ON PARATHION

By KENNETH HELRICH (New Jersey Agricultural Experiment Station, New Brunswick, N. J.), *Associate Referee*

Some analysts still prefer the "spot test" titration of O'Keefe and Averill as it appeared in the parathion method originally adopted as first action (*This Journal*, **35**, 64 (1952)). It has been suggested that this titration be added to the present procedure as an alternate method of determining the end point.

The Associate Referee's laboratory has begun work on an infrared method for parathion, but it is not yet known whether the method will be suitable for control use. We hope to have more to report at the next meeting.

It is recommended* that the first action method for parathion, **5.166**, be adopted as official and that the "spot test" end point of O'Keefe and Averill be included as an alternate method of titration, as follows:

At end of first par., **5.167(e)**, add: "or following spot test, adding the $NaNO_2$ in 4 drop portions near end point: Dip glass rod into soln being titrd and touch rod quickly to piece of KI-starch paper, **34.128(c)**. End point is reached when intense blue-black color appears immediately and can be obtained repeatedly during 1 min. period without further addn of $NaNO_2$."

* For report of Subcommittee A and action of the Association, see *This Journal*, **40**, 23, 24 (1957).

REPORT ON PYRETHRINS

By DAVID KELSEY (Pesticide Regulation Section, Plant Pest Control Branch, Agricultural Research Service, U. S. Department of Agriculture, Washington 25, D. C.), *Associate Referee*

At the 1955 meeting of this Association, it was recommended that the investigation of analytical methods for the determination of the pyrethrins be continued (1). The program of the investigation for 1956, to be carried out jointly with the Insecticide Chemical Analysis Committee of the Chemical Specialties Manufacturers' Association in the collaborative program previously reported (2), was planned around a chromatographic purification step designed to remove from a sample of pyrethrum extract being analyzed (3) certain extraneous materials, the so-called "false" pyrethrins, which are biologically inactive but which react positively with Denige's reagent (active Pyrethrin I).

Brown and Phipers, the authors of this chromatographic procedure, had reported no loss of biological activity in pyrethrum extract that had been passed through a suitably prepared column of alumina. It was believed that a purification of this type might eliminate the objections to the mercury reduction method (in which the saponifying alkali is neutralized by sulfuric acid in the dollar areas of the world and by hydrochloric acid in the sterling areas) as the official method of analysis for Pyrethrin I and II.

It was apparent very early in the program, however, that the chromatographic procedure being used by the collaborators was not satisfactory, even though it was the exact procedure followed by Brown and Phipers and the chromatographic alumina being used had been specially imported from England. The collaborators were unable to get uniform results with this alumina; some reported excessive reduction in pyrethrin content of the test extract as a result of the chromatographic separation, while others reported little or no reduction in the pyrethrin content of the official samples. Furthermore, one of the laboratories reported reduced biological activity in test insecticides made from the official sample of pyrethrum extract after chromatographing. This result indicated that the alumina was removing active material as well as the "false" pyrethrins. Such a finding, of course, was contrary to that reported by the authors.

Accordingly, the collaborative program was suspended until the cause of the difficulty could be determined. As the outcome of extensive correspondence with the authors, it was recognized that Moore (4) had specified Brockmann Grade III alumina as the only grade that provided the proper degree of activity for chromatographic separation of polymerized pyrethrins and other extraneous material from pyrethrum extract; and that Cornelius (5) had used Sudan Yellow and Sudan Red to standardize alumina for chromatographic work with pyrethrum extracts to what was

essentially Brockmann Grade III, although he did not specifically mention the Brockmann scale. The activity of the alumina had not previously been considered. In fact, the Associate Referee had gone to considerable effort to obtain some of the English alumina specified by Brown and Phipers, rather than attempting to use a domestic grade. It was also disclosed that the authors had very carefully standardized their alumina to Grade III activity before they used it for their chromatographic columns.

In view of this information, therefore, it was decided to defer any more collaborative work until a suitable domestic grade of chromatographic alumina could be obtained and enough of it standardized to Grade III activity to furnish each collaborator with a quantity sufficient for the contemplated work. The alumina will be placed in hermetically sealed tin cans to prevent any undesirable hygroscopic action during shipment. It is anticipated that the samples of standardized alumina, with fresh samples of commercial pyrethrum extract, will be furnished to the collaborators early in 1957.

Concurrently with this work, a simplified procedure for standardizing the alumina is being investigated so that each analyst can prepare and standardize to Grade III activity any chromatographic alumina he may obtain. The standardization procedure being investigated has been developed by Dr. J. B. Wilkie, Food and Drug Administration, as a result of extensive practical work in the standardization of the alumina adsorbents used for Vitamin A chromatography (6). This modified and simplified procedure, although not yet published, has been furnished to the Associate Referee for use in the collaborative program. Details of this method will probably be published in the near future.

It is recommended* that the investigation of analytical methods for the determination of pyrethrins be continued.

ACKNOWLEDGMENTS

The Associate Referee is indebted to Howard A. Jones of the Fairfield Chemical Division, Food Machinery and Chemical Corporation, for his continued interest and assistance in this program; to J. B. Wilkie, Food and Drug Administration, for furnishing the standardization procedure for the chromatographic alumina; and to R. A. Caswell, Pesticide Regulation Section, Plant Pest Control Branch, Agricultural Research Service, Beltsville, Md., who is preparing the standardized alumina for distribution to the collaborators.

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* For report of Subcommittee A and action of the Association, see *This Journal*, **40**, 23, 24 (1957).

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REPORT ON ROTENONE

By R. PAYFER (Laboratory Services, Plant Products Division, Department of Agriculture, Ottawa, Ontario, Canada),
Associate Referee

After the Associate Referee's report on rotenone was published (*This Journal*, **37**, 630 (1954)), some chemists reported that they could not duplicate the standard curve. The original experiments were therefore repeated. It was found that in order to duplicate the standard curve, the rotenone must be purified by the method in *Official Methods of Analysis*, 8th Edition, **5.107**, as many times as necessary until the same melting point (161–163°) is obtained after two consecutive purifications. The experiment was also repeated with the alcoholic solution which was more than two years old at that time, and the standard curve could be duplicated.

It is recommended* that, if enough collaborators are willing to participate, a collaborative study be conducted during the coming year.

* For report of Subcommittee A and action of the Association, see *This Journal*, **40**, 23, 24 (1957).

REPORT ON VOLATILITY OF ESTER FORMS OF HORMONE TYPE HERBICIDES

By EDWARD P. CARTER (Fungicide and Herbicide Unit, Pesticide Regulation Section, Plant Pest Control Branch, U. S. Department of Agriculture, Beltsville, Md.),
Associate Referee

The term "volatile" as applied to hormone type herbicides, such as 2,4-D and 2,4,5-T formulations, refers to the effect of the vapors of the material upon various plants, rather than to the actual magnitude of the vapor pressure of the herbicide itself. In this light, a volatile herbicide produces, upon vaporization, a formative concentration of a physiologically active compound recognized by such responses in affected plants as downward curvature of leaves (epinasty), leaf malformations, stem curvatures, and severe distortion.

Several methods are currently available for determining the qualitative

response of esters of 2,4-dichlorophenoxyacetic acid (2,4-D) and related compounds. Three of these methods qualitatively estimate herbicidal vapor activity on the above-ground vegetative structures of such test plants as beans or tomatoes. Good agreement has been observed between these methods in assigning vapor hazard ratings to various ester forms of the herbicides. The tests are all sensitive enough to detect 1.0 per cent or less of a volatile 2,4-D herbicide present as contamination in an otherwise low-volatile product.

Marth and Mitchell (1) enclosed young, rapidly growing bean and tomato plants in bell jars and cellophane bags, together with 2,4-dichlorophenoxyacetic acid or various salts and esters of this acid. Their method could distinguish between the relative volatilities of these compounds. The acid, sodium salt, triethanolamine salt, and amide forms were nonvolatile. All of the esters tested were found to volatilize and to produce growth effects on the test plants. The methyl, ethyl, butyl, and isopropyl esters appeared to be more volatile than the longer chain esters.

Hitchcock, *et al.* (2) used essentially the same technique in exposing tomato plants to ester vapors of 2,4-D and 2,4,5-T. A measured quantity of test solution (0.001–0.1 ml) was placed on filter paper; the paper was then suspended over the test plant incased in a No. 20 paper bag and kept at temperatures between 70° and 80°F. Curvatures of leaves and stems were evaluated at the end of the exposure period or within 24 hours after the plants were removed from the bags. Responses were evaluated by measuring stem and leaf curvature, modification, and stem proliferation. Esters of high and low volatility were distinguished by applying 0.01 ml of the formulations (equivalent to 4 lb 2,4-D or 2,4,5-T acid per gallon) and exposing for 24 hours. On the basis of these tests, isopropyl and pentyl esters were considered to be highly volatile and longer chain esters to be low volatile.

These two methods of testing volatility in closed systems have resulted in a proposed Federal Specification test for volatility as follows:

4.2.4. *Volatility Test—Relative Vapor Activity.* The vapor activity test is conducted with gastight cellophane cases approximately $3\frac{1}{2} \times 3\frac{1}{2} \times 16$ inches in size. Young rapidly growing Pinto bean plants about 4 inches in height are used as test plants. A single bean plant growing in a 3 inch pot is placed in each cellophane case just prior to testing the ester.

Two mg of acid equivalent as the ester is dissolved in 10 ml of 95% ethyl alcohol, and a Whatman No. 1 filter paper (9 cm diameter) is thoroughly moistened by dipping in the solution. (Do not reuse the container used in this impregnation.) The alcohol is then allowed to evaporate and the filter paper impregnated with the ester is inserted into the cellophane case containing the bean plant and fastened to the inside of the case 6 inches above the leaves of the test plant. The open end of the case is then sealed.

The case containing the test plant and treated filter paper is then placed in a dark room for a period of 24 hours. The temperature range of the room should be 80°–90°F. Control plants are also sealed in separate cases. The experimental design

is a randomized block with three replications and each test is repeated three times. The evaluations shall be made following an exposure period of 24 hours.

Observations of the effect of the vapors on test plants should take into consideration whether or not the plant is slightly, moderately, or severely injured, including such symptoms as degree of stem curvature, terminal bud inhibition and degree of leaf curl. The relative vapor activity of an ester can be numerically designated as follows: 0—no visible effects; 1, 2, 3—slight injury, plant usually recovered with little or no reduction in growth, slight epinasty present, stem curvature slight; 4, 5, 6—moderate injury, plant usually recovered, moderate epinasty, moderate terminal bud inhibition and moderate stem curvature present; 7, 8, 9—severe injury, plant usually does not recover, pronounced epinasty, together with pronounced stem curvature; 10—plant killed.

Chemically pure 2,4-D acid and the butyl ester of 2,4-D are used as standards. The 2,4-D acid under most conditions is rated 0 while the butyl ester has a high vapor activity with a rating of 9.0. Esters receiving the following ratings would be classed as follows: 0—no vapor activity; 1, 2, 3—very low vapor activity; 4, 5, 6—low to moderate vapor activity; 7, 8, 9—high vapor activity; 10—very high vapor activity.

Esters must receive a vapor activity rating of less than 4 to be designated low volatile.

Baskin and Walker (3) reported a method in which tomato plants were confined under paper bags and exposed to a stream of air that had been passed over 10 ml of undiluted product in a test tube placed in a water bath at 70°F. Exposure periods were 2, 4, and 16 hours; observations were recorded at the end of the exposure period, and 24, 48, and 72 hours later. Response was measured by curvature of leaf and stem and other growth effects. On the basis of these studies it was concluded that methyl, ethyl, propyl, butyl, and pentyl esters were highly volatile, while longer chain esters were low volatile. In another paper (4) these workers showed that the esters which were low volatile at 70°F. became progressively more volatile as the temperature of the ester in the test tube was raised to 90° and to 120°F.

This method stresses the importance of air turbulence in disseminating ester vapor. It has become the test method of the Pesticide Regulation Section of the Plant Pest Control Branch, Agricultural Research Service, U. S. Department of Agriculture. It is also being used by the pesticide law enforcement agencies of certain states and is recognized by the National Agricultural Chemicals Association. This method as presently used is as follows:

Material and Method.—Pipet 10 ml of the undiluted product to be tested into a 200×25 mm Pyrex test tube fitted with a 2-hole No. 5 neoprene stopper. Into one hole place a right angle glass tube with 1 arm extending 50 mm into test tube, and with rubber tubing attach outside arm of tube to the compressed air line (which runs thru a water bath). Into second hole place a glass tube, extending 5 mm into test tube, with arm about 170 mm long bent at about 80° angle above the stopper. Use test tube, 2-hole stopper, and glass tubing coming in contact with the ester vapor only once for herbicidal tests; do not re-use.

Support test tube in a water bath at 90°F.; pass long arm of tube through hole

punched in side of large paper sack ($\frac{1}{8}$–$\frac{1}{4}$ barrel size) that covers each unit of the system (replace with new bag for each exposure). Place test plant (Rutgers tomato, grown to 4 or 5-leaf stage in a disposable container such as a clean, used 1 qt oil can with the top cut away and drainage holes punched in the bottom) under the paper bag.

(To test several products simultaneously, split main air line with T-tubes and adjust with screw clamps or appropriate lengths of capillary bore glass tubing so that air flow emerges from test tube at rate of approximately 150 ml/min.)

In each exposure prepare standard of 10 ml of a saturated aqueous sodium 2,4-D solution and use as check in each test by comparing with a volatile ester (butyl 2,4-D) and a low volatile formulation (butoxyethanol 2,4-D). Use exposure intervals of 2, 4, and 16 hr. Make first observations when plants are removed from under the paper bag and again at 24, 48, and 72 hr after exposure.

Evaluate volatility in terms of plant response, based on epinasty and formative effects, in 6 reaction classes designated as follows: (1) no apparent response; (2) epinasty 1–20° compared to normal, no curling; (3) epinasty 21–40° compared to normal, slight curling; (4) epinasty 41–60° compared to normal, moderate curling; (5) epinasty 61–80° compared to normal, moderately severe curling; and (6) epinasty 81° to greater than 90°, severe formative effects apparent (twisting of main stem, distortion of petioles and leaflets). Measure angles of epinasty with a protractor.

Test any given ester for each of the three exposure intervals at least three successive times; use a fresh 10 ml aliquot of the undiluted product for every test. Calculate average response, R, as follows: $R_i = NC/r$, where C is reaction class, i is exposure interval, N is number of times a given class is obtained for each exposure interval, and r is number of replications.

A deviation of not more than one reaction class for successive tests at a given exposure interval would be tolerable, since a high reading at one response might well overlap a low reading of the next higher response. In this case, $[R_i = N(C_1) + N(C_2)]/r$. Responses falling in classes 1–2 are considered to be low volatile esters; responses falling in classes 3–6 are considered volatile esters.

RECOMMENDATION

The original findings were first presented at the 67th meeting of this Association in 1953, and previously published copies of the method are available for review. The Buskin-Walker method has been investigated further and tested to prove its effectiveness under normal and high temperatures during the last three years. It is believed that the method is now ready for a collaborative test.

It is recommended* that the methods for the extent of volatility of ester formulations of 2,4-D and 2,4,5-T acids and related compounds used as herbicides, described in this report, be studied collaboratively, and that further consideration be given to biological methods.

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* For report of Subcommittee A and action of the Association, see *This Journal*, **40**, 23, 24 (1957).

No reports were given on aldrin, chlordane and toxaphene, dieldrin, dithiocarbamates, piperonyl butoxide, quaternary ammonium compounds, rodenticides, or systemic insecticides.

REPORT ON DISINFECTANTS

By L. S. STUART (Plant Pest Control Division, Agricultural Research Service, U. S. Department of Agriculture, Washington 25, D.C.), *Referee*

During the past year a number of investigations and collaborative studies of existing methods and proposed new methods have been completed. Two studies were begun and will be completed by the 1957 meeting. Some of the results of the completed studies indicate that minor changes in some existing methods might be desirable. However, additional collaborative study is necessary.

C. W. Chambers has published a minimum performance test to evaluate germicidal and sanitizing rinses for equipment and utensils in dairies, restaurants, and food plants (*J. Milk and Food Technol.*, **19**, 183 (1956)). This method was developed at the Robert A. Taft Sanitary Engineering Center of the U. S. Public Health Service. Regulatory agencies are already using the method, and Public Health Service officials have asked this Association to consider adopting it. A collaborative study was begun in August 1956. It is hoped that enough data will be available by the 1957 meeting to warrant a recommendation for its adoption.

Germicidal chemicals are currently being incorporated in textiles, rubber, plastics, and paints. The possibility that the germicides are adsorbed on the surfaces of articles fabricated from these materials makes it necessary to develop an official method for evaluating self-sterilizing, self-disinfecting, and self-sanitizing activity claims for such surfaces. At present, only one controlled laboratory method proposed for measuring such activity can be expected to yield a reasonably valid result. This method was developed at Camp Dietrick, Maryland, and has been published by R. K. Hoffman, S. B. Yeager, and S. Kaye (*Soap Chem. Specialties*, **31**, 135 (1955)). The details of this method are being standardized so that it can be accepted as official.

During the past year interest in studies to standardize disinfectant performance tests has been more active than in any previous year in the history of the Association. However, the Referee and Associate Referees have had considerable difficulty in finding State laboratory workers who can participate in the necessary collaborative studies, and thus no recommendation can be made at this time.

RECOMMENDATIONS

It is recommended*—

(1) That additional studies be made to determine the desirability of amending the present method for the sporicidal test to provide for use of an alternate hard-surface carrier with a secondary control chemical, the latter to consist of a lower concentration of hydrogen chloride than constant boiling hydrochloric acid.

(2) That the present Associate Referees be reappointed for the coming year.

* For report of Subcommittee A and action of the Association, see *This Journal*, **40**, 20, 21 (1957).

REPORT ON FUNGICIDES AND SUBCULTURE MEDIA

AVAILABLE CHLORINE GERMICIDAL EQUIVALENT CONCENTRATION TEST

By L. F. Ortenzio (Plant Pest Control Division, U. S. Department of Agriculture, Agricultural Research Center, Beltsville, Md.), *Associate Referee*

When the available chlorine germicidal equivalent concentration test was adopted as first action (1), the following minimum standards were set up for disinfectants used on previously cleaned non-porous surfaces in dairies, restaurants, food plants, and similar places: (*a*) concentrations of 50, 100, and 200 ppm of available chlorine as hypochlorite; (*b*) *p*H 8.5; (*c*) temperature of 20°C.; (*d*) an exposure period of 1 minute. Because of the historic pattern of acceptance of hypochlorites by Public Health officials, a standard of three concentrations was thought to be necessary. This acceptance pattern requires that a concentration of 200 ppm of available chlorine be recommended for general application on previously cleaned non-porous surfaces except for solutions in which the concentration of available chlorine can be controlled so that it will not fall below 50 ppm at any time. In this case, starting solutions must provide 100 ppm of available chlorine. The *p*H, time, and temperature factors in the test were selected as those most likely to assure the practical result desired.

After this first action method had been adopted, the U. S. Public Health Service revised Appendix F of the 1953 Recommendations of the Public Health Service Milk Ordinance and Code (2). By this revision, bactericides containing bromine and iodine and other formulations are accepted provided they give a bactericidal result *in use* at least equivalent to 50 ppm of available chlorine as hypochlorite at *p*H 10.0 and 75°F.

in an exposure period of 2 minutes; other products are accepted *without* direct comparison with chlorine, provided the product will give a 99.999 per cent kill of 75–125 million *E. coli* and 75–125 million *M. pyogenes* var. *aureus* within 30 seconds at 70–75°F. in the Chambers modification (3) of the Weber and Black test (4).

Therefore data obtained with some known chemicals was needed for both the first action available chlorine equivalent concentration test and the Chambers test for two reasons: (*a*) to clarify the minimum concentration standard permitted for solutions in use, and (*b*) to recommend the proper concentrations of starting solutions of germicides to assure effective working solutions in dairies, restaurants, and food plants.

The Chambers method (3) was used to study sodium hypochlorite preparations that provide 50 ppm available chlorine at *p*H 8.5 and 20°C., the conditions specified in the first action method, and at *p*H 10.0 and 75°F., the conditions specified in the revised Appendix F of the Milk Ordinance and Code. The following are typical results:

Conditions	*No. E. coli cells Exposed*	*% Reduction after Exposure for:*		
		30 sec.	*1 min.*	*2 min.*
*p*H 8.5, 20°C.	87 million	100.0	100.0	100.0
*p*H 10.0, 75°F.	94 million	99.999+	100.0	100.0

These results show clearly that the minimum permitted concentration standards of hypochlorite both in the revised Appendix of the 1953 Code and in the first action A.O.A.C. method (5) will give the end result specified in the revised code as acceptable for use without reference to chlorine as a standard, namely, a reduction in a count of at least 99.999 per cent of 75–125 million *E. coli* within 30 seconds.

Results of further studies of hypochlorite solutions at higher *p*H values and exposures at lower temperatures, and with the additional test organism *M. pyogenes* var. *aureus*, indicate that, within the range of experimental error (subject to statistical confirmation), the 99.999 per cent kill within the 30 second period is the correct end point for 50 ppm available chlorine as sodium hypochlorite at 75°F. and *p*H 10.00 in the Chambers test.

Thus no serious discrepancy seems to exist between the results for the minimum permitted standard for hypochlorite use by the Chambers test and those by the first action A.O.A.C. method. This conclusion is confirmed for the most part by results obtained by the A.O.A.C. method using hypochlorite solutions that provide 50 ppm of available chlorine at *p*H 6.7, 8.5, 10.2, and 10.7. These results are given in Table 1; they indicate that within the range of experimental error, the result is the same over the *p*H range of 6.7–10.7.

The Chambers test does not provide a means for making direct measurements of starting solution concentrations equivalent in germicidal activity to 200 and 100 ppm of available chlorine as hypochlorite. The

TABLE 1.—*Results by the first action method with 50 ppm available chlorine solution as hypochlorites at various pH levels*

pH	INCREMENT EXPOSED FOR 1 MINUTE										NO. NEGATIVE INCREMENTS
	1	2	3	4	5	6	7	8	9	10	
6.7	−	−	+	+	+	+	+	+	+	+	2
8.5	−	−	+	+	+	+	+	+	+	+	2
10.2	−	+	+	+	+	+	+	+	+	+	1
10.7	−	+	+	+	+	+	+	+	+	+	1

first action A.O.A.C. method does provide such a measurement, and at present it is the only available guide to such concentration activity equivalents.

Data obtained with Chloramine T preparations (U.S.P.) at *p*H 6.7, 8.3, and 9.6 illustrate quite clearly the type of comparisons that may be drawn (see Table 2).

TABLE 2.—*Results of tests of U.S.P. Chloramine T preparations by the first action available chlorine germicidal equivalent concentration method, using S. typhosa*

AVAILABLE CHLORINE CONCENTRATIONS	NO. OF CONSECUTIVELY NEGATIVE INCREMENTS			
	NaOCl CONTROL, *p*H 8.5	CHLORAMINE T		
		*p*H 6.7	*p*H 8.3	*p*H 9.6
ppm				
50	1	0	0	0
100	3	10	0	0
200	5	10	0	0

These data show that available chlorine provided by Chloramine T at *p*H 8.3 and 9.6 does not give a result equivalent to available chlorine as hypochlorite at *p*H 8.5. At *p*H 6.7, however, a superior result is obtained with the organic chlorine preparation at the 100 and 200 ppm levels. Thus, the acceptability of the recommended starting solutions for products of the Chloramine T type depend on the *p*H of the use solutions. If the *p*H is relatively high, concentrations providing considerably more available chlorine than 100 and 200 ppm must be recommended.

In another phase of the study, timing schedules were modified to provide 30 second and 2 minute exposure intervals instead of the 1 minute specified. Results of these studies showed only minor differences in available chlorine as hypochlorite (that is, within the range of experimental variation of ±1 increment, determined for the method in 1955 collaborative studies); however, they did indicate that the timing schedule is a critical factor in results for germicides that act at a slower rate than hypochlorites do. Chloramine T was not observed to kill when the 30 second

timing schedule was used with *p*H 6.7, 8.3, or 9.6 and available chlorine concentrations of 50, 100, and 200 ppm, respectively. On the other hand, when a 2 minute exposure interval was employed, at *p*H 6.7 Chloramine T killed completely in all 10 increments at these three concentrations. The increased exposure interval did not improve killing at *p*H 8.3 and 9.6.

DISCUSSION AND RECOMMENDATION

No serious discrepancy exists between the minimum standards for solutions *in use* as germicidal rinses for previously cleaned non-porous surfaces in dairies, restaurants, and food plants by the first action available chlorine germicidal equivalent concentration method and those indicated as acceptable by the recent revision of Appendix F of the 1953 Recommendations of the U. S. Public Health Service Milk Ordinance Code.

For preparations containing compounds of iodine, bromine, and organic chlorine, the acceptance pattern in the revised Appendix F is based on activity equivalent to available chlorine as hypochlorite; the first action A.O.A.C. test provides a convenient and workable procedure for determining concentrations of these compounds equivalent to the commonly accepted recommendations for starting solutions of hypochlorites, namely, solutions providing 100 and 200 ppm of available chlorine.

The 1 minute exposure periods employed in the present procedure appear to give results more in line with those obtained in the Chambers test as recommended by the U. S. Public Health Service than do the modified timing schedules employing 30 second and 2 minute exposure periods.

It is recommended* that the study be continued.

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* For report of Subcommittee A and action of the Association, see *This Journal*, **40**, 20, 21 (1957).

REPORT ON MEDIUM FOR DISINFECTANT TESTING

By Michael J. Pelczar, Jr. (Department of Bacteriology, University of Maryland, College Park, Md.), *Associate Referee*

Previous work in our laboratory had indicated that a medium of the following composition would be suitable for use in the A.O.A.C. method

for determination of phenol coefficients (*Official Methods of Analysis*, 8th Ed., 1955, **5.177–5.180**):

	per cent
Peptone (peptic digest of animal tissue; a bacteriological peptone, U.S.P.)	2.0
NaCl	0.5
K_2HPO_4	0.2
Glucose	0.05
*p*H 6.8	

Accordingly, at the December 1955 meeting the Scientific Disinfectant Committee of the Chemical Specialties Manufacturers' Association decided to undertake a collaborative study to evaluate this new medium. Furthermore, they requested two commercial laboratories to prepare experimental lots of a dehydrated medium conforming to this formula for use in the investigation.

Two test compounds were submitted to each laboratory participating in the study, and phenol coefficients were determined by both the experimental medium and the A.O.A.C. medium. Eight laboratories participated in this study. The results obtained with the experimental medium compared very favorably with those obtained with the A.O.A.C. formula.

Plans have been made to continue collaborative testing. L. S. Stuart, the Referee, will supply test compounds appropriate for such work. The manner of performing the test and reporting results will be standardized to allow for a statistical evaluation of the completed study.

It is recommended* that the study be continued.

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 20, 21 (1957).

REPORT ON SPORICIDAL TESTS

By J. L. Friedl (Plant Pest Control Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Md.), *Associate Referee*

A number of complaints have been received from bacteriologists to the effect that the first action sporicide test (1) is too severe to be a practical index to sporicidal concentrations in all types of applications. Undoubtedly the present procedure is highly critical, but this was considered essential for such a test because of the very critical uses for which such products are commonly recommended. However, some of the provisions of the procedure specifically objected to have been re-evaluated to determine whether any of the suggested changes should be studied collaboratively.

It has been alleged that the present method should be amended to specify use of a definite number of spores in each instance. It is well known that resistance in laboratory dilution tube tests increases as the number of exposed spores increases. The present carrier procedure is standardized only with respect to the number of spores occurring in a specific type of culture that has a prescribed resistance to a control chemical in which vegetative cells cannot survive. It has also been alleged that this procedure may cause an unreasonably large number of spores to be used in many instances, particularly because the silk-suture loop carrier tends to adsorb more spores from the culture than a hard surface carrier does.

In studying this factor, spore counts were made on the standardized silk-suture loops and on individual porcelain penicillin rings with 6 spore-forming bacteria. Brewer anaerobic Petri dishes and anaerobic agar were used with the anaerobes; standard 110 mm Petri dishes and nutrient agar were used with the aerobes. The suture loops and porcelain rings were contaminated and dried as prescribed, placed in 100 ml individual sterile water blanks, and heated to 80°C. for 1 hour to kill the vegetative cells. The water blanks were then shaken in a mechanical shaker for 30 minutes, and dilution plate counts were made from them. All plates were incubated at 37°C. for 48 hours.

The results given in Table 1 show that the contamination on the prescribed suture loop carrier may vary with the organism from 320 thousand spores to as many as 14 million spores. However, within the range of experimental error, there seems to be no substantial difference between the number of spores found on the standard suture loop carrier and on the hard surface porcelain penicillin ring carrier.

Since spore numbers appear to be characteristic of the specific organism, rather than other factors, standardization of a fixed number of spores for all species might be almost impossible to achieve. It would certainly introduce an additional complicating factor, and could be considered seriously only if this factor is eventually shown to be highly critical in obtaining the final result in this test.

With liquid germicides, it has been stated that the surface of the silk

TABLE 1.—*Spore counts found on suture-loop carriers and porcelain rings*

ORGANISMS	NUMBER OF SPORES	
	BY SUTURE LOOP	BY PORCELAIN RING
Cl. sporogenes	14,100,000	7,600,000
Cl. botulinum	320,000	520,000
Cl. tetani	1,020,000	10,100,000
Cl. perfringens	720,000	1,940,000
B. subtilis	1,600,000	3,900,000
B. globigii	3,500,000	4,400,000

suture exerts a significantly greater protective influence on the adsorbed spores than hard surfaces do.

A study was therefore made with peracetic acid, a chemical commonly acknowledged to have practical sporicidal values, in which spores of 7 organisms were exposed to varying concentrations after adsorption on the standard suture loop and on the hard surface of porcelain penicillin rings. The first action method was followed exactly, except for the introduction of the alternate carrier and the inclusion of a 10 per cent hydrogen chloride control in addition to the constant boiling hydrochloric acid control.

The results, summarized in Table 2, show that the protective action of the silk-suture loop carrier against the cidal effects of both hydrogen chloride and peracetic acid solutions on the adsorbed spores is much greater than the effect of the surface of the porcelain ring carrier. The effect on the anaerobic spores is so great that the spores of only one organism, *B. subtilis*, showed a measurable resistance to the constant boiling hydrochloric acid control when adsorbed on the hard surface carrier, although all 7 organisms showed measurable resistance to this control when absorbed on the silk-suture loop. Five of the 7 test organisms did produce spores of measurable resistance to 10 per cent hydrogen chloride when adsorbed on the hard surface rings. Peracetic acid was sporicidal against all of the spores tested when exposed after adsorption on the suture-loop carriers, but concentrations had to be much lower and exposure intervals shorter to obtain a sporicidal result with the hard surface porcelain carrier.

DISCUSSION AND RECOMMENDATIONS

The protective influence of various surfaces for bacteria exposed to liquid germicidal chemicals has been discussed at some length by Flannery, Friedl, Ortenzio, and Stuart (2) and by Stedman, Kravitz, and Bell (3). It is not surprising, therefore, that the protective effect of the surface of silk sutures for adsorbed spores can be measured. However, the magnitude of this effect as compared to that of a hard surface carrier and the fact that it sometimes appears to be more specific for anaerobic spores than for aerobic spores is somewhat disturbing. The results found do not seem to invalidate the method for determining practical sporicidal concentrations and exposure times for applications on uncleaned surfaces or porous absorbent surfaces, but they indicate that the method may be too severe for use as an index to sporicidal concentrations and exposure times in the case of relatively clean, hard-surface applications. The problem cannot be solved by amending the method to include the hard surface porcelain ring carrier as an alternate to the silk suture loop carrier to provide an index to values on hard surfaces; it is doubtful whether all the various species that might have to be tested could produce spores showing measurable resistance to the control chemical—constant boiling hydro-

TABLE 2.—*Resistance of spores to hydrogen chloride and peracetic acid when exposed on silk-suture loops and procelain ring carriers*

ORGANISMS	SILK-SUTURE EXPOSURES						PORCELAIN RING EXPOSURES					
	HCl		PERACETIC ACID				HCl		PERACETIC ACID			
	CONSTANT BOILING	10%	0.2%	0.4%	2.0%	4.0%	CONSTANT BOILING	10%	0.2%	0.4%	2.0%	4.0%
	SURVIVAL TIME IN MINUTES											
Cl. sporogenes	>60	>60	>60	60	10	10	<5	5	10	<5	<5	<5
Cl. botulinum	>60	>60	>60	60	20	20	<5	< 5	5	5	<5	<5
Cl. tetani	20	30	>60	60	30	20	<5	< 5	10	5	<5	<5
Cl. perfringens	30	30	>60	30	20	20	<5	10	5	<5	<5	<5
B. subtilis	10	20	>60	30	10	10	20	>60	10	<5	<5	<5
B. coagulans	30	>60	20	5	5	<5	<5	>60	5	<5	<5	<5
B. globigii	10	>60	< 5	< 5	< 5	<5	<5	>60	5	<5	<5	<5

chloric acid—when exposed after adsorption on a hard surface. Spaulding (4) suggested that 1*N* hydrochloric acid be used as the control chemical for sporicidal tests on instrument germicides. The results with 10 per cent hydrogen chloride (approximately 2.8*N* hydrochloric acid) and the hard surface carrier suggest that this might be the highest concentration feasible for a secondary standard, and possibly, that a lower concentration would be more practical.

It is recommended* that additional studies be made to determine the desirability of amending the present method to provide for use of an alternate hard-surface carrier with a secondary control chemical, the latter to consist of a lower concentration of hydrogen chloride than constant boiling hydrochloric acid. The actual concentration selected should probably be within the range of 1*N* to 2.8*N* hydrochloric acid; the most practical concentration within this range must be determined experimentally.

The results obtained in these studies with peracetic acid should be compared with a report of practical sporicidal use values recently published by Trexler (5). He reported the successful use of 1 and 2 per cent solutions of peracetic acid for sterilizing the uniforms in the personnel lockers of sterile rooms used to maintain germ-free animal colonies at the Lobund Institute. Since 2.0 per cent peracetic acid was found to be an effective sporicide for all of the test spores examined at intervals considerably shorter than one hour in the first action method, the present method appears to give a result indicative of practical sporicidal values in general applications. It is doubtful if the more favorable results obtained with the ring carrier at concentrations of 0.2 and 0.4 per cent in these investigations would provide a reliable index to practical values in this type of application. Thus the recommendation for additional studies to amend the present method for special types of applications should not be construed as meaning that the existing procedure is not accurate enough for general applications.

ACKNOWLEDGMENT

The peracetic acid used in these studies was furnished by the Becco Chemical Division, Food Machinery and Chemical Corporation, Buffalo, N.Y.

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* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 20, 21 (1957).

REPORT ON MEAT AND MEAT PRODUCTS

By R. M. MEHURIN (Meat Inspection Branch, Agricultural Research Service, U. S. Department of Agriculture, Washington 25, D.C.), *Referee*

Because of the shortage of qualified technical assistants, the Associate Referee on Serological Tests for Identification of Meats has been unable to undertake collaborative work on the method he previously discussed (*This Journal*, **37**, 843 (1954)). He expects to be able to do so during the coming year.

A considerable improvement has been made in the method for lactose in meat food products. Previously, a yeast specific for maltose had been tried out, but this work was suspended when it appeared that the readily obtainable bakers' yeast could be made acceptable by the maltose treatment described by the Associate Referee.

The Associate Referee on Moisture and Fat in Meat Products has presented an interesting and informative comparison of the official method with rapid methods for fat determination. While good agreement in results was reported for the types of meat products examined, it is believed that for all types of product and for all ranges of fat content, the official method is more dependable.

Definite progress has been made by the newly appointed Associate Referee on Starch in Meat Products. He has apparently located the chief cause of the occasional wide fluctuations previously reported by collaborators. Collaborative work on the modified procedure appears fully justified.

RECOMMENDATIONS

It is recommended*—

(1) That the modified method for lactose in meat products (i.e., nonfat dry milk) be adopted as first action and that collaborative work be continued.

(2) That collaborative work be carried out on the modified method for starch in meat products.

(3) That the serological method for the detection of horse meat, described by the Associate Referee in 1954, be studied collaboratively.

(4) That studies of chemical methods for detection of horse meat, including spectrophotometric procedures, be continued.

(5) That work on fat in meat products be discontinued.

* For report of Subcommittee C and action of the Association, see *This Journal*, 40, 33, 34 (1957).

REPORT ON RAPID METHODS FOR DETERMINATION OF FAT IN MEAT PRODUCTS

By E. S. WINDHAM (Division of Veterinary Medicine, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington 12, D.C.), *Associate Referee*

The official method for determination of fat in meats is not suitable for all applications where a knowledge of fat content is desired. It is of necessity a laboratory test and considerable time is required to obtain the results. Several rapid methods have been recommended for use when the fat content must be known immediately. The Associate Referee investigated a number of methods and selected three for further study.

METHODS

Steinlite Method.—This method uses an instrument, instructions, and calibration charts furnished by the Fred Stein Laboratories, Achison, Kansas. The fat from a weighed 50 or 75 g sample is extracted with 100 ml of standardized *o*-dichlorobenzene by a high speed blender. The extract is filtered, the filtrate is placed in a special condenser cell in the instrument, and a scale reading is made. After temperature correction, the scale reading is converted to per cent fat by using calibration charts, furnished with the instrument, that were prepared by taking readings on the solvent containing various known amounts of fat.

Modified Babcock Method.—This method was previously reported by the Associate Referee (*This Journal*, **38**, 210 (1955). Sulfuric acid is used to dissolve a weighed sample in a Paley-Babcock test bottle. The method is similar to the Babcock fat test for cream, except the 5 minute centrifugation step is omitted.

Perchloric-Acetic Acid Babcock Method.—The method was reported by Harold Salwin, I. K. Bloch, and J. H. Mitchell, Jr., of the Armed Forces Food and Container Institute Laboratory (*J. Agr. and Food Chem.*, **3**, 588 (1955). The sample is placed in the Paley-Babcock bottle and is digested by heating in a boiling water bath with a mixture of 60 per cent perchloric acid and glacial acetic acid (1+1). The time required for dissolving the meat is 10 to 20 minutes. The fat layer is brought up into the graduated portion of the bottle by adding more perchloric-acetic acid mixture. The test sample is centrifuged for 2 minutes and the per cent fat is read as in the Babcock cream test.

RESULTS AND RECOMMENDATION

A total of 18 samples of meat products were analyzed for fat content by each of the three rapid methods and by the official method in which petroleum ether is used as the extracting solvent. Triplicate analyses were per-

TABLE 1.—*Comparison of methods of analysis for fat in meat products*

PRODUCT	OFFICIAL		STEINLITE		MODIFIED BABCOCK		PERCHLORIC-ACETIC ACID-BABCOCK	
	AVERAGE % FAT	RANGE	AVERAGE % FAT	RANGE	AVERAGE % FAT	RANGE	AVERAGE % FAT	RANGE
Ground beef	13.7	0.3	14.6	0.1	13.7	0.2	14.3	0.4
	22.8	1.1	24.1	1.1	23.0	0.0	24.0	0.8
	22.2	0.8	21.7	0.2	22.2	0.0	23.0	0.0
	21.5	0.5	22.2	1.2	21.8	0.5	23.1	0.2
	15.5	0.7	17.0	0.7	15.4	0.3	16.3	0.3
	17.6	0.2	19.1	0.6	17.2	0.4	18.3	0.3
	26.2	0.0	26.5	0.5	26.2	0.3	27.5	0.5
	25.5	0.2	26.0	0.2	25.4	0.2	27.0	0.4
	22.2	0.2	21.5	0.7	22.2	0.0	23.2	0.5
	18.0	0.6	18.0	1.0	18.3	0.2	19.5	0.3
Pork sausage	47.8	1.1	48.2	0.3	48.5	0.6	49.1	0.2
	52.9	1.2	52.8	1.0	52.8	2.4	54.6	1.0
	52.2	0.1	52.7	0.3	52.3	0.4	52.7	0.2
Frankfurter	29.1	0.3	28.4	0.9	30.4	0.8	30.9	0.2
	31.4	0.1	31.1	0.4	31.4	0.3	33.5	0.5
	22.2	0.3	21.8	0.1	22.2	0.1	23.3	0.5
Beef and gravy	9.8	0.2	10.8	0.2	10.4	0.2	10.5	0.1
	6.6	0.1	6.8	0.1	6.6	0.0	6.7	0.0
Averages	25.40	0.44	25.73	0.53	25.55	0.38	26.53	0.38

formed by each method except the Steinlite procedure. For the Steinlite procedure, duplicate samples were prepared and duplicate instrument readings were made on each sample preparation.

A comparison of the results by the three methods and by the official method is given in Table 1. The reproducibility of the rapid methods was comparable to that of the official method, as shown by the average range of values found among replicate determinations. The fat percentages determined by the Steinlite procedure and by the modified Babcock procedure are in close agreement with those obtained by the official method.

The perchloric-acetic acid Babcock method tends to give a higher percentage of fat in any sample than the official procedure does. The average per cent fat on all samples by this method was 104.5 per cent of the average official value. Investigation showed that the fat separated by the perchloric-acetic acid method contained about 5 per cent acid, calculated as acetic acid. Thus, when the per cent fat determined by this method is multiplied by a factor of 0.95, the corrected values approximate the fat percentage obtained by the official method.

It is recommended* that study of these methods be discontinued.

For those who use the perchloric-acetic acid method, it is suggested that a correction factor of 0.95 be appropriately applied to the per cent fat found.

Each of the three rapid methods gives sufficiently accurate results to serve for plant control, market survey, or screening uses.

* For report of Subcommittee C and action of the Association, see *This Journal*, **40**, 33, 34 (1957).

REPORT ON STARCH IN MEAT PRODUCTS

By J. F. BLANCHARD (Food and Drug Laboratories, Department of National Health and Welfare, Winnipeg, Canada), *Associate Referee*

Two methods for the estimation of starch in meat products have been reported by Stevens and Chapman (*This Journal*, **36**, 292 (1953); **38**, 202 (1955)). In both methods the soluble reducing substances were extracted in the presence of a precipitate of zinc ferrocyanide. After hydrolysis, the starch was estimated by two methods: with modified Fehling's reagent, and colorimetrically with anthrone.

The collaborative results of the Fehling's method showed average recoveries varying from 103.5 to 110.3 per cent. The anthrone method gave low recoveries, ranging from 93.9 to 96.3 per cent.

Preliminary work was done on the anthrone method. However, we found quite a variation in blanks. Another objection to the method is that the anthrone must be recrystallized before the reagent is made up. Moreover, since the anthrone is dissolved in sulfuric acid of high concentration, it is somewhat hazardous to use for routine analyses. For these reasons it was decided to defer further work on this method until the potentialities of the Fehling's method had been explored.

The high values obtained were confirmed and it was also noted that the results of analyses varied considerably. It was discovered that the high values were due to the breakdown of the filter paper. Table 1 shows results obtained on hydrolysis of starch with and without the addition of filter paper and, also, the apparent starch obtained by hydrolysis of paper alone.

The extraction method outlined by Stevens and Chapman in 1953 was therefore modified to eliminate the filter paper, in the following manner: After centrifuging and filtering, the funnel containing the filter paper was transferred to the centrifuge bottle. From a cylinder containing 90 ml hot 1.5 *N* HCl (about 70°C.) 40 ml was poured into the filter paper to melt the adhering fat and free the starch. A hole was poked in the tip of the fil-

TABLE 1.—*Apparent content of starch with and without the addition of filter paper*

	WITH FILTER PAPER	WITHOUT FILTER PAPER	DIFFERENCE
	per cent	*per cent*	*per cent*
Meat and starch extracted and hydrolyzed	3.32	2.90	0.42
	3.96	3.45	0.51
	4.12	3.60	0.52
	3.67	3.52	0.15
Starch hydrolyzed	3.73	3.44	0.29
	3.78	3.30	0.48
	3.93	3.42	0.51
	3.96	3.38	0.58
Meat extracted and hydrolyzed	0.26	0.07	0.19
Filter paper hydrolyzed	0.28	—	—
	0.38		
	0.31		

ter paper and the acid was allowed to run into the centrifuge bottle. The filter paper was then washed with the remainder of the acid.

The sample was then hydrolyzed for 90 minutes instead of 105 minutes and the reducing sugars were determined by the modified Fehling's method. This method was used in subsequent analyses.

Table 2 shows the effect of hydrolysis of starch with and without meat on unextracted and extracted samples. It can be seen that although the apparent starch content of a mixture of starch with meat on hydrolysis is higher than that obtained without meat, the reverse is noted when the samples are extracted before hydrolysis. Starch was then added to meat in varying amounts, and was extracted and hydrolyzed. The results are shown in Table 3. It will be observed that the recoveries increase as the amount of starch increases; this suggests that a constant factor is depressing the recovery of the starch.

Since experiments showed that neither the zinc ferrocyanide nor the phosphotungstic acid, used as the protein precipitant, had any effect on the analysis of starch, meat was hydrolyzed with and without extraction and dextrose was added to the hydrolysate. The results obtained are shown in Table 4. There is an apparent loss of dextrose in both cases. It is thought that this may be due to residual protein present in the hydrolysate, as insufficient phosphotungstic acid is used for complete precipitation.

The modified Fehling's method described in this report is not considered

TABLE 2.—*Effect of hydrolysis on starch content of unextracted and extracted samples without and with meat*[a]

HYDROLYZED WITHOUT MEAT		HYDROLYZED WITH MEAT		EXTRACTED AND HYDROLYZED WITHOUT MEAT		EXTRACTED AND HYDROLYZED WITH MEAT	
STARCH FOUND, GRAMS	PER CENT RECOVERY	STARCH FOUND, GRAMS	PER CENT RECOVERY	STARCH FOUND, GRAMS	PER CENT RECOVERY	STARCH FOUND, GRAMS	PER CENT RECOVERY
Casco Potato Starch							
3.365 (Av. of 14)	97.7	3.39	98.4	3.36	97.6	3.21 (Av. of 14)	93.2
		3.33	96.7	3.36	97.6		
		3.42	99.3	3.28	95.2		
				3.36	97.6		
Bullmeat Flour Binder							
3.05	92.2	3.29	99.5	3.04	91.9	3.07	92.8
3.03	91.6	3.15	95.3	3.04		2.95	89.2
		3.28	99.2				
No Starch							
—	—	0.14	—	—	—	−0.10	—
		0.04				0.08	
		0.12				0.00	
		0.13					
		0.18					

[a] Total carbohydrate calculated as starch.

TABLE 3.—*Effect of hydrolysis on extracted starch and meat mixtures*

CASCO POTATO STARCH ADDED, PER CENT	PER CENT FOUND	PER CENT RECOVERY
2	1.46	84.8
3	2.43	94.1
4	3.35	97.3
5	4.18	97.1

TABLE 4.—*Recovery of dextrose added to hydrolyzed unextracted and extracted meat*

PER CENT DEXTROSE ADDED	UNEXTRACTED		EXTRACTED	
	PER CENT DEXTROSE FOUND	PER CENT RECOVERY	PER CENT DEXTROSE FOUND	PER CENT RECOVERY
2	1.82	91.0	1.77	88.5
3	2.86	95.3	2.93	97.6
4	3.98	99.5	4.02	100.5

satisfactory enough for collaborative study. It is recommended* that the study be continued.

ACKNOWLEDGMENTS

Acknowledgment is given to J. Dorozynsi who did the bulk of the analyses and also to E. J. Beverly, A. B. Tennenhouse, and L. A. Moroz for their analytical assistance.

* For report of Subcommittee C and action of the Association, see *This Journal*, 40, 33, 34 (1957).

REPORT ON LACTOSE IN MEAT PRODUCTS

DETERMINATION OF LACTOSE IN PRESENCE OF MALTOSE AND DEXTROSE

By H. R. Cook (Meat Inspection Branch, Agricultural Research Service, U. S. Department of Agriculture, Washington 25, D.C.), *Associate Referee*

The principal obstacle in the determination of lactose in meat products is the interference of maltose. The present method utilizes selective fermentation of the dextrose from the lactose and subsequent analysis for lactose by a copper reduction procedure, using Benedict's solution. In the past this method has been adequate for controlling the use of non-fat milk solids in meat food products. However, in recent years the use of additives containing maltose, such as corn sirup solids, has become widespread, and thus a better procedure is needed to separate lactose from other reducing sugars. A method has been developed for efficient separation of lactose from maltose and dextrose, the most common reducing sugars encountered in meat products.

Details of this method have been published in *This Journal*, **40**, 57 (1957).

Three samples were submitted to four laboratories for collaborative study of the method. By analysis, the samples were composed as follows: Sample A, 1.50 per cent lactose and 0.50 per cent dextrose; Sample B, 1.50 per cent maltose and 0.50 per cent dextrose; Sample C, 1.20 per cent lactose and 0.80 per cent maltose.

RESULTS

Individual results are shown in Table 1.

A few collaborators criticized the time required to acclimate the yeast but no one had difficulty in applying the method. The collaborative re-

TABLE 1.—*Collaborative results for per cent lactose in meat*

SAMPLE	PER CENT LACTOSE FOUND									
	COLL. 1	COLL. 2	COLL. 3	COLL. 4	COLL. 5	COLL. 6	COLL. 7	COLL. 8	COLL. 9	AV.
A	1.41	1.47	1.53	1.51	1.47	1.51	1.47	1.48	1.48	1.49
	1.45	1.41	1.51	1.53	1.50	1.65	1.47	1.46	1.48	
									1.43	
B	0.24	0.09	0.00	0.00	0.09	0.02	0.04	0.03	0.08	0.07
	0.24	0.07	0.00	0.00	0.09	0.09	0.04	0.03	0.01	
									0.10	
C	1.21	1.21	1.27	1.27	1.15	1.35	1.33	1.27	1.30	1.28
	1.24	1.27	1.26	1.27	1.25	1.37	1.34	1.27	1.33	
									1.30	

sults show that 6.6 per cent of the maltose remained after fermentation and 99.3 per cent of the lactose was recovered.

RECOMMENDATIONS

It is recommended*—

(1) That further work be done to better acclimate the yeast and reduce the time required for acclimating.

(2) That collaborative study be continued.

* For report of Subcommittee C and action of the Association, see *This Journal*, **40**, 33, 34 (1957).

No report was given on serological tests for identification of meats.

REPORT ON CEREALS

By V. E. MUNSEY (Division of Food, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D.C.), *Referee*

It is recommended*—

(1) That the study of the determination of starch in cereals be continued.

(2) That the study of the ferricyanide method for determination of sugar in baked products be continued.

(3) That the study of the determination of lactose in bread be continued.

* For report of Subcommittee D and action of the Association, see *This Journal*, **40**, 35 (1957).

(4) That the first action qualitative test for iodates and bromates and for iodates alone, **13.44** and **13.45**, be made official.

(5) That the first action method for the quantitative determination of bromates in white flour and whole wheat flour, *This Journal*, **39**, 87 (1956), be made official.

(6) That the study of moisture determinations be continued and include collaborative study of the 103° air oven method and Karl Fischer method suggested by the Associate Referee.

(7) That the first action method for diastatic activity of flour, **17.5**, **17.6**, and **17.7**, *This Journal*, **38**, 562 (1955), be made official.

REPORT ON SUGARS IN BAKED PRODUCTS

By R. P. SMITH (National Biscuit Co., New York, N. Y.), *Associate Referee*

At the recommendation of the Association (1), study was continued on the adaptation of the alkaline ferricyanide method for reducing and non-reducing sugars in flour (2) to the determination of these sugars in baked products.

The collaborative results of sugar determinations by the alkaline ferricyanide method, obtained on a standard cookie formula and reported by the Associate Referee (3), indicate that the method is feasible in spite of the discrepancies between values obtained by this method and those by the standard Munson-Walker method (4).

As mentioned in last year's report on sugars in baked products (5), the difficulty appeared to be in the reducing effect of non-sugar constituents on the ferricyanide itself. A study was made of several clarification reagents to reduce or eliminate this effect, and it was found that a combination of two reagents, together with a shorter time in the boiling water bath, lowered the reducing effect of the non-sugar constituents. The two clarification reagents used were sodium tungstate and the Somogyi reagent (6). The use of the two reagents involved an additional step in the procedure but did appear to give improved results.

Further study has shown that satisfactory results could be obtained when the Somogyi reagent alone was used and the sodium tungstate, the original clarification reagent in the method from which this is adapted, was omitted. By using the Somogyi reagent alone and the usual 20 minutes' reduction in the water bath, values were obtained which were closest to those obtained by the Munson-Walker method (4). The acid buffer solution used in the original method for sugars in flour was eliminated be-

cause the Somogyi reagent functions most efficiently in a neutral medium, whereas the sodium tungstate reacts most effectively in a medium at about *p*H 1.1. When the acid buffer solution was omitted, there was no difficulty in completely extracting the sugars from the standard cookie samples. In addition, by conducting the clarification in a nearly neutral medium, the effect of any inversion due to acid buffer was eliminated. Inversion for the determination of sucrose was accomplished by adding 1 :10 hydrochloric acid just before heating in the water bath. The excess hydrochloric acid did not have to be neutralized because adding the alkaline ferricyanide itself brings the *p*H to 9.4, the proper range for reduction of the ferricyanide by the sugars.

Standard cookie samples containing various sugar concentrations were prepared and tested by the method; the Somogyi reagent alone was used as the clarifying agent and the acid buffer solution was omitted. The results obtained for seven separate analyses are shown in Table 1. These results, although not as accurate as desired, were quite promising and a definite improvement over previous work.

Before further refinements of the method were attempted, it was decided to submit the method to collaborative study to determine whether

TABLE 1.—*Comparison of results for sugars in standard cookie samples by Munson-Walker method and by alkaline ferricyanide method*

METHOD	SAMPLE A (DIFF.)	SAMPLE B (DIFF.)	SAMPLE C (DIFF.)
	Invert Sugar		
Munson-Walker, **29.38**	1.45	4.96	8.37
Alkaline ferricyanide method	1.57(+0.12)	4.97(+0.01)	8.22(−0.15)
	1.57(+0.12)	4.96	8.20(−0.17)
	1.62(+0.17)	4.95(−0.01)	8.20(−0.17)
	1.61(+0.16)	4.90(−0.06)	8.24(−0.13)
	1.55(+0.12)	5.04(−0.08)	8.45(+0.08)
	1.59(+0.14)	4.93(−0.03)	8.20(−0.17)
	1.55(+0.12)	4.99(+0.03)	8.41(+0.04)
	Sucrose		
Munson-Walker, **29.38**	2.52	6.92	10.19
Alkaline ferricyanide method	2.57(+0.05)	6.89(−0.03)	10.51(+0.32)
	2.57(+0.05)	6.82(−0.10)	10.47(+0.28)
	2.57(+0.05)	6.94(+0.02)	10.46(+0.28)
	2.50(−0.02)	7.00(+0.08)	10.10(−0.09)
	2.57(+0.05)	6.83(−0.09)	10.67(+0.48)
	2.58(+0.06)	6.81(−0.11)	10.73(+0.54)
	2.58(+0.06)	6.88(−0.04)	10.63(+0.44)

TABLE 2.—*Collaborative study of the alkaline ferricyanide method*

METHOD	SAMPLE A (DIFF.)	SAMPLE B (DIFF.)	SAMPLE C (DIFF.)
	Invert Sugar		
Munson-Walker, **29.38**	1.45	4.96	8.37
Alkaline ferricyanide method			
Collaborator 1	1.58(+0.13)	4.97(+0.01)	8.30(−0.07)
Collaborator 2	1.53(+0.08)	4.84(−0.12)	7.96(−0.41)
	Sucrose		
Munson-Walker, **29.38**	2.52	6.92	10.10
Alkaline ferricyanide method			
Collaborator 1	2.57(+0.05)	6.88(−0.04)	10.47(+0.37)
Collaborator 2	2.58(+0.06)	6.36(−0.52)	9.88(−0.22)

it was reproducible. Time did not permit extensive collaborative work but results obtained to date are given in Table 2.

A study of the work done on this method so far indicates that it should prove feasible with additional modifications.

It is recommended* that study of this method be continued to eliminate the remaining sources of error, and that the modified method be submitted to additional collaborative study.

ACKNOWLEDGMENT

The author wishes to express his appreciation to W. R. Moran of this laboratory for his cooperation in this study.

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No reports were given on bromates in flour, egg content of cereal foods, milk solids and butterfat in bread, or starch in raw and cooked cereals.

* For report of Subcommittee D and action of the Association, see *This Journal*, **40**, 35 (1957).

REPORT ON NUTS

By A. M. HENRY (Food and Drug Administration, Department of Health, Education, and Welfare, Atlanta 3, Ga.), *Referee*

Studies of the methods for moisture, crude fat, crude protein, crude fiber, and ash of nut products indicate that the proposed methods are satisfactory. Collaborative samples for these determinations will be sent out in the near future.

The methods for sucrose and reducing sugars and for sodium chloride have been studied further. D. C. Healton, Atlanta District, has done considerable work on these methods but his results are not ready for submission at this time.

No work has been done on the method for hydrogenated oil in nuts.

Associate Referee Griffin has studied additional methods for propylene glycol, glycerine, and sorbitol in coconut. He has reported as follows:

PROPYLENE GLYCOL AND GLYCERINE IN DESICCATED COCONUT

"The method proposed by Neish (*Can. J. Research*, **28(B)**, 535 (1950) for propylene glycol and glycerine in fermentation residues has been applied to the determination of glycols in coconut with some success.

"In knowns containing 0.40 per cent propylene glycol and 0.41 per cent glycerine, the recoveries were 0.47 per cent propylene glycol and 0.44 per cent glycerine after correction was made for blanks of 0.21 per cent for propylene glycol and 0.07 per cent for glycerine.

"It is believed that the method can be applied successfully to coconut. This year it was tried on imitation vanilla flavors; this application should solve many difficulties in regard to using the method for coconut, since the problems are similar.

"The *p*H of the solution added to the column is critical. It was found that if the ratios of propylene glycol and glycerine were varied beyond 3:1 or 1:3, the column failed to give a quantitative separation. This problem was solved by adjusting the *p*H of the solution to 8–10. It is believed that such treatment of the solution extracted from the coconut will decrease the blank.

"The apparatus for the distillation method for propylene glycol and glycerine in coconut has been modified, and the method will be studied further.

"It is recommended* that the distillation method and the application of the Neish method to glycols in coconut be further studied."

The Referee recommends* that all studies under this topic be continued.

* For report of Subcommittee C and action of the Association, see *This Journal*, 40, 33 (1957).

REPORT ON SPICES AND OTHER CONDIMENTS

By E. C. Deal (Food and Drug Administration, Department of Health, Education, and Welfare, New Orleans 16, La.), *Referee*

Two reports were received by the Referee this year. Associate Referee Roe submitted a progress report covering work done on the determination of volatile oil in brown mustard and commercial components prepared from it. He tried out a modification of the A.O.A.C. method; it gave higher and more consistent results on coarsely ground mustard seed. Previously reported results of assays by the official method (*This Journal*, **39**, 645 (1956)) had indicated a volatile oil content of less than half that of the flour prepared from the same seed.

Associate Referee Carson reported the results of work in which he used a modification of the Clevenger method to determine volatile oil in spices. He studied the effect of varying the heating mantle—i.e., varying the temperature in the distillation flask—on the recovery of volatile oil. He also studied other variables, such as ratio of sample weight to size of distillation flask and the effect on volume readings of letting the distilled volatile oil stand overnight. The Associate Referee compared the efficiency of the official Clevenger trap with that of a modified trap described by Lee and Ogg (*Ibid.*, **39**, 806 (1956)). He concluded that the method is very empirical and that consistent results can be obtained only by adhering closely to the prescribed conditions.

Last year Subcommittee C recommended that studies be conducted on the determination of caramel, tartrates, free mineral acids, and sorbitol in vinegar, and that the study of methods for the determination of ash and pungent principles in prepared mustards and mustard flour and for volatile oil in spices be continued (*Ibid.*, **39**, 75 (1956)). The Referee recommends* that these studies be continued and that methods for the determination of sugar in prepared mustards be studied as a separate topic.

* For report of Subcommittee C and action of the Association, see *This Journal*, **40**, 33, 34 (1957).

REPORT ON VOLATILE OIL IN SPICES

By N. Aubrey Carson (Food and Drug Administration, Department of Health, Education, and Welfare, St. Louis 1, Mo.), *Associate Referee*

In 1953 a study was made of the A.O.A.C. method for the determination of volatile oil in spices. All-glass connections and heating mantles as sources of heat were proposed as modifications to improve the precision

(1). These modifications were tested collaboratively in 1954 (2). The results for nutmeg and allspice were as precise as or better than those of previous collaborative studies of the official method; the collaborative results on fennel varied widely. The Associate Referee did not present a report in 1955.

In 1955 Lee and Ogg presented a modification of the A.O.A.C. method and a modified trap for collecting the oil (3). These modifications included the two changes proposed in 1953 plus a magnetic stirrer for mixing the suspension in the distilling flask. Lee and Ogg followed the practice of adding a measured amount of xylene to the trap at the beginning of the analysis if previous experience had shown that the oil from that particular spice would separate into two fractions—one lighter and one heavier than water. They reported that the magnetic mixer permitted the use of higher distillation temperatures with no hot spots, scorching, or boiling over. The collaborative study made with their modified trap and their method gave more precise results than any previously reported by the official method. In 1955 Subcommittee C recommended that a further study be made of the method (4).

In this year's study of the method, all-glass connections, a heating mantle, and a magnetic stirrer were used, and xylene was placed in the trap to prevent separation of the oil into two fractions. Two ground nutmegs were used; one that had been held in a freezer in separate ½ gallon jars for three years (Nutmeg I and II); and one that had been held in a freezer for seven years (Nutmeg III).

Table 1 shows the effect on the yield of oil when the voltage and distillation time are varied and Clevenger's trap is used. The hot readings were taken without stopping the distillation. The cold readings were taken after stopping and cooling the oil in the trap. On 11/21/55 the distillation was run four hours; then the oil was cooled and the volume was read. During the last half-hour of distillation the hot reading indicated that oil was still distilling over (increase of 0.1 ml), and the distillation was continued for an additional four hours. At the end of this time there was no increase in oil over a one-hour period and the determination was considered complete. Thus four hours' distillation time did not give a complete yield of oil. The precision for eight hours' distillation (heating mantle at 80 volts) was fairly good. When the voltage was increased to 90 (Part B, Table 1), the yield of oil was higher and precision was excellent. When one distillation was run at 100 volts, the yield decreased. This was not investigated further.

Table 2 shows that the yield of oil is directly related to the ratio of the weight of spice to the volume of distilling liquid. The lower results, obtained with a 2 liter flask, are probably due to the solubility of the oil in the water. Thus if half the sample weight is taken, half the volume of dis-

TABLE 1.—*Effect on volatile oil recoveries of varying the voltage and distillation time*

DATE		PER CENT VOLATILE OIL										
		TIME, HOURS										
		1	2	2.5	3	3.5	4	5	6	7	7.5	8
A: Nutmeg I, 80 volts, 1 liter flask, 1 ml xylene												
11/8	Cold						7.20					
11/18	Hot		7.40		7.80		8.20					
	Cold						7.16					
11/21	Hot	7.00	7.60		8.00	8.20	8.30				8.50	
	Cold						7.40				7.84	
11/22	Hot		7.88		8.25		8.50				8.75	
	Cold										7.75	
11/28	Hot		7.25		7.88		8.25	8.25		8.30		
	Cold										7.75	
B: Nutmeg I, 90 volts, 1 liter flask, 1 ml xylene												
11/29	Hot		8.25		8.75		8.75		9.00			
	Cold								7.93			
12/7	Cold						7.68					7.95
12/15	Hot	7.50		8.25				8.75	9.00			
	Cold								7.90			
12/16	Cold										7.95	
12/19	Cold										7.95	
12/21	Cold										7.90	
12/23	Cold										7.95	

A: three 8 hr runs, read cold: Average %, 7.78
Std. Dev., ±0.13, 1.67%

B: 7 cold readings: Average %, 7.93

TABLE 2.—*Relationship between yield of oil and ratio of sample weight to volume of distilling liquid*[a]

NUTMEG I[b]		NUTMEG II[c]	
1 LITER FLASK	2 LITER FLASK	40 G SAMPLE, 1 LITER FLASK, 1 ML XYLENE	20 G SAMPLE, 500 ML FLASK, 0.5 ML XYLENE
per cent	*per cent*	*per cent*	*per cent*
7.95	7.75	8.00	8.00
7.90	7.75	8.00	8.00
7.95	7.75		

[a] Nutmeg was from same lot and held same length of time but in separate jars.
[b] 40 g distilled 7.5 hr at 90 volts.
[c] Distilled 7.5 hr at 90 volts.

tilling liquid must be used to yield the same percentage of oil (*see* Nutmeg II, Table 2).

Table 3 shows that if the volume of oil is read on the day of distillation and then is left overnight in a closed system and read again, the volume definitely decreases. The oil is still cloudy when read the same day but is clear if left overnight.

Table 4 shows the comparative results obtained with the two traps on the two differently aged nutmegs (II and III). In both cases the Clevenger trap gave very precise results (standard deviation of less than 0.3 per cent); the Lee and Ogg trap did not give comparable results.

The discrepancy of results between the two traps may have been caused by the age of the spice. Experience has shown that when nutmeg ages, its oxidation products give both a lighter-than-water and a heavier-than-water fraction of oil. The oil from allspice separates even when fresh.

The smaller bore in the Lee and Ogg trap increases the oil's tendency to

TABLE 3.—*Effect of time of reading on yield of oil, using 1 liter flask, 1 ml xylene, and 90 volts*

NUTMEG II, 40 G SAMPLE		NUTMEG III, 50 G SAMPLE[a]	
READ SAME DAY	READ NEXT DAY	READ SAME DAY	READ NEXT DAY
per cent	*per cent*	*per cent*	*per cent*
7.95	7.75	4.88	4.80
7.98	7.75	4.88	4.80
7.98	7.75	4.86	4.80
7.98		4.86	4.78
8.00		5.00[b]	4.80
Av. 7.98	7.75	4.87	4.80
Std Dev.: ±0.02, 0.25%	0.00	±0.01, 0.21%	±0.01, 0.21%

[a] Ground nutmeg held in freezer 7 years.
[b] Not used in average; water droplets were seen in oil.

TABLE 4.—*Comparison of Clevenger's trap and Lee's modified trap*

NUTMEG II		NUTMEG III	
CLEVENGER TRAP, 40 G SAMPLE, 1 LITER FLASK, 1 ML XYLENE[a]	LEE TRAP, 12 G SAMPLE, 500 ML FLASK, 0.5 ML XYLENE[b]	CLEVENGER TRAP, 50 G SAMPLE, 1 LITER FLASK, 1 ML XYLENE[a]	LEE TRAP, 20 G SAMPLE 500 ML FLASK, 0.5 ML XYLENE[b]
per cent oil	*per cent oil*	*per cent oil*	*per cent oil*
7.75	6.25	4.83	3.58
7.75	4.92	4.80	4.42
7.75	4.83	4.80	4.73
7.75	6.42	4.80	4.50
		lost	4.75
		4.80	4.90
		4.78	5.10
		4.80	5.00
Av. 7.75	5.61	4.80	4.62
Std Dev.:	±0.85, 15.2%	±0.01, 0.21%	±0.48, 10.4%

[a] Volumes read next day.
[b] Volumes read after centrifuging.

separate into two layers. Even the addition of xylene did not stop this separation. Once the oil separates and the heavier fraction returns to the distilling flask, the yields are lower and more variable. One of the advantages of the Clevenger trap is that if the oil separates into two fractions, the heavier fraction can be drawn off through the stopcock as it comes down, then cooled, and read. The transfer of oil introduces errors, but they are smaller than the errors caused by the heavier fraction returning to the distilling vessel.

CONCLUSIONS

The method is very empirical and the study has shown that slight changes affect the yield of oil. Thus there is a direct relationship between the yield of oil and the ratio of sample to the volume of distilling liquid. The amount of heat applied also affects the yield of oil. The use of a magnetic mixer has definitely increased the precision of the volatile oil method if all variables are kept constant. Use of a magnetic mixer and increased voltage did not decrease the distillation time required for a complete yield of oil. Precise results may be obtained in a shorter time if all variables are kept constant, but the yield of oil may not be complete. A sealed stirrer with a two-hole distillation flask should work as well as the magnetic mixer and give less trouble (this was not investigated).

The average recovery obtained by combining the 14 determinations from Nutmegs I and II (Clevenger trap) was 7.96 per cent, with a standard deviation of ±0.03, or 0.4 per cent. These nutmegs were from the same lot but were held in separate jars for three years; results were read the same day of distillation. All seven readings for Nutmeg II were 7.75 per cent when the cleared oil was read the next day.

SUMMARY AND RECOMMENDATION

Use of a magnetic mixer, as proposed by Lee and Ogg, has permitted higher heating temperatures and very precise results, when all variables are constant. The Lee and Ogg trap did not give as precise results as did the Clevenger trap for the determination of volatile oil in nutmeg that had been ground and held in a freezer from three to seven years.

It is recommended* that the modified method be studied collaboratively.

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* For report of Subcommittee C and action of the Association, see *This Journal*, **40**, 33, 34 (1957).

REPORT ON PUNGENT PRINCIPLES IN MUSTARD

By J. E. ROE (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D.C.), *Associate Referee*

This year work has been done on the distribution of volatile oils in brown mustard seed, brown mustard flour, and various by-products obtained in the manufacture of brown mustard flour.

Last year's report mentioned that when the present A.O.A.C. method was used, the volatile oil content of brown mustard seed, ground in a laboratory mill, was found to be less than half as much as that obtained from the flour produced from the same seed. In a private communication J. M. Fogelberg, Technical Director, The R. T. French Company, stated that higher and more uniform results were obtained by using a modification of the present A.O.A.C. method for mustard seed of comparatively coarse grind.

In this modification the glucoside is extracted by adding the coarsely ground seed to boiling water. The boiling is continued for a few minutes, and the glucoside, which is soluble in water, is leached from the mustard. The water suspension is cooled, and a small amount of pure yellow mustard flour is added to provide the enzyme, myrosin, that releases the volatile oil from the glucoside. The A.O.A.C. method is followed from this point.

The following samples were furnished by the R. T. French Company, Rochester, New York: Canadian brown mustard seed (*Brassica juncea*), brown bran, brown mustard flour, brown tailings, brown seconds, and brown cake flour. Duplicate determinations of volatile oil in these prod-

ucts showed that the cake flour had the largest amount of volatile oil. The other products in decreasing order of volatile oil found were as follows: tailings, flour, seed, seconds, and bran. The French Company's modification was used in all determinations except the cake flour and flour. The tailings and seconds are by-products containing flour with some bran. The cake flour is the product obtained by further milling after some of the fixed oil is removed.

When this modification was used, the volatile oil content of brown mustard seed was found to be almost the same as that of the flour from the seed. Also, the results of the determination of volatile oil content of coarsely-ground mustard seed were much more uniform.

It is proposed that the Corran and Terry method, *Analyst*, **64**, 164 (1939), be used to analyze known mixtures of botanically pure brown and yellow mustard flours. If this method is satisfactory, studies should then be made to determine the applicability of these methods to commercial mustard flours and prepared mustard.

It is recommended* that the study be continued.

No report was given on vinegar.

* For report of Subcommittee C and action of the Association, see *This Journal*, **40**, 33, 34 (1957).

REPORT ON PRESERVATIVES AND ARTIFICIAL SWEETENERS

By Andrew W. Breidenbach (Robert A. Taft Sanitary Engineering Center, Public Health Service, Cincinnati 26, Ohio), *Referee*

The number of artificially sweetened food items of low caloric content has increased during the past year. While increased sales may partly reflect greater usage of artificially sweetened products by diabetics, the growing emphasis in this country on weight reduction probably contributes more than any other single factor.

An excellent comprehensive discussion of the nutritional aspects of the use of cyclohexylsulfamate has appeared in a recent report (1). Sorbitol has been incorporated in a variety of food products including jams, jellies, puddings, beverages, salad dressings, imitation maple sirup, and cake mixes (2). Sorbitol cannot be considered strictly non-nutritive, since it is slowly converted into glucose or fructose *in vivo*. The slowness of this conversion has led to the suggestion that sorbitol could be used effectively by those who suffer from diabetes mellitus (3).

Improved methods are needed for the rapid identification of these sweeteners, particularly for a mixture of them in food products. The Associate Referee on Artificial Sweeteners has studied the paper chromatographic method of Mitchell (4). This method shows promise for qualitative work.

Each year of the past decade has seen increased emphasis on the use of antibiotics to retard the spoilage of fresh foods. The clearance of aureomycin for use on fresh poultry by the Food and Drug Administration (5) has spurred interest in the use of antibiotics for other foods. The success of aureomycin in extending the shelf life of chickens up to two weeks (6) has resulted in trials of the antibiotic as a dip for salad greens (7). Campbell and O'Brien (8) have published an excellent review of the use of antibiotics as food preservatives. There is a serious and immediate need for qualitative and quantitative information about these new agents on specific foods.

The oxidation of fats and oils, culminating in off-flavors and finally rancidity, has stimulated research for several decades. The relatively recent popularity of freezing meats and seafood has directed attention to the problem of the flavor changes in frozen pork fat and fish. Some progress has been reported in the inhibition of oxidative off-flavors in ground pork by antioxidants of the butylated hydroxyanisole type (9). Guaiaretic and nordihydroguaiaretic acids have been reported to inhibit the off-flavors in beer (10). Phenolic antioxidants have been used successfully on cereals (11). The value of some spices in aiding in the preservation of foods has been shown to be due to their antioxidative action (12).

Sorbic acid for the inhibition of mold has also received much attention. The use of this popular fungistat has been increasing rapidly. New ideas for application appear regularly. Recently suggestions were made for the use of sorbic acid on strawberries (13), tomatoes (13), and apple juice (14). A recent innovation is the use of acetoglycerides to inhibit mold in such products as wieners by forming a waxy coat over them (15). A similar treatment for packaged frozen meats has been reported (16).

Research in the newest field of preservatives has dominated the literature again this year: many investigations have been concerned with the application of radiation to food preservation. It appears, however, that any widespread use of this technique in commercial food processing is still in the future. Efforts at present seem to be directed in two channels:

A considerable amount of research is related to the use of lower levels to effect a "pasteurizing dose" of radiation which would not necessarily sterilize food, but would increase its refrigerated shelf life. Concurrently, other work is proceeding in the search for additives that will absorb the oxidative flavor changes associated with higher doses of radiation. The elevated costs associated with this new type of processing still hamper plans for general commercial application (17). The Associate Referee on

this subject has recently published an excellent review (18). Further clarification of the attitudes of the Food and Drug Administration have also been informally reported (19).

The older, more classical preservative problems have not diminished in significance. Such additives as hydrogen peroxide for milk are still of importance in the regulatory field (20). The Associate Referee on this subject studied a method in which the Referee served as a collaborator. Sodium nitrite in combination with ascorbic acid has been suggested for use in the curing of meat (21). Boric acid, a preservative which has been used for meat, was studied by the Associate Referee during 1956. A study of the available methods for benzoates and hydroxybenzoates in meat was begun by the Associate Referee during the year.

ACKNOWLEDGMENT

The Referee wishes to thank those Associate Referees who, in spite of pressure of routine work, contributed valuable efforts to methodology.

RECOMMENDATIONS

It is recommended*—

(1) That the method proposed by the Associate Referee for the determination of dulcin in non-alcoholic beverages be adopted as first action.

(2) That the method proposed by the Associate Referee for the qualitative determination of hydrogen peroxide in dairy products be adopted as first action.

(3) That the methods for the determination of cyclohexylsulfamate of sodium and of calcium as given in *This Journal*, **38**, 559 (1955) be adopted as first action.

(4) That work be continued on methods for the determination of quarternary ammonium compounds in milk.

(5) That work be continued on a qualitative method for hydroxybenzoate.

(6) That work be continued on the detection of fluorides.

(7) That work be continued on the methods for the determination of thiourea in food.

(8) That work be continued on the determination of benzoates in meat.

(9) That work be continued on methods for the determination of boric acid in meat.

(10) That work on methods for the determination of dimethyldichlorosuccinate and dehydroacetic acid be continued.

(11) That work on the paper chromatographic detection and identification of saccharin, cyclohexylsulfamate, and dulcin be continued.

(12) That work on the methods for the detection of sorbic acid be continued.

* For report of Subcommittee D and action of the Association, see *This Journal*, 40, 37, 38 (1957).

(13) That Associate Referees be appointed to work on the following:

Microbiological method for the detection of antimicrobial preservatives.

Methods for the detection and determination of the radical acceptors or other additives used in radiation preservation of foods.

(14) That the first action method for the determination of P-4000 in non-alcoholic beverages be made official.

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REPORT ON ARTIFICIAL SWEETENERS

By WILLIAM S. COX (Food and Drug Administration, Department of Health, Education, and Welfare, Atlanta 3, Ga.),
Associate Referee

As reported by the Associate Referee last year (*This Journal*, **39**, 652 (1956)) a spectrophotometric method for the determination of dulcin (*p*-phenetylurea) was studied collaboratively. The results of this study were inconclusive, but an analysis of the data indicated that small amounts of P-4000 (propoxy-2-amino-4-nitrobenzene) interfered strongly and were the probable cause for the discrepancy in results.

A collaborative study was made this year with the same type of sample as in 1955 but on a much simpler scale. Since P-4000 was not present in the 1956 sample, the main purpose of this study was to demonstrate that

P-4000 was the interfering substance and also to determine if the method were satisfactory without this interference.

The 1956 method (*see* Recommendations) was the same as last year's, with two exceptions: the drying time of the dulcin residue, after extraction with ethyl ether, was increased from 10 to 30 minutes to insure the absence of any moisture which might cloud the spectral solvent, and secondly, *redistilled* ethyl acetate was specified as the spectral solvent.

The composition of the sample solution, except for the omission of P-4000, was essentially the same as that of Sample A of the 1955 study: an orange-flavored soft drink made from a base consisting of dextrose, citric acid, orange flavor, and certified color, to which was added known amounts of dulcin, sodium benzoate, and propylene glycol; in 1955, P-4000 was also added in known amounts to the sample solution.

RESULTS AND DISCUSSION

Table 1 shows the results reported by this year's collaborators and compares their percentage recoveries for the two years.

TABLE 1.—*Comparison of collaborative results for dulcin in beverages, 1955 and 1956*

COLLABORATOR	1956 SAMPLE (4.25 MG DULCIN ADDED/50 ML)		1955 SAMPLES: AV. RECOVERY, %	
	FOUND, MG	AV. RECOVERY, %	A (5.25 MG DULCIN ADDED/50 ML)	B (2.63 MG DULCIN ADDED/50 ML)
A	4.38 4.29	102.1	95.2	123.8
B	4.30 4.30	101.2	105.2	147.6
C	5.12 5.12	120.5	167.0	254.3
D	4.05 4.10	95.9	109.4	111.4
E	3.95 4.04	94.1	102.4	112.4
F[a]	4.81	113.2	110.0	150.5
G	4.80 4.75	112.5	110.5	152.5

$\bar{x}$ (mean)[b] = 4.30; std. deviation = 0.43; coeff. of variation = 10.0%; range = 1.17 mg

[a] One determination only.
[b] 1956 data.

Even though the data do not indicate the method to be of high precision, the results reported this year agree well enough so that the method can be recommended for adoption.

The results definitely indicate that the P-4000 present in the 1955 samples resulted in extremely high "recoveries," most of which were in excess of 100 per cent; some were as high as 250 per cent.

RECOMMENDATIONS

It is recommended*—

(1) That the following method for the determination of dulcin in non-alcoholic beverages be adopted as first action:

Pipet 50 ml sample into separator. If 5-nitro-2-propoxyaniline (P-4000) is present, ext. with four 50 ml portions petr. ether, shaking 2 min. each time, and discard petr. ether. Make aq. phase alk. to litmus with 10% NaOH soln and ext. with four 100 ml portions ether, shaking 2 min. each time. Combine exts, wash with 10 ml H_2O, and discard H_2O. Evap. ether in 400 ml beaker and dry residue 30 min. at 110°. Dissolve residue in ca 50 ml redistd Et acetate, transfer to 100 ml vol. flask, dil. to vol. with 4 or 5 washings of the Et acetate, and mix. Read absorbance of soln in spectrophotometer at 294 mμ against redistd Et acetate. Det. quantity of dulcin in final soln from previously prepd std curve and calc. to mg/l.

(2) That further work on the determination of dulcin in non-alcoholic beverages be discontinued.

ACKNOWLEDGMENT

Sincere thanks are extended to the following collaborators, all of the Food and Drug Administration, whose kind cooperation made this report possible: Charles E. Beisel, Baltimore; Mary C. Harrigan, Boston; Benjamin Krinitz, New York; Mary A. McEniry, St. Louis; D. W. McLaren, Buffalo; Donald M. Taylor, Denver; and Floyd E. Yarnall, Kansas City.

* For report of Subcommittee D and action of the Association, see *This Journal*, 40, 37, 38 (1957).

REPORT ON CYCLAMATE SODIUM (SUCARYL ®)

By JOHN B. WILSON (Division of Food, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

Two collaborative samples of beverages containing Sucaryl were prepared this year and analyzed by three collaborators. Since both sodium and calcium cyclamate are commercially available, one of the samples contained the sodium salt, the other the calcium salt. The composition of the samples was as follows:

	Sample 3	*Sample 4*
Sucaryl	3.00 g (sodium salt)	1.00 g (calcium salt)
Sugar	30 g	70 g
Citric acid	3 g	1 g
Sodium benzoate	1 g	1 g
Artificial raspberry flavor	0.3 ml	0.3 ml
Color solution	q.s.	q.s.
Anhydrous sodium sulfate	0.00	0.50 g
Water, q.s.	1000 ml	1000 ml

The sodium sulfate was added to Sample 4 to show the effect, if any, of the presence of a substantial quantity of precipitate that had to be filtered off before sodium nitrite was added to decompose the cyclamate. David C. Wimer, Abbott Laboratories, collected and weighed the barium sulfate precipitated at this point. He reported 0.0352 g of sulfate per 100 ml, which corresponds exactly to the 0.05 g of anhydrous sulfate added.

Collaborators were instructed to analyze the samples by the method given in the 1954 report (*This Journal*, **38**, 559 (1955)), to use 100 ml of sample, and to disregard the direction to use a quantity of sample containing 10–100 mg of Sucaryl. The results are given in Table 1.

TABLE 1.—*Collaborative results on Sucaryl ® in beverages*

SUCARYL PRESENT	SUCARYL FOUND, G/100 ML			
	COLL. 1	COLL. 2	COLL. 3	AV.
Sample 3, 0.300 g/100 ml	0.300	0.302	0.307	0.3035
	0.300	0.302	0.310	
Sample 4, 0.100 g/100 ml	0.100	0.100	0.102	0.1003
	0.099	0.100	0.101	

In view of the excellent results, it is recommended* that the following method be adopted as first action for solutions containing 10–300 mg Sucaryl ® per 100 ml:

To 100 ml soln contg 10–300 mg Na or Ca cyclohexylsulfamate add 10 ml HCl and 10 ml 10% $BaCl_2$ soln. Stir and let stand 30 min. If ppt forms, filter and wash with H_2O. To filtrate or clear soln add 10 ml 10% $NaNO_2$ soln, stir, cover with watch glass, and heat on steam bath at least 2 hr. Stir up ppt 3 times at 0.5 hr intervals. Remove from steam bath and leave in warm place overnight. Collect ppt on tared gooch, wash, and dry on asbestos mat over flame at least 10 min. Ignite, cool in desiccator, and weigh. Wt $BaSO_4 \times 0.8621$ = Na cyclohexylsulfamate; $\times 0.9266$ = Ca cyclohexylsulfamate.

ACKNOWLEDGMENTS

Thanks are extended to David C. Wimer, Abbott Laboratories, and George Yip, Division of Food, Food and Drug Administration, for acting as collaborators.

* For report of Subcommittee D and action of the Association, see *This Journal*, 40, 37, 38 (1957).

REPORT ON HYDROGEN PEROXIDE IN MILK

By WILLIAM H. MUNDAY (Food and Drug Administration, Department of Health, Education, and Welfare, Minneapolis 1, Minn.), *Associate Referee*

Hydrogen peroxide has been used for many years as a preservative for laboratory samples of milk and cream. It is possible that it could be used as a preservative for market milk; if so, the milk would be rendered adulterated under present food laws. Thus it is desirable to have an official method for its detection.

The Associate Referee has surveyed numerous methods for the detection of hydrogen peroxide in aqueous solutions to determine if any of these methods can be adapted for use with milk. Of the methods tested, the most sensitive and easiest to use was the Arnold-Mantzel method (*The Merck Index*, 5th Ed.). According to this method, when the proper amount of a vanadic acid reagent solution is added to liquids containing hydrogen peroxide, a permanent red color appears. The method is sensitive to 0.0006 per cent.

A solution of vanadic acid was prepared by dissolving 1 gram of vanadium pentoxide in 100 ml of 10 per cent sulfuric acid. When 1–20 drops of this solution was added to milk containing hydrogen peroxide, a light pink to dark brick red color was formed in the milk, depending on the concentration of hydrogen peroxide present. In milk containing a high concentration of hydrogen peroxide (more than 1000 ppm) the color "bleached out." However, when more vanadic acid solution was added, the color reappeared. This color was stable; it lasted from 1 to 8 hours depending on the type of milk, the concentration of hydrogen peroxide, and the amount of vanadic acid reagent used.

Raw and pasteurized milk were both tested with the vanadic acid reagent. The test proved equally sensitive in both types of milk; however, the hydrogen peroxide is not as stable in the raw milk.

This test was used to determine the stability of hydrogen peroxide in milk. Results are summarized in Table 1.

On the basis of the findings shown in Table 1, a collaborative study was made of the vanadic acid method. Because of the instability of hydrogen peroxide, prepared samples were not sent to the collaborators; instead they were instructed to obtain their own samples.

INSTRUCTIONS TO COLLABORATORS

PREPARATION OF SOLUTIONS

(a) *Milk.*—Sweet milk (pasteurized and raw) known to be free of hydrogen peroxide (local retail milk was found to be satisfactory in the Minneapolis area).

(b) *Hydrogen peroxide.*—3% H_2O_2. Analyze to find actual H_2O_2 content (U.S.P. XV method is satisfactory). *Note:* If only 30% H_2O_2 is available, dilute to 3% before adding to the milk.

TABLE 1.—*Stability of hydrogen peroxide in milk at various concentrations*

H_2O_2 PRESENT, PPM	HOURS AFTER PREPARATION										
	0	1	4	8	16	24	48	72	96	120	168
Raw Milk											
10	−	−	−	−	−	−	−	−	−	−	−
100	+	+	+	+	−	−	−	−	−	−	−
1,000	+	+	+	+	+	+	+	−	−	−	−
10,000	+	+	+	+	+	+	+	+	+	+	+
Pasteurized Milk											
10	−	−	−	−	−	−	−	−	−	−	−
100	+	+	+	+	+	+	+	+	−	−	−
1,000	+	+	+	+	+	+	+	+	+	+	−
10,000	+	+	+	+	+	+	+	+	+	+	+

(c) *Vanadic acid reagent.*—Dissolve 1 g vanadium pentoxide in 100 ml dilute H_2SO_4.

PREPARATION OF H_2O_2-TREATED MILK

Prepare two series of samples, using both pasteurized and raw milk. Add a sufficient volume of the analyzed 3% H_2O_2 solution to a 100 ml volumetric flask so that exactly 0.1 g H_2O_2 is present, and dilute to volume with sweet milk (Solution 1, 0.001 g/ml, 1000 ppm). Pipet 10 ml of Solution 1 into a second 100 ml volumetric flask and dilute to volume with milk (Solution 2, 0.0001 g/ml, 100 ppm). In same manner, use 10 ml Solution 2 to prepare Solution 3 (0.00001 g/ml, 10 ppm) and 10 ml Solution 3 to prepare Solution 4 (0.000001 g/ml, 1 ppm).

DETECTION OF HYDROGEN PEROXIDE

Prepare a series of 5 clean test tubes of convenient size (20–30 ml). To one, add 10 ml untreated milk for the blank; to each of the others, add, respectively, 10 ml each of Solutions 4, 3, 2, and 1. To each tube add ca 10 drops of vanadic acid reagent and give the tube a quick, hard shake to mix (the acid causes the milk to curdle); then observe the tubes for development of orange-pink to brick-red color (color develops almost immediately). If red color bleaches out, due to high concentrations of H_2O_2, add more vanadic acid reagent to restore color. Note the lowest concentration of H_2O_2 that develops pink color.

Let tubes stand overnight and again examine, this time for greenish-yellow color in the curd layer. Note the lowest concentration of H_2O_2 in which this color is observed. Determine sensitivity as follows:

Prepare fresh solutions of milk (pasteurized and raw) comparable to the lowest concentration of H_2O_2 that gave a positive test in the first series. From this solution prepare second series of dilutions in steps of 10 ppm by pipetting (*a*) 1 ml into a test tube and diluting to 10 ml; (*b*) 2 ml in the second test tube and diluting to 10 ml, etc., up to 10 tubes. Test as before. Again let tubes stand overnight and examine for the yellow color in curd layer.

Example: If a pink color is detected in Solution 2 but not in Solution 3, H_2O_2 is detectable in concentrations of 10–100 ppm. To determine the lowest detectable

concentration, test concentrations of less than 100 ppm but not less than 10 ppm, by preparing blank tube, No. 1, and other tubes as follows:

To tube No. 2, add 1 ml Solution 3 and 9 ml milk (10 ppm); to tube No. 3, add 2 ml Solution 3 and 8 ml milk (20 ppm); to tube No. 4 add 3 ml Solution 3 and 7 ml milk (30 ppm), etc., up to 100 ppm.

Report the lowest concentrations of H_2O_2 detected by both techniques and describe the colors observed.

RESULTS AND DISCUSSION

Table 2 lists the lowest concentrations at which a positive test for hydrogen peroxide was obtained by the Associate Referee and the collaborators.

TABLE 2.—*Collaborative results for lowest concentration of hydrogen peroxide detected in milk*

COLLABORATOR	H_2O_2 CONCENTRATION, PPM	
	RAW MILK	PASTEURIZED MILK
Associate Referee	30	30
A	20	20
B	50	50
C	70	60
D	80	30
E	100[a]	70

[a] Not reported between 10–100 ppm.

The alternate technique of letting the milk stand overnight with the vanadic acid reagent did not substantiate the Associate Referee's findings. Table 3 lists the results obtained by using the alternate procedure.

Hydrogen peroxide was detected in milk serum from hydrogen peroxide-treated milk by the vanadic acid reagent. The test was found to be even more sensitive for milk serum than for whole milk (to less than 10 ppm).

TABLE 3.—*Collaborative results by alternate method*

COLLABORATOR	H_2O_2 CONCENTRATION, PPM	
	RAW MILK	PASTEURIZED MILK
Associate Referee	10	10
A	20	30
B	50	50
C	100	100
D	No color change	1000
E	No color change	No color change

A spectrophotometric curve was made from milk serum containing 10 ppm hydrogen peroxide. Peak absorbance was found at 460 mμ. A curve

was prepared by plotting concentration of hydrogen peroxide from 0 to 700 mg per 25 ml of milk serum *versus* absorbance at 460 mμ. Essentially a straight line was obtained. Because of the difficulty of obtaining the milk serum free of interfering substances, the quantitative procedure was not pursued further, since the problem was primarily qualitative.

SUMMARY AND RECOMMENDATION

When a 1 per cent solution of vanadic acid is added to milk containing hydrogen peroxide, a pink to brick-red color is produced. The color developed by the vanadic acid reagent is sensitive to less than 80 ppm hydrogen peroxide in milk. Hydrogen peroxide decomposes rapidly in milk; this decomposition is faster in raw milk than in pasteurized milk.

If hydrogen peroxide has been added to milk in concentrations of 300–1000 ppm (1–4), it can be detected by the vanadic acid reagent 16–72 hours after its addition. Vanadic acid reagent also offers a means of quantitative measurement of hydrogen peroxide in milk when samples of clarified milk serum are used.

It is recommended* that the following qualitative test for the detection of hydrogen peroxide in milk be adopted as first action:

(a) *Reagent.*—Dissolve 1 g V_2O_5 in 100 ml H_2SO_4 (6+94).

(b) *Test.*—Add 10–20 drops reagent to ca 10 ml sample and mix. Pink or red color indicates H_2O_2.

ACKNOWLEDGMENTS

The Associate Referee wishes to express appreciation to A. W. Breidenbach, Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio, and the following collaborators, all of the Food and Drug Administration, for their assistance in this work: H. E. Theper, St. Louis, Mo.; J. E. Weeks, Jr., New Orleans, La.; F. H. Collins, Cincinnati, Ohio; and R. L. Stephens, Chicago, Ill.

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No reports were given on benzoates and hydroxybenzoates, benzoates in meats, boric acid in meats, dimethyldichlorosuccinate and dehydroacetic acid, fluorides, monochloroacetic acid, quaternary ammonium compounds, radiation preservation of foods, sorbic acid, or thiourea.

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* For report of Subcommittee D and action of the Association, see *This Journal*, 40, 37, 38 (1957).

REPORT ON VEGETABLE DRUGS AND THEIR DERIVATIVES

By PAUL S. JORGENSEN (Food and Drug Administration, Department of Health, Education, and Welfare, San Francisco 2, Calif.), *Referee*

Method II for monobromated camphor in tablets has been in first action status for some time. Subcommittee B recommended that "the method of Emory (Method I) for the estimation of monobromated camphor in tablets be studied cooperatively, and that the method of Eaton (Method II) be studied further" (*This Journal*, **5**, 337 (1921–1922)). The results of this study were discussed in a report by Wright (*Ibid.*, **5**, 587 (1921–1922)) in which collaborative results by four analysts were presented for both methods. At this time the Associate Referee recommended that both methods be made tentative and that Method II be studied further in order to simplify it.

The following year Subcommittee B recommended that the tentative methods adopted at the 1921 meeting be made the subject of collaborative study during the coming year (*Ibid.*, **7**, 272 (1923–1924)).

In his 1926 report on drugs, Paul recommended that Method I be made official (*Ibid.*, **9**, 269 (1926)) and this recommendation was adopted by the Association (*Ibid.*, **10**, 67 (1927)). However, he stated that Method I was preferred to Method II and that it would be undesirable to suggest any change in the status of Method II. He further stated that there appeared to be no urgent need to continue these topics, except to recommend, in due course, that Method II be made official. This later action has never been taken by the Association.

Since Method I was preferred at the time and two methods are not needed for this drug, the Referee believes that, in the interest of reducing duplication, Method II should be deleted from *Official Methods of Analysis*.

RECOMMENDATIONS

It is recommended*—

(1) That the method for aminophylline and phenobarbital, **32.21–32.24**, be amended as recommended by the Associate Referee, and that the topic be closed.

(2) That the first action method for monobromated camphor (*Method II*), **32.278**, be deleted, as recommended by the Referee.

(3) That the method for reserpine and related alkaloids be adopted as first action and that the topic be continued.

(4) That the method for the reserpine-rescinnamine group alkaloids in *Rauwolfia serpentina* be adopted as first action and that the topic be continued.

* For report of Subcommittee B and action of the Association, see *This Journal*, **40**, 24, 25 (1957).

(5) That study of the following topics be continued:

Quinine and strychnine
Digitoxin and related glycosides

REPORT ON DIGITOXIN AND RELATED GLYCOSIDES

By DANIEL BANES (Division of Pharmaceutical Chemistry, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

The preceding report on this subject presented the results of a collaborative assay of digitoxin tablets (1). In the method studied, digitoxin was eluted from a formamide-siliceous earth column, and the glycoside was determined colorimetrically after treatment with sodium picrate. The method was adopted as first action (2).

Since its adoption by the Association, the method has been investigated critically by a Canadian group, Mannell, Lavallee, Carioto, and Allmark, who recommended an increase in eluent volume to ensure complete recovery of the digitoxin from the column (3). The modified assay procedure was made official in the U.S.P. XV monographs on Digitoxin, Digitoxin Injection, and Digitoxin Tablets (4). The officially recognized substance now contains not less than 90 per cent of the pure glycoside. However, neither *Official Methods of Analysis* nor the *United States Pharmacopeia* provides criteria for ascertaining whether the dosage forms contain digitoxin of the specified purity.

The chief contaminants of digitoxin are the other naturally-occurring digitalis glycosides, chiefly gitoxin. Both the original papers describing the chromatographic separation of digitoxin from other glycosides (5) and the paper by Mannell and his co-workers (3) mention the extraction of more polar digitoxosides with chloroform after the removal of digitoxin from the column. The proposed test for other digitoxosides is based upon the earlier chromatographic investigations. It employs the Keller-Kiliani test for digitoxose, as modified by Rowson (6), for the colorimetric estimation of these digitoxosides.

METHOD

Reagent.—Mix 60 ml glacial acetic acid with 5 ml concentrated H_2SO_4, add 2 ml 5% $FeCl_3$ solution, mix, and cool.

Determination.—After digitoxin separates, elute other digitoxosides with 200 ml $CHCl_3$, collecting eluate in separator. Shake with 100 ml H_2O. Transfer lower layer to beaker, extract H_2O with 30 ml $CHCl_3$, and add $CHCl_3$ washings to the beaker. Evaporate to dryness. Pipet 5 ml of the diluted digitoxin standard solution into second beaker and evaporate to dryness. Add 4 ml of the reagent to each of the cooled residues and mix, swirling occasionally. After 20 min., determine absorbance

of sample and standard, relative to reagent blank, at 590 mμ, repeating measurements at 5 min. intervals until maximum values are attained. Calculate content of other digitoxosides in sample.

DISCUSSION AND RECOMMENDATION

Mixtures of digitoxin and gitoxin in known proportions were incorporated in formamide-Celite columns, and the digitoxin was eluted with 280 ml of (3+1) benzene-chloroform mixture. An additional 50 ml of the solvent was percolated through the column, and the residue after evaporation was tested with picrate reagent. No discernible color developed. Other glycosides were then eluted and estimated by the proposed method. Recoveries by comparison with a gitoxin standard and with the digitoxin standard are shown in Table 1.

TABLE 1.—*Recoveries of glycosides from known mixtures*

SAMPLE	DIGITOXIN		OTHER DIGITOXOSIDES		
			ADDED AS GITOXIN	RECOVERED	
	ADDED	RECOVERED		GITOXIN AS STANDARD	DIGITOXIN AS STANDARD
	mg	*mg*	*mg*	*mg*	*mg*
1	1.92	1.91	0	0.03	0.03
2	1.94	1.90	0.11	0.11	0.10
3	1.96	1.95	0.19	0.18	0.16
4	1.98	1.99	0.27	0.25	0.22
5	2.00	2.00	0.35	0.33	0.29

The recoveries of other digitoxosides from known mixtures by comparison with a gitoxin standard are excellent. When the digitoxin standard is used as a reference, recoveries are somewhat lower because the color developed with digitoxin is about 1.1 times as intense as that due to the same weight of gitoxin. Nevertheless, digitoxin was selected as the standard because it is readily available as a homogeneous reference of known purity, whereas gitoxin is not. Moreover, digitoxosides other than gitoxin may be present in the fraction analyzed (3). It should be noted that Samples 2 and 3 would have been found acceptable by the proposed method whereas Sample 5 would have been rejected, if the presence of more than 10 per cent of other digitoxosides had been the decisive criterion. The borderline case, Sample 4, would have been classified as doubtful.

Recoveries of digitoxin from the known mixtures ranged from 97.9 to 100.5 per cent of the quantities added, indicating a reliable separation of the two glycosides in the U.S.P. modification of the procedure.

It is recommended* that the subject be continued for the collaborative study of both the modified digitoxin procedure and the proposed limiting test for other digitoxosides.

* For report of Subcommittee B and action of the Association, see *This Journal*, 40, 24, 25 (1957).

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REPORT ON RESERPINE

By DANIEL BANES (Division of Pharmaceutical Chemistry, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

Several methods have been proposed for the assay of reserpine in its dosage forms. These methods include ultraviolet spectrophotometric analysis after electrophoresis (1), extraction (2), or chromatographic separation (3); colorimetric determinations in which vanillin (4), bromophenol blue (5), mineral acids (6), or nitrous acid (7) is used as the chromogenic reagent; and fluorimetry (6, 8).

Reserpine possesses a characteristic ultraviolet absorption spectrum. Theoretically the spectrophotometric methods are the most specific; as applied to pharmaceutical preparations, however, they are the most subject to interference. The vanillin method, studied collaboratively last year (9), presents technical disadvantages. Of the remaining methods, the colorimetric procedure, based on the reaction of reserpine with nitrous acid, gave the most reliable and reproducible results when it was applied to a variety of commercial samples.

A modification of the procedure originally published by Szalkowski and Mader (7) was sent to collaborators. They were instructed to analyze crystalline reserpine standard containing at least 97 per cent of the pure alkaloid (Sample A) and a simulated tablet granulation (Sample B) containing reserpine, 0.128 per cent, in a mixture of cornstarch, lactose, talc, stearic acid, magnesium stearate, sodium alginate, and tragacanth. Details of the method have been published in *This Journal*, **40**, 63 (1957).

INSTRUCTIONS TO COLLABORATORS

(1) Protect reserpine, and especially its solutions, from direct strong light. Complete analyses as rapidly as possible to avoid decomposition of reserpine. If necessary, analyses may be interrupted after the chloroform extraction or the evaporation of solvents. The developed colors are stable for at least an hour.

(2) Extract duplicate 5 ml aliquots of the reserpine standard solution, as under

Crystalline Reserpine, for procedural standards. On the same day, analyze Collaborative Sample B (about 1.7 g) in duplicate as under *Reserpine Tablets*. The symbols in the formulas (S and S_0) should be the averages for these procedural standards.

(3) Use the procedural standard solution as Collaborative Sample A. Dilute a 5 ml aliquot of the reserpine standard solution (0.4 mg reserpine/ml ethanol) to 100 ml with ethanol. Treat two 5 ml aliquots of this diluted solution as blanks and two as samples in the nitrite color development.

DISCUSSION AND RECOMMENDATION

Collaborators' results are presented in Table 1. Recoveries of reserpine from the crystalline sample ranged from 91.5 per cent to 99.3 per cent with an average recovery of 96.6 per cent. Except for the low result of 0.113 per cent reserpine in the tablet mixture reported by Collaborator 5, the values of reserpine found in Sample B ranged from 0.121 per cent to 0.130 per cent, or 94.5–101.6 per cent of the quantity added.

Although several of the known Rauwolfia alkaloids yield a color with nitrous acid similar to that given by reserpine, all but rescinnamine are

TABLE 1.—*Recoveries of reserpine by the proposed method*

COLLABORATOR	RESERPINE FOUND	
	SAMPLE A	SAMPLE B
	per cent	*per cent*
1	96.9	0.124
	96.6	0.124
2	95.2	0.128
	95.9	0.124
3	98.0	0.125
		0.126
	99.3	0.125
		0.124
		0.125
4	97.4	0.126
	97.2	0.127
5		0.127
	92.5	0.113
		0.128
		0.130
6	98.9	0.128
	98.7	0.128
7	96.0	0.122
	91.5	0.121
Average:	96.6	0.125

excluded by the preliminary extraction. Excessive quantities of rescinnamine in reserpine preparations may be detected by spectrophotometric measurements (10) or by chromatographic separations on paper (3). When the proposed method is combined with these identification tests, it should provide a reliable assay for reserpine in pharmaceutical preparations.

It is recommended* that the proposed nitrite procedure be adopted as first action for reserpine, and that the subject be continued.

ACKNOWLEDGMENT

The cooperation of the following collaborators, all of the Food and Drug Administration, is gratefully acknowledged: Jonas Carol, Division of Pharmaceutical Chemistry; Matthew L. Dow, St. Louis District; Donald W. Johnson, Chicago District; Arthur Kramer, New York District; Lewis H. McRoberts, San Francisco District; and Jacob Wolff, Division of Pharmaceutical Chemistry.

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* For report of Subcommittee B and action of the Association, see *This Journal*, **40**, 24, 25 (1957).

REPORT ON *RAUWOLFIA SERPENTINA*

By DANIEL BANES (Division of Pharmaceutical Chemistry, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

Two methods have been proposed for the determination of the potent alkaloids reserpine and rescinnamine in *Rauwolfia serpentina* preparations. In one of these methods[1] a feebly basic alkaloidal fraction is isolated by column chromatography, and the biologically active alkaloids are determined individually by ultraviolet spectrophotometry.

[1] CAROL, J., BANES, D., WOLFF, J., and FALLSCHEER, H. O., *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 200 (1956).

TABLE 1.—*Assay of Rauwolfia serpentina preparations*

COLLABORATORS	RESERPINE-RESCINNAMINE GROUP ALKALOIDS	
	POWDERED ROOT	TABLET
	mg/100 g	*mg/100 g*
1	229	203
	232	203
2	226	196
	231	195
3	233	203
	235	209
4	238	208
	233	205
5	238	204
	230	
	227	206
	231	
	232	205
6	242	200
	234	207
7	235	207
	234	206
Average:	233	204

In the other method[2] the sample is extracted as described in the preceding report (p. 796) and treated with nitrous acid; then reserpine and rescinnamine together are determined colorimetrically. Results by the two procedures show good correlation.[2]

The colorimetric procedure was selected for collaborative study. A sample of powdered *Rauwolfia serpentina* root, tablets fabricated from it, and a crystalline reserpine standard were forwarded to collaborators with directions for applying the proposed method of assay, details of which have been published in *This Journal*, **40**, 64 (1957).

INSTRUCTIONS TO COLLABORATORS

(1) Powder about 60 tablets, weigh about 2.5 g portions of the powdered material, and analyze in duplicate. Report as mg reserpine-rescinnamine group alkaloids per 100 g sample.

(2) Weigh about 2.5 g sample of powdered *Rauwolfia serpentina* root and analyze in duplicate. Report as mg reserpine-rescinnamine group alkaloids per 100 g root.

[2] BANES, D., WOLFF, J., FALLSCHEER, H. O., and CAROL, J., *ibid.*, 45, 708 (1956).

(3) Complete assays as rapidly as possible, preferably within a day. If it is necessary to interrupt the analysis, the alcoholic extracts of the root are stable in the dark for a day or two, and the alcohol-chloroform solutions are stable for several hours when protected from the light.

DISCUSSION AND RECOMMENDATION

Collaborators' results, shown in Table 1, indicate that the average content of reserpine-rescinnamine group alkaloids is 233 mg/100 g of the powdered root and 204 mg/100 g of the tablets. The ratio of these values agrees closely with the manufacturing formula and with other criteria (total alkaloids; strong alkaloids) obtained by independent analyses.

The data show remarkably good reproducibility, and all values reported were within ± 4 per cent of the averages. Although the proposed method does not differentiate between the potent alkaloids reserpine and rescinnamine, it provides a reliable criterion for estimating their total concentration in *Rauwolfia serpentina* preparations.

It is recommended* that the proposed method be adopted as first action, and that the subject be continued.

ACKNOWLEDGMENT

The cooperation of the following collaborators, all of the Food and Drug Administration, is gratefully acknowledged: Jonas Carol, Division of Pharmaceutical Chemistry; Herman O. Fallscheer, Bureau of Field Operations; Donald W. Johnson, Chicago, Ill.; Arthur Kramer, New York, N. Y.; Lewis H. McRoberts, San Francisco, Calif.; and Jacob Wolff, Division of Pharmaceutical Chemistry.

* For report of Subcommittee B and action of the Association, see *This Journal*, 40, 24, 25 (1957).

REPORT ON AMINOPHYLLINE AND PHENOBARBITAL

By RUPERT HYATT (Food and Drug Administration, Department of Health, Education, and Welfare, Cincinnati 2, Ohio),
Associate Referee

It was reported that, when tablets containing stearates were analyzed by the first action method for aminophylline and phenobarbital, **32.21–32.24**, the phenobarbital solution was cloudy after filtration (Communication to Division of Field Operations). The method was therefore investigated to see if it should be modified.

Two mixtures of the following compositions were prepared and examined by **32.21–32.24**:

Mixture A		*Mixture B*	
Phenobarbital	5.05%	Phenobarbital	4.95%
Aminophylline	37.75%	Aminophylline	30.68%
Stearic acid	5.00%	Stearic acid	1.00%
Starch	54.20%	Starch	43.57%
		Talc	9.90%
		Magnesium carbonate	9.90%

Mixture A was examined by method **32.21–32.24** and by a modification. The alkaline filtrate was slightly cloudy; apparently some of the stearate material was of colloid size and came through the filter. At 240.5 mμ, the absorbance of this solution was 0.466.

A portion of the solution was acidified by adding hydrochloric acid, and the acidified solution was filtered (the increase in volume was negligible); the stearic acid agglomerated and the filtrate was clear. The absorbance of this solution was 0.431. Apparently the slight cloudiness of the first solution caused the higher reading; if dilute solutions of phenobarbital are acidified, the filtrate will be clear.

According to available tablet formulas, the stearates were present as magnesium stearate to the extent of 0.5 per cent or less.

Mixture B was examined by **32.21–32.24** and by a method that included an acid filtration (*see* Recommendations).

The Associate Referee and two collaborators examined Mixture B with the following results:

	Associate Referee	*Coll. 1*	*Coll. 2*
$A_{240.5}$ by method **32.21–32.24**	0.380	0.370	0.370
$A_{240.5}$ by modified method	0.380	0.370	0.366

Since a smaller amount of stearates was present, filtration was not needed. The small amount of ammonium chloride formed had no effect on the reading. Even though talc was present, the aminophylline solutions gave the same readings regardless of whether the solutions were filtered.

RECOMMENDATIONS

It is recommended* that the following alternative method for the determination of phenobarbital in aminophylline-phenobarbital preparations, applicable in the presence of stearates, be added to **32.23** and be adopted as official:

Proceed as in **32.23**, dissolving residue in ca 100 ml of the dil. NH_4OH and dilg to ca 190 ml with the NH_4OH. Acidify with HCl, testing with litmus paper; dil. to vol. with H_2O, mix, and filter. Transfer 10 ml aliquot to 100 ml vol. flask, add 1 drop NH_4OH (1+1), and dil. to vol. with dil. NH_4OH, **32.21**(a). Proceed as in **32.23**, beginning "Det. absorbance, *A*, at 240.5 mμ . . . ."

* For report of Subcommittee B and action of the Association, see *This Journal*, 40, 24, 25 (1957).

ACKNOWLEDGMENTS

The cooperation of Chester R. Ball and James F. Langston, both of the Food and Drug Administration, who performed the collaborative work, is gratefully acknowledged.

REPORT ON QUININE

SEPARATION OF QUININE AND STRYCHNINE

By David J. Miller (Food and Drug Administration, Department of Health, Education and Welfare, Buffalo 3, New York), *Associate Referee*

In 1955 the Associate Referee recommended that Banes' spectrophotometric method for quinine and strychnine be further studied to determine its applicability to old elixirs (*This Journal*, **39**, 614 (1955)). This year collaborators were asked to re-analyze the sample left from the previous year by the Banes method and by the official method, **32.60**. Three collaborators did not have reserve samples, and the Associate Referee sent them material from his reserve.

The method was essentially the same as that used in 1955; the wording of some directions was clarified.

Results obtained are shown in Tables 1 and 2. Comparative results for 1955 and 1956 are shown in Table 3.

DISCUSSION

Collaborators were asked again this year to report the absorbance of the strychnine solutions at 347.5 mμ; at this wavelength strychnine absorbs very weakly. The absorbances reported ranged from 0 to 0.154. These absorbances may be due to the elution of a very small amount of quinine but are more likely due to the presence of ferric iron which absorbs strongly both at 347.5 and 254 mμ. High absorbances at 347.5 mμ in general correlated closely with high recoveries of strychnine, as follows:

	Coll. 5	*Coll. 2*	*Coll. 4*
Absorbance at 347.5 mμ	0.123; 0.154	0.046; 0.063	0.035; 0.040
Recovery, per cent	135; 154	111; 116	119; 123

It is difficult to evaluate the results of this year's study. Two out of six collaborators who participated both years report a significant increase in the strychnine content. The remaining 4 either show no increase, or the increase is not beyond the variations common to a spectrophotometric method. Four collaborators this year reported the color of the strychnine

TABLE 1.—*Collaborative results for strychnine and quinine in Elixir of Iron, Quinine, and Strychnine, N.F.*[a]

COLLABORATOR	STRYCHNINE		QUININE			
			BANES METHOD		A.O.A.C. METHOD	
	MG/10 ML	PER CENT RECOVERY	MG/10 ML	PER CENT RECOVERY	MG/10 ML	PER CENT RECOVERY
1	1.63	119	67.0	103	66.5	102
	1.68	123	66.4	102		
2	1.52	111	65.8	101	65.8	101
	1.59	116	65.8	101		
3	1.46	107	66.5	102	66.7	102
			66.8	103		
			66.1	102		
			67.3	103		
			66.7	103		
4	1.43	104	66.9	103	66.8	103
	1.42	104	66.9	103		
5	1.85	135	64.9	100		
	2.11	154	66.0	101		
6	1.55	113	64.7	99	67.6	104
	1.51	110	65.4	100		
7	1.40	102	67.4	104	68.0	104
	1.51	110	68.0	104		
	1.46	107				

[a] Contains 1.37 mg strychnine and 65.08 mg quinine/10 ml.

TABLE 2.—*Collaborative results for strychnine and quinine in Elixir A*[a]

COLLABORATOR	APPARENT STRYCHNINE, MG/10 ML	APPARENT QUININE, MG/10 ML
1	0	1.9
2	0.10	3.7
3	0.06	1.8
4	0	1.7
5	0.06	1.8
6	0.03	0.4
7	0.01	2.1

[a] An elixir containing all of the ingredients of the Elixir of Iron, Quinine, and Strychnine, N.F., except the alkaloids.

TABLE 3.—*Comparison of 1955 and 1956 results for strychnine and quinine in mixtures*

COLLABORATOR	ELIXIR OF IRON, QUININE, AND STRYCHNINE, N.F.				ELIXIR A	
	STRYCHNINE, % RECOVERY		QUININE, % RECOVERY		APPARENT STRYCHNINE, MG/10 ML	
	1955	*1956*	*1955*	*1956*	*1955*	*1956*
1	104	119	103	103	0.02	0.00
	104	123	104	102		
2	111	111	100	101	0.01	0.10
	110	116	101	101		
3	96	107	101	102	0.01	0.06
	99		101	103		
	101		101	102		
				103		
				103		
4	102	104	100	103	0.04	0.00
	102	104	100	103		
5	113	135	94	100	0.04	0.06
	108	154	96	101		
6[a]	118	113	99	99	0.00	0.03
	109	110	102	100		
7[b]		102		104		0.01
		110		104		
		107				

[a] Results were received after submission of 1955 report and are not included in the Associate Referee's report of 1955.
[b] Did not collaborate in 1955.

solutions to be yellowish; this indicates some contamination with iron. Two of the collaborators stated that the strychnine was more difficult to elute this year than last, and that the column required more pressure for elution.

CONCLUSIONS AND RECOMMENDATION

Six collaborators, using the Banes method, found the quinine content of a year-old Elixir of Iron, Quinine, and Strychnine, N.F., to be essentially the same as that of the freshly prepared Elixir. Two collaborators found the strychnine content significantly higher when reassaying the Elixir after one year; however, it is not certain that aging has a disturbing effect on the strychnine analysis. The Associate Referee and several of the collaborators who used the method obtained good recoveries for both

the old and the freshly prepared elixirs. Contamination with iron appeared to be more of a problem this year than in 1955.

The results obtained this year do not warrant recommending adoption of the method. The high recoveries do not appear to be due to a basic defect in the method; they do indicate, however, that directions for preparation of the column and elution of the strychnine must be more explicit to eliminate possible contamination. A positive step to eliminate iron in the strychnine solution may have to be included in the method.

It is recommended* that the subject be continued.

ACKNOWLEDGMENT

The Associate Referee is grateful for the collaboration of the following chemists, all of the Food and Drug Administration: Rupert Hyatt and James F. Langston, Cincinnati; Harry Rogavitz, New York; Harold E. Theper, St. Louis; Curt Rupke, Buffalo; R. Edge, Denver; Charles E. Beisel, Baltimore.

* For report of Subcommittee B and action of the Association, see *This Journal*, 40, 24, 25 (1957).

REPORT ON SYNTHETIC DRUGS

By F. C. SINTON (Food and Drug Administration, Department of Health, Education, and Welfare, New York 14, N. Y.), *Referee*

Phenylpropanolamine Hydrochloride.—The Associate Referee reports development of a method involving a chromatographic separation and spectrophotometric determination. The procedure offers an advantage in recovery over the extraction method described in this year's report of the Associate Referee on Phenylethylamines. The Referee concurs with the recommendation* that the subject be continued.

Mannitol Hexanitrate in Mixtures.—Work was continued this year to overcome the background interference which caused difficulty in the determination of phenobarbital in mixture with mannitol hexanitrate. The Referee concurs with the recommendation that the subject be continued.

Amphetamines.—It is recommended that this subject be discontinued since the report of the Associate Referee on Phenylethylamines covers the topic adequately.

Amobarbital Sodium and Secobarbital Sodium (Tuinal ®).—The Associate Referee has suggested that certain corrections be made in the method, and the Referee concurs. The corrections do not involve the accuracy of the method. It is recommended that the method be made official.

* For report of Subcommittee B and action of the Association, see *This Journal*, 40, 25 (1957).

Acetophenetidin and Caffeine.—The Associate Referee recommends that the method be adopted as official, and the Referee concurs.

It is recommended that studies on *p*-aminosalicylic acid and isonicotinyl hydrazine, antihistamines, sulfonamide derivatives, steroid estrogens, and synthetic estrogens be continued.

REPORT ON ACETYLSALICYLIC ACID, ACETOPHENETIDIN, AND CAFFEINE

By Gordon Smith (Food and Drug Administration, Department of Health, Education, and Welfare, New York 14, N. Y.),
Associate Referee

The method adopted as first action by the Association last year (*This Journal*, **39**, 99 (1956)) has been used to analyze 14 typical commercial samples of tablets and capsules in this laboratory. Twelve of these samples were straight APC mixtures. The results obtained for all of them were close to the declared amounts of all three ingredients. Each of the remaining two samples contained a fourth active ingredient. Acetylsalicylic acid in one of these samples had to be determined by the older A.O.A.C. gravimetric method, because the acetylsalicylic acid had been partially hydrolyzed during the separation of the fourth ingredient, codeine. When any substantial fraction of the acetylsalicylic acid has been hydrolyzed, the gravimetric method is subject to much smaller error than the spectrophotometric method. With this exception, results for these two samples by the method under consideration were also close to the declared amounts. The method may not be satisfactory for all samples which contain additional active ingredients.

Two lots of isopropyl ether, one checked by the Associate Referee and one in another laboratory, could not be purified to produce the low absorbance specified in the method. When the first of these two lots was received, however, its absorbance was not far above the specified limit.

One supplier has done some experimental work on this subject, and is contemplating production of a spectrophotometric grade. The absorbance limit could be raised from 0.100 to 0.200 without appreciably affecting accuracy, but this does not appear to be necessary.

No other difficulties with the method have been encountered at this laboratory, and none have been reported elsewhere.

It is recommended* that the method be adopted as official, and that the subject be closed.

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* For report of Subcommittee B and action of the Association, see *This Journal*, 40, 25 (1957).

REPORT ON p-AMINOSALICYLIC ACID AND ISONICOTINYLHYDRAZINE IN ADMIXTURE

By LLEWELLYN H. WELSH (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.), *Associate Referee*

p-Aminosalicylic acid (PAS) has been used in the treatment of tuberculosis since its bacteriostatic action against tuberculosis bacilli was observed in 1946. *New and Nonofficial Remedies* first listed the acid and its sodium salt in the 1951 edition (1a), and in 1955 the U.S.P. XV (2) recognized the calcium salt in addition to these two substances.

The antitubercular activity of isonicotinylhydrazine (isoniazide, INH) was demonstrated in 1952, and the drug has been recognized in N.N.R. (1b) and in the U.S.P. XV (2).

It has been found advantageous to employ PAS and INH jointly in the treatment of tuberculosis. A basic daily regimen includes the administration of 12 grams of PAS and 300 mg of INH. Tablets containing the drugs in this ratio (500 mg PAS, 12.5 mg INH) are being marketed, but the dosage form is not listed in N.N.R. or the official compendia, and a method of analysis applicable to such tablets apparently has not been described in the original literature.

The U.S.P. method for the assay of PAS, its sodium and calcium salts, and tablets of the three drugs is a titration with standard sodium nitrite. This might be applied to tablets of PAS-INH; although the primary amino group of the hydrazine would consume nitrous acid, a correction could be made on the basis of the INH assay result. There is, however, another consideration which speaks against the general applicability of the nitrite titration of PAS-INH tablets: these tablets frequently contain a buffering agent, one component of which is glycine or its basic aluminum salt (dihydroxy aluminum aminoacetate). It would therefore be necessary to determine the quantity of either of these substances and correct for the contribution of its amino group toward the consumption of nitrite.

The N.N.R. determination of PAS (1a) is a spectrophotometric method that depends on measuring the absorbance of its sodium salt at the maximum at 265 mμ in aqueous solution buffered at *p*H 7. In the same system, the specific absorbance of INH at this wavelength is about one-third that of PAS (Fig. 1). In dealing with tablets containing PAS and INH in the ratio previously cited (40:1) this would cause no serious error in the PAS determination, even without application of a correction factor, since the contribution of the INH to the total absorbance would be only about 0.8 per cent.

The N.N.R. method (1b) for the assay of INH as the pure drug, or in capsules, tablets, and sirups, depends on measurement of the absorbance

at the maximum at 266 mμ in 0.1 *N* hydrochloric acid. Carol (3) has described a method for tablets which involves measuring the ultraviolet absorption spectra in 0.1 *N* acid and in 0.1 *N* alkali. The U.S.P. XV method for INH and its tablets is based on the reaction of the hydrazide with standard iodine in the presence of bicarbonate. None of these methods is suitable for determining INH directly in the presence of a forty-fold quantity of PAS.

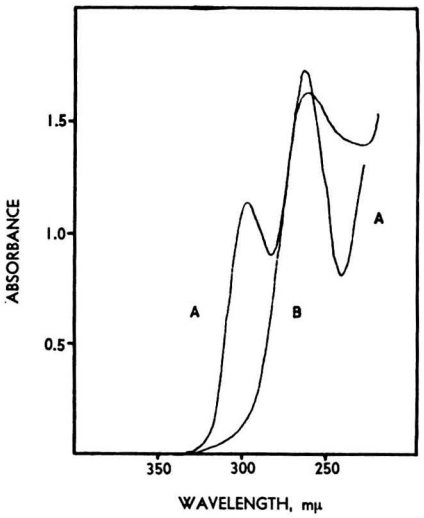


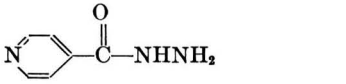
FIG. 1.—Ultraviolet absorption spectra, recorded with a Cary Model 11 spectrophotometer (1 cm cell, pH 7 buffer): A: *p*-aminosalicylic acid (20 mg/liter), $a_{265}=86.3$, $a_{299}=56.3$; B: isonicotinylhydrazine (54 mg/liter), $a_{264}=30.3$.

Biffoli (4) has reported a method for determining INH in the presence of calcium PAS. The procedure involves hydrolysis of the hydrazide by boiling the sample under reflux with about 20 per cent hydrochloric acid to liberate hydrazine, which is titrated with standard potassium iodate. With this method the Associate Referee obtained excellent results for mixtures containing PAS and INH only, although to complete the hydrolysis it was necessary to reflux 60–90 minutes instead of the 10–12 minutes specified. However, when the method was applied to commercial tablets containing starch, dihydroxy aluminum aminoacetate, and calcium carbonate, recoveries were very low and the end point was somewhat obscured by the brown color that developed during refluxing. Apparently the carbohydrate component was responsible for the interference.

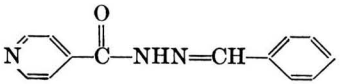
Consideration was then given to the possibility of determining INH after separating it from other substances associated with it in tablets. The hydrophilic nature of INH renders impracticable its quantitative isolation by extraction from aqueous systems with immiscible solvents. However, the basic properties of the substance, in contrast to the predominately acidic nature of PAS, would appear to make feasible its separation by a technique involving the use of ion-exchange resins. An alternative would be to convert the substance to a derivative whose solubility characteristics would permit its quantitative isolation by partition. It was decided to explore the possibilities of the latter alternative.

The primary amino group is the most reactive unit in the INH molecule (I) and the first choice as a point of attack in seeking to prepare a useful

derivative. Easily prepared derivatives of primary amines include acylation products and Schiff bases.



I



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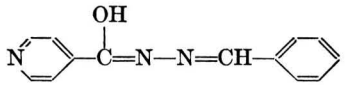
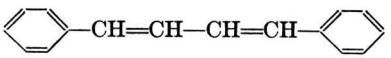


III



IV

The acetylation reaction has been used in this laboratory to form quantitatively, in dilute aqueous solution, derivatives that have desirable physical and chemical properties (5). When the reaction was applied to a 0.1 per cent solution of INH, only a small fraction of the calculated amount of product was obtained after several extractions with chloroform. This was not entirely unexpected in view of the literature report of the great solubility of acetyl INH in water (6).

The formation of Schiff bases with aromatic aldehydes was then investigated. The identification test in the N.N.R. monograph (1b) depends on the formation of a benzylidine derivative, m.p. 197–200°, from benzaldehyde; that of the U.S.P. (2) employs vanillin to prepare the vanillal derivative, m.p. 228–231°. The condensations are carried out in hot methanol and water, respectively; concentrations of INH are of the order of 1 per cent. The derivatives precipitate from the reaction mixtures, and are separated by filtration and purified by recrystallization. Though these reactions are useful qualitative tests, the experimental conditions cannot be directly applied to the quantitative isolation of derivative. In any practical quantitative method, the reaction must proceed to completion in an aqueous system (extract of tablets) in which the concentration of INH is substantially less than in the qualitative tests. Furthermore, the solubility characteristics of the derivative must favor its passage from the aqueous medium into an immiscible solvent.

Benzaldehyde was found to react quantitatively with INH in about 0.1 per cent aqueous solution at room temperature. The derivative may be readily extracted into chloroform along with excess reagent, the volatility of which permits it to be easily removed from the derivative. The possible utility of vanillin was not investigated; it may work as well as benzaldehyde, but its lower volatility would complicate the removal of excess reagent.

On the basis of the foregoing considerations and others arising from additional experimental work a scheme of analysis was devised. The Associate Referee obtained recoveries of PAS and INH in the range of 98–99 per cent by this method. The principles are as follows:

Powdered tablets are treated with an aqueous solution of sodium bicarbonate to dissolve the PAS and INH, and the solution is separated from insoluble constituents by filtration. An aliquot of the solution, containing 15 mg INH, is shaken with benzaldehyde. After reaction is complete, the mixture is extracted with chloroform, and solvent and excess reagent are removed from the extract by heating on the steam bath. The residue of crude benzylidine INH is taken up in chloroform, and the solution is washed with aqueous bicarbonate to remove benzoic acid formed during evaporation of the extracts. After the solvent is evaporated, the residue of purified derivative is dissolved in alcohol, and the absorbance of the solution is measured at the peak at 303 mμ (Fig. 2).

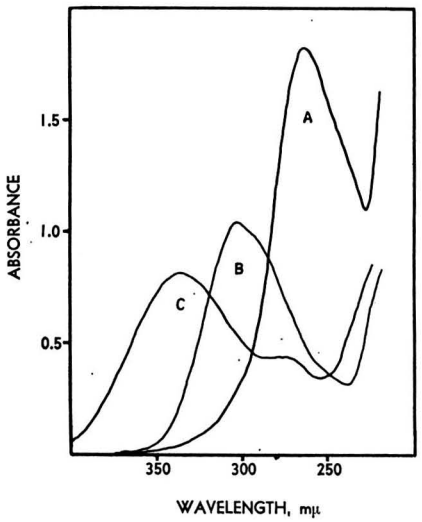


FIG. 2.—Ultraviolet absorption spectra, recorded with a Cary Model 11 spectrophotometer (1 cm cell, 95% ethanol): A: isonicotinylhydrazine (55 mg/liter), $a_{264}=33.5$; B: benzylidine isonicotinylhydrazine (11 mg/liter), $a_{303}=94$; C: benzylidine isonicotinylhydrazine (11 mg/liter, in presence of KOH), $a_{335}=74$.

For the determination of PAS, an aliquot of the bicarbonate extract of tablets is suitably diluted with *p*H 7 buffer, and the absorbance of the dilution is measured at the peak at 299 mμ. Although the absorbance at this maximum is only two-thirds that at the 265 mμ peak (Fig. 1), the measurement is made at this point to diminish the error due to the absorbance of INH. As stated previously, the error at 265 mμ amounts to about 0.8 per cent when the ratio of PAS to INH is 40:1. It is reduced to less than 0.3 per cent at 299 mμ, since the absorbance of INH at this wavelength is about one-tenth that at 265 mμ.

EXPERIMENTAL

STANDARDS

Benzylidine isonicotinylhydrazine (BINH).—One g U.S.P. INH was dissolved in 10 ml H_2O in a small flask, 1 ml N.F. benzaldehyde was added, and the flask was was stoppered and shaken vigorously. In about a minute the derivative precipitated out. Shaking was continued 4 minutes more; then the precipitate was filtered off on a Büchner funnel, washed with water, and sucked fairly dry on the filter. The derivative was dissolved in 8–9 ml boiling ethanol, and the hot solution was filtered. Crystallization was completed in a freezing unit, and the crystals were filtered off and washed with cold alcohol, then with a little ether, and finally with petroleum ether. After standing 24 hours *in vacuo* over silica gel the product weighed 1.16

grams (71%): m.p. 199–200°. Its ultraviolet spectrum is shown in Fig. 2, curve B.

p-Aminosalicyclic acid.—A solution of 40 g 95% NaOH in 500 ml H_2O was added slowly, with stirring, to 150 g of a 5-year-old, highly discolored commercial sample of PAS. When no more solid appeared to dissolve, 5 g $NaHCO_3$ was added and the system was stirred until solution was complete. Darco® G-60 (15 g) was then added to the almost black solution, stirring was continued for 5 min., and the system was filtered. The filtrate was a clear, light-brown; the intensity of the color did not appear to be diminished significantly by additional charcoaling. The solution was acidified with 100 ml glacial acetic acid and cooled, and the precipitated PAS was filtered off and washed with cold H_2O. Most of the color remained in the mother liquor. The air-dried acid was dissolved in 750 ml boiling ethanol, stirred 5 min. with 10 g Darco® G-60, and filtered. A small amount of decarboxylation evidently took place in the hot ethanolic solution. Crystallization in the filtrate was allowed to proceed as the solution cooled spontaneously to room temperature, and was completed in a refrigerator freezing unit. The product was filtered off, and washed with cold ethanol, benzene, and finally petroleum ether. The PAS thus purified amounted to 58 g (39% recovery) after drying 48 hr *in vacuo* over silica gel. It was very pale ivory in color, and satisfied the specifications of the U.S.P. monograph (2). It consisted of plates and lath-like crystals ranging in length up to 2 mm. In this state of aggregation the substance exhibits much greater resistance to discoloration than it does in the powdered state. No change was evident in its appearance after it had been stored 6 months in a brown glass bottle. Its ultraviolet spectrum is shown in Fig. 1, curve A.

Simulated tablet mixture.—The mixture employed in the present work was prepared to contain 75.24% PAS (finely powdered before mixing), 1.88% INH, and a quantity of *m*-aminophenol (MAP) equivalent to 0.26% of the amount of PAS present. The reason for incorporating the MAP will be explained in subsequent discussion. The remainder of the mixture consisted of a diluent of the following percentage composition: calcium carbonate, 49.3; dihydroxy aluminum aminoacetate, 32.9; starch, 7.9; acacia, 2.0; magnesium stearate, 5.3; talc, 2.6.

METHOD

Accurately weigh a sample of tablet powder, containing 35–40 mg INH, and transfer it to a 150 ml beaker. While stirring the sample with 20 ml H_2O, add 1.5 g $NaHCO_3$, and continue stirring until effervescence has ceased. Filter the suspension with suction through a medium porosity fritted glass filter (ca 3.5 cm diameter is a convenient size). Rinse the beaker thoroughly with 5 ml H_2O, break the suction, and transfer the rinsings to the filtering funnel so as to wash down the inside wall. Stir the mixture in the funnel so as to secure a uniform suspension and reapply suction. For quantitative transfer and filtration, repeat washing of the beaker and funnel with 3 additional 5 ml portions of H_2O. Quantitatively transfer the filtrate to a 50 ml volumetric flask with small portions of H_2O, dilute to volume, and mix. Proceed immediately with the determination of PAS. Determine INH as soon as practicable, preferably not later than 4 hours after preparation of the bicarbonate solution.

p-AMINOSALICYLIC ACID

From the aqueous bicarbonate extract of tablet powder, select an aliquot containing ca 150 mg PAS, transfer it to a 500 ml volumetric flask, and dilute to the mark with H_2O. Transfer a 10 ml aliquot of this dilution to a 250 ml volumetric flask, add 12.5 ml of concentrated pH 7 buffer,[1] and dilute to volume with H_2O. With a Beckman Model DU spectrophotometer or equivalent (1 cm cell, pH 7 buffer as blank), measure the absorbance of the dilution at 299 mμ (max.), 244 mμ

[1] Dissolve 34 g potassium dihydrogen phosphate in 136 ml 1.0*N* NaOH and dilute to 1 liter with H_2O.

(min.), and 325 mμ.[2] From these data calculate the baseline absorbance, A_b, by means of the equation: $A_b = A_{299} - (0.3210\ A_{244} + 0.6790\ A_{325})$.

Accurately weigh ca 300 mg finely powdered pure PAS, dissolve it in 2–3 ml 1*N* NaOH, and dilute the solution to exactly 1000 ml with H_2O. Transfer a 10 ml aliquot of the solution to a 250 ml volumetric flask, add 12.5 ml concentrated pH 7 buffer, and dilute to volume with H_2O. Measure the absorbance of this dilution at 244 mμ, 299 mμ, and 325 mμ, as above.[2] Determine A_b, and from this value and that obtained from the solution of the sample, calculate the quantity of PAS in the sample originally taken for analysis.

ISONICOTINYLHYDRAZINE

Transfer a 20 ml aliquot of solution to a 125 ml separatory funnel, add 0.5 ml N.F. benzaldehyde, shake the system for 15 minutes, and let it stand 10 minutes more. Extract with six 20 ml portions of $CHCl_3$, filter the extracts through a compact pledget of absorbent cotton into a 150 ml beaker, and evaporate the filtrate on the steam bath in a current of air until the residue is odorless or has, at the most, a faint odor of benzoic acid (there must be no sweet odor or odor of benzaldehyde). Dissolve the residue in $CHCl_3$ and transfer it quantitatively to a separatory funnel with more solvent. Add enough $CHCl_3$ to make the volume 20–30 ml, shake the solution with 10 ml freshly prepared 5% $NaHCO_3$, and filter the $CHCl_3$ layer through a compact pledget of absorbent cotton. Wash the bicarbonate solution with three 10 ml portions of $CHCl_3$, passing each wash through the filter, and evaporate the combined $CHCl_3$ extracts to dryness on the steam bath in a current of air.

Dissolve the residue of BINH in ethanol without the aid of heat, and dilute to exactly 100 ml with ethanol. Dilute a 5 ml aliquot of this solution to exactly 200 ml with ethanol, and measure the absorbance, *A*, of the dilution with a Beckman DU spectrophotometer, or its equivalent (1 cm cell, ethanol blank), at 303 mμ (max.), 237 mμ (min.), and 355 mμ (see footnote 2). From these data, calculate the baseline absorbance, A_b, by means of the equation: $A_b = A_{303} - (0.4407A_{237} + 0.5593\ A_{355})$.

Dissolve an accurately weighed 50–60 mg portion of pure BINH in ethanol and dilute to exactly 200 ml. Dilute a 5 ml aliquot of this solution to exactly 200 ml with ethanol, and measure the absorbance of this dilution at 237 mμ, 303 mμ, and 355 mμ, as above (footnote 2). Determine A_b; using this value and that given by the sample, calculate the quantity of BINH corresponding to the sample originally taken for analysis: BINH × 0.60888 = INH.

DISCUSSION AND RECOMMENDATION

Under the experimental conditions, the primary amino group of PAS apparently does not react with benzaldehyde. After BINH and excess reagent were extracted from the reaction mixture, acidification of the aqueous system yielded a precipitate of PAS. After the PAS was separated by filtration, boiling the acid filtrate produced no trace of an odor of benzaldehyde, which would be formed from any benzylidine PAS that might be present.

PAS decomposes to form *m*-aminophenol (MAP), particularly in acid solution. The U.S.P. (2) tolerates a maximum of 0.2 per cent of this toxic substance in PAS. In a preparation containing PAS and INH in the ratio

[2] With instruments that yield optimum absorbance readings in the range 1.0–1.5, use a 2 cm cell or modify the dilution so that the concentration of substance is twice that specified.

of 40:1, the quantity of MAP representing 0.2 per cent of the PAS would correspond in weight to about 8 per cent of the weight of INH present. It is conceivable that its presence as such, as its benzylidine derivative, or as its oxidation products in the residue of BINH could seriously affect the spectral characteristics of that material.

To investigate the effect of MAP on the INH assay, the procedure was applied to 20 ml of a solution containing 70 mg of bicarbonate and 1.3 mg of MAP (these values correspond, respectively, to the weight of excess bicarbonate and about 0.2 per cent of the weight of PAS, 0.6 gram, present in the aliquot taken to determine INH). The residue from the chloroform extracts consisted of 2 mg of brownish resin. In ethanolic solution, at a concentration of 1 mg/liter, its absorbance varied almost linearly from 0.10 at 215 mμ to virtually zero at 400 mμ (Cary Model 11 spectrophotometer, 1 cm cell). At 303 mμ the absorbance was 0.02, or about 2 per cent of that expected in the INH assay. This error is not particularly serious, and is reduced to about 1 per cent in the assay calculations, which are designed to minimize error due to background absorption by employing baseline absorbance instead of total absorbance.

Replacement of the hydrogen atoms of the primary amino group of INH by a benzylidine group shifts the absorption peak from 264 mμ to 303 mμ, and raises the specific absorbance, a, from 33.5 to 94 (Fig. 2). In the presence of alkali, the absorption peak of the benzylidine derivative shifts in the direction of the visible spectrum to 335 mμ, a virtual plateau makes its appearance between 275 and 290 mμ, and a_{max} decreases about 20 per cent. Benzylidine isonicotinylhydrazine may be represented (see p. 809) as an amide (keto form, II) or as a hydroxyimine (enol form, III). The presence of alkali would be expected to favor a shift toward the enol form, in which there is established a system of conjugated double bonds throughout the length of the molecule. A shift in the wavelength of the absorption maximum in the direction of the longer wavelengths would be expected to accompany such a structural change. It is of interest to note that the enol structure, III, is analogous to that of 1,4-diphenyl-1,3-butadiene, IV, which exhibits maximum absorption in the vicinity of 330 mμ (7).

The spectral characteristics of BINH serve to establish the presence of INH in the tablets analyzed. The presence of PAS may be confirmed by observing the absorption spectrum of the solution used in the assay, and by applying U.S.P. identity tests to the PAS which precipitates on acidification of the bicarbonate extracts of the tablets.

It is recommended* that the subject be continued and that the proposed method be submitted to collaborative study.

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* For report of Subcommittee B and action of the Association, see *This Journal*, 40, 25 (1957).

REFERENCES

(1) (a) *New and Nonofficial Remedies*, J. B. Lippincott Co., Philadelphia, Pa., 1951; (b) *ibid.*, 1953.
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REPORT ON TUINAL ® (AMOBARBITAL SODIUM AND SECOBARBITAL SODIUM)

By GEORGE E. KEPPEL (Department of Health, Education, and Welfare, Food and Drug Administration, Minneapolis 1, Minn.), *Associate Referee*

An error is contained in the preparation of the standard curve for sodium secobarbital, as published in *This Journal*, **38**, 630 (1955), and *Official Methods of Analysis*, Eighth Edition, **32.146**. Instead of 25 mg sodium secobarbital to prepare the standard solution, 100 mg is required. This is the quantity used in development work, and the amount specified in directions to collaborators. One analyst reported that in the determination of total barbiturates, **32.147**, he was not able to obtain a clear filtrate of the sample solution. He suggested using a quantitative type of paper.

The reference to "0.1 *N* $KBrO_3$", **32.148**, should be changed to "0.1 *N* KBr-$KBrO_3$".

It is recommended* that the method be corrected as follows:

PREPARATION OF STANDARD CURVE, **32.146**, line 1, change "25 mg" to "100 mg."

DETERMINATION OF TOTAL BARBITURATES, **32.147**, line 3, beginning "Filter thru . . . " change to "Filter, using a quantitative paper sufficiently retentive to produce a clear solution, discarding first 10–15 ml filtrate."

DETERMINATION OF SODIUM SECOBARBITAL, **32.148**, line 5, change "$KBrO_3$" to "KBr-$KBrO_3$."

* For report of Subcommittee B and action of the Association, see *This Journal*, **40**, 25 (1957).

REPORT ON MANNITOL HEXANITRATE IN MIXTURES

By Evelyn Sarnoff (Food and Drug Administration, Department of Health, Education, and Welfare, New York 14, N. Y.), *Associate Referee*

In accordance with the recommendation of Subcommittee B, the study of this topic was continued in order to eliminate the inconsistencies in last year's collaborative study of the assay of phenobarbital in tablets containing mannitol hexanitrate (*This Journal*, **39**, 630 (1956)).

The average of the collaborative results for phenobarbital in a tablet mixture simulating a commercial preparation of mannitol hexanitrate and phenobarbital was 108 per cent of the theoretical amount. The high results indicated that the interference might be due to the excipients used in preparing this mixture. The method previously studied involved diluting the solution, making it ammoniacal, and reading the amount of phenobarbital directly on the spectrophotometer.

Measurements made with a Beckman *p*H meter showed that the *p*H of the final solution varied by 0.1 units for each 0.5 ml of ammonia used. The *p*H of the final solutions, sample, standard, and blank must be the same. To insure this the directions should read: "add exactly 10.0 ml of ammonia," instead of "add 10 ml of ammonia."

Extraction procedures to separate the contaminant from the phenobarbital were tried but proved unsuccessful.

When the technique was modified in this way and calculations were made by the baseline method, reproducible and reliable results were obtained. The average of twelve determinations was 99.4 per cent recovery of phenobarbital from a control mixture.

It is recommended* that collaborative studies of this topic be continued.

* For report of Subcommittee B and action of the Association, see *This Journal*, **40**, 25 (1957).

REPORT ON PHENYLPROPANOLAMINE HYDROCHLORIDE

By Gordon Smith (Food and Drug Administration, Department of Health, Education, and Welfare, New York 14, N. Y.), *Associate Referee*

The previous Associate Referee, A. W. Steers, obtained satisfactory results for capsules and aqueous solutions of phenylpropanolamine hydrochloride by extracting and titrating phenylpropanolamine base (*This Journal*, **37**, 683 (1954). F. A. Rotondaro has also worked on the problem, using a single extraction method. In the work reported here, a

chromatographic-spectrophotometric method has been used to separate and determine the base. The Banes procedure (*J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 680 (1954)) was used, with some modification, to set up the chromatographic column. The sample was taken up in dilute ammonia and Celite, and completely transferred to the tube by sweeping the beaker with portions of Celite. Chloroform was used as the eluent. Thus the chromatographic process could be substituted for a shakeout extraction.

The absorbance of the eluate was read in the spectrophotometer. The phenylpropanolamine curve has two rather sharp peaks, the higher one at 258.5 mμ. A relatively high concentration, about 40 mg per 100 ml, gives suitable readings.

METHOD

Fix a pledget of glass wool in the outlet of a 25×200 mm chromatographic tube. Thoroly mix 3 g Celite 545 and 2 ml H_2O in a beaker. Transfer to tube with a metal spatula and press down evenly with a suitable packing rod.

To a 150 ml beaker, transfer an accurately weighed quantity of sample containing ca 50 mg phenylpropanolamine.HCl. Add 4 ml 5% NH_3 solution and mix. Add 5 g Celite and stir with a spatula until mixture appears uniform. Transfer to tube without loss thru a powder funnel, in 4 or more portions, pressing down each portion evenly with moderate pressure. When removing funnel from tube each time, tap it lightly in tube to remove loosely adhering particles; then hang it in a beaker of such size that it does not touch bottom. After using packing rod, scrape off most of adhering material into tube with spatula, and tap rod and spatula lightly over mouth of tube. When laying down these implements place them in such position that their ends do not touch anything. Finally use a smooth, intact rubber policeman to sweep adhering material from beaker and funnel into tube. Rub beaker, spatula, and packing rod with 3 successive 1 g portions of Celite, sweeping each portion thru funnel into tube, using the rubber policeman. Press down each portion with packing rod.

With a 100 ml volumetric flask as receiver, wash down inside of tube with $CHCl_3$, adding enough (ca 25 ml) to moisten column and produce only a few drops of eluate, and elute with 95 ml $CHCl_3$. Dilute to volume with $CHCl_3$. Place aliquot in silica cell, and within 3–6 min., measure absorbance at 258.5 mμ, using as blank a portion of same $CHCl_3$ used for elution.

To a 150 ml beaker transfer 50.0 mg pure phenylpropanolamine.HCl. Treat and read absorbance in same manner as for sample. Calculate phenylpropanolamine HCl content of sample.

RESULTS AND RECOMMENDATION

Recoveries of 101.3 and 101.5 per cent were obtained on capsule mixtures simulating the commercial product. The method without modification should be applicable to tablets and aqueous solutions. (No tablets are now being marketed, as far as is known.) Application to elixirs and nasal jellies, which are more complex mixtures, may require modification.

It is recommended* that the application of the method to other dosage forms be studied, with a view to submitting it to collaborative study.

ACKNOWLEDGMENT

The writer wishes to thank Dr. A. W. Steers for supplying useful data.

* For report of Subcommittee B and action of the Association, see *This Journal*, 40, 25 (1957).

REPORT ON SULFONAMIDE DERIVATIVES

By F. E. YARNALL (Food and Drug Administration, Department of Health, Education, and Welfare, Kansas City 6, Mo.),
Associate Referee

Committee B recommended (1) that studies of sulfonamides be continued. Upon the Referee's suggestion, the Associate Referee studied the use of column chromatography to separate and determine these compounds.

Various literature reports on column chromatography described the use of alumina (2), Celite (3), and ion-exchange resins (4) as adsorbents. The alumina and Celite methods required undesirably large volumes of solvent to elute the compounds. The exchange resins described were of the sulfonic or carboxylic types and their use depended upon a free NH_2 group; several unofficial sulfonamides do not contain this group. Successful separation of sulfanilamide and sulfadiazine was reported. The Associate Referee did not have an opportunity to test this method on other compounds.

A limited and unsuccessful attempt was made to use Solka-Floc (a cellulose fiber material) in a column to separate sulfadiazine from sulfamethazine; the butanol-ammonia solvent used in paper chromatography was employed. Other solvent systems were not tried.

The Associate Referee had considerable experience with paper chromatographic methods for the separation of sulfonamides; he used methods essentially the same as the method later adopted by the U.S.P. XV and the Tenth Edition of the National Formulary. Many of the nonofficial sulfonamide mixtures separated satisfactorily. Separation on paper appears to be much more efficient and convenient than column chromatography. The paper method is to be preferred for both identification and quantitative determinations wherever recoveries are sufficiently accurate.

A review of 69 sulfonamide mixtures used in human medicine, currently listed in the Sixth Edition of *Modern Drug Encyclopedia*, showed only one product which might not be suitable for analysis by the U.S.P.-N.F. method: a mixture of sulfathiazole and sulfamethazine. The Associate Referee could not separate these two compounds satisfactorily with the U.S.P.-N.F. solvent system, but a different solvent system might separate this mixture. Other commercial products may require special treatment to remove interfering vehicles.

The field of veterinary medicine includes a variety of sulfonamide products. Some products contain mixtures of as many as four sulfonamides and include compounds, such as phthalylsulfacetamide, that must be hydrolyzed before chromatography to be detected by the reagents commonly used for quantitative measurements. Phthalylsulfacetamide has been successfully separated from mixtures and identified. The hy-

drolyzed sample was compared with a known mixture subjected to the same treatment.

It is suggested that further work on this subject include tests of the ability of the U.S.P.-N.F. method both to separate the components of sulfonamide mixtures, other than official mixtures, and to make accurate quantitative determinations of them.

It is recommended* that study be continued of the paper chromatographic separation of therapeutically important sulfonamide mixtures.

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No reports were given on amphetamines, antihistamines, or synthetic estrogens.

* For report of Subcommittee B and action of the Association, see *This Journal*, 40, 25 (1957).

REPORT ON MISCELLANEOUS DRUGS

By I. SCHURMAN (Food and Drug Administration, Department of Health, Education, and Welfare, Chicago 7, Ill.), *Referee*

Mercury Compounds.—The Associate Referee has submitted pure mercury compounds, solutions, ointments, and tablet material to collaborative study. The results obtained are considered satisfactory.

It is recommended* that the method for mercury in pharmaceutical preparations be adopted as first action; that further work be done on other organic mercurials; and that the method be studied for the lowest limit of concentration of mercury in the sample.

Infrared Spectrophotometric Methods for Drugs.—The Associate Referee has continued his study of quantitative determinations of several drugs by infrared spectrophotometric methods. This year's work was done on ethinyl estradiol and ethinyl testosterone with good results. The Associate Referee recommends that the infrared analysis of all drugs reported in 1953, 1954, and 1956 be submitted to collaborative study. The Referee concurs.

Ultraviolet Spectrophotometric Methods for Drugs (Phenylethylamines).—

* For report of Subcommittee B and action of the Association, see *This Journal*, 40, 25, 26 (1957).

The Associate Referee has submitted an excellent report on the quantitative determination of phenylethylamines based upon their absorptivity in 0.1 *N* sulfuric acid. The Associate Referee recommends that the method be adopted as first action, and that study be made of the application of spectrophotometric procedures to oxygenated phenylethylamines. The Referee concurs in both recommendations.

Nitrites.—The Associate Referee, R. F. Heuermann, has reported that no adverse comments have been received on the first action hydrazine method for nitrites. He therefore recommends that the method be adopted as official. The Referee concurs.

REPORT ON GENERAL METHOD FOR DETERMINATION OF MERCURY IN PHARMACEUTICAL PREPARATIONS

By R. L. Herd (Department of Health, Education, and Welfare, Food and Drug Administration, St. Louis 1, Mo.),
Associate Referee

Last year samples of mercury preparations were submitted for collaborative study by the strychnine method (*This Journal*, **38**, 645 (1955)). Results were reported at the 1955 meeting of the Association. Some of the results were not acceptable, which indicated that the instructions to collaborators were ambiguous. On this basis it was recommended that study of the same method be continued.

The method was revised and sent to collaborators with the following samples: (1) Phenyl mercuric chloride (recrystallized); (2) Mercuric iodide (recrystallized); (3) Nitromersol (commercial); (4) Mercuric oxide ointment, 1.000 per cent HgO; (5) Calomel tablet material, 17.00 per cent Hg_2Cl_2 with aloes, capsicum, ginger, starch, and talc; and (6) Merbromin solution, N.F., 2.00 g per 100 ml.

Nitromersol solution is unstable. To insure uniformity of sample the collaborators were requested to prepare a solution according to N.F. instructions. The merbromin solution was prepared by the Associate Referee to reduce the sampling error created by the hygroscopic nature of the dried merbromin. As a result of the collaborators' comments, two minor changes have been made in the method. This modified method is as follows:

METHOD

REAGENTS

(a) *Strychnine sulfate soln.*—Approx. 0.01*M*; 4.3 g/500 ml.

(b) *Valser's reagent.*—Dissolve 10 g KI in H_2O and dil. to 100 ml. Sat. with HgI_2 (ca 14 g) and filter.

APPARATUS

(a) *Digestion flask.*—Round bottom or acetylation; 100 ml capacity fitted to H_2O-cooled straight tube condenser with $\overline{\$}$ joint.

(b) *Gooch crucibles.*—Fitted with 21 mm filter paper disks, covered with thin layer of asbestos, and dried at 105°. Use to filter and weigh ppt of strychnine. $HI.HgI_2$.

PREPARATION OF SAMPLES

Weigh (avoid use of metal containers) or measure accurately quantity of sample contg 20–100 mg Hg (optimal ca 50 mg) and treat as follows:

(a) *Solutions of organic mercurials.*—Transfer sample to beaker and evap. just to dryness with low heat (60–70°) and current of air. Dissolve residue in ca 5 ml 10% NaOH soln and transfer to digestion flask. Rinse beaker with four 3–4 ml portions H_2O and add rinsings to digestion flask. Add excess liquid Br to soln and connect flask to condenser. Boil 4–5 min. and add 3 ml HCl thru top of condenser. Continue to heat soln until Br collects in condenser tube. Remove heat and cool until Br returns to soln in digestion flask. Alternately heat and cool until Br has almost completely dissipated. (After 3 intervals of heating, flow of H_2O thru condenser may be discontinued to aid in removing Br.) Allow flask to cool, and rinse inside of condenser with ca 5 ml H_2O. Disconnect flask and rinse tip of condenser with small stream of H_2O from wash bottle. Filter thru 9 cm paper into 150 ml beaker, and rinse flask and filter with four 5 ml portions H_2O.

(b) *Ointments.*—Transfer sample to digestion flask and add 5 ml HCl (1+3) followed by 5 ml satd Br-H_2O. Place small pieces of porcelain, SiC, or few glass beads in flask to prevent bumping. Connect flask to condenser and fit flask over hole cut in asbestos board so that bottom of flask just extends below under-surface of board. Heat over low flame, maintaining slow and continuous ebullition ca 10 min., and then cool to room temp. Disconnect flask and decant aq. portion thru 9 cm paper into 150 ml beaker. Take precautions to retain all ointment base in flask. Rinse neck of flask into filter with few drops of H_2O from wash bottle. Add 1 ml HCl (1+3), 1 ml satd Br-H_2O, and 8 ml H_2O to flask and reflux. Again cool contents of flask and decant aq. phase thru filter. Repeat refluxing and decanting with two 10 ml portions H_2O and finally rinse condenser tube into flask with ca 5 ml H_2O. Disconnect flask, rinse condenser tip, and decant rinsings thru filter. Rinse filter with 2 small portions H_2O from wash bottle. Test for complete removal of Hg by adding 5 ml H_2O and 2 drops HCl (1+3) to digestion flask and refluxing as before. Pass this soln thru original filter into 50 ml beaker. To filtrate add 1 drop of 10% KI soln and 1 drop of the strychnine sulfate soln. No turbidity should be produced. If extn is incomplete, repeat refluxings with H_2O until all Hg is removed. Reserve all test solns showing presence of Hg to add to major portion after pptn of Hg.

(c) *Calomel tablets.*—Weigh at least 20 tablets and det. av. wt. Grind to fine powder and transfer accurately weighed portion to digestion flask. Add 10 ml satd Br-H_2O and 5 ml HCl (1+3). Connect flask to reflux condenser and boil contents gently until most of Br vapors collect in condenser. Discontinue heating until Br returns to soln in flask. Repeat alternate heating and cooling until Br vapors are dissipated. Cool flask and contents to room temp. and rinse condenser tube with ca 10 ml H_2O. Disconnect flask and rinse condenser tip into flask. Filter soln thru gooch into 150 ml beaker. Rinse flask with three 5 ml portions H_2O and pass rinsings thru crucible, and finally rinse crucible with fine stream of H_2O.

(d) *Tablets containing purgative drugs.*—If tablets contain purgative drugs, add 10 ml alcohol to weighed sample in flask. Heat on steam bath with gentle agitation until alcohol begins to boil. Remove flask, cool under tap, and filter supernatant

liquid thru gooch fitted with asbestos mat. Retain as much of insol. residue in flask as possible. Rinse flask and contents with three 10 ml portions alcohol and two 5 ml portions H_2O, and decant thru crucible as above. Remove asbestos mat with fine wire or needle and transfer to the flask. Rinse crucible with 10 ml satd Br-H_2O and 5 ml HCl (1+3), and add rinsings to flask. Connect flask to condenser, and treat as in (c).

DETERMINATION

Add 10 ml 10% KI soln to filtrate, and if necessary, evap. on steam bath under current of air to ca 50 ml. If soln has not previously been acidified, add 3 ml HCl (1+3). Add 1% $NaHSO_3$ soln until I color is discharged, and keep soln free from I color by addn of the $NaHSO_3$ soln until final filtration is made. Add strychnine sulfate soln, (a), slowly from buret or pipet until ppt coagulates and settles rapidly. (Strychnine sulfate soln may be added as rapidly as it will flow from buret if theoretical quantity is used, based on 1 ml soln for each 4 mg Hg expected to be present.) Avoid undue excess of strychnine because of slight solubility of its hydriodide. Allow ppt to settle and test for complete pptn by adding 2–3 drops of the strychnine sulfate soln to clear supernatant liquid. If pptn is incomplete, indicated by cloudiness around the drops, add strychnine sulfate soln in 1 ml increments until pptn is complete. Allow ppt to remain in beaker, with occasional stirring, 0.5–1 hr. Decant supernatant liquid thru tared gooch. Wash ppt into crucible with fine stream of H_2O. Completely transfer ppt to crucible, and wash residue and crucible with three 5 ml portions H_2O. Scrub beaker thoroly with policeman. Transfer crucible and holder to another small suction flask and wash residue with 2–3 ml H_2O. Test filtrate for complete removal of strychnine by addn of Valser's reagent, (b). If necessary, continue washing ppt with small portions of H_2O until last washings give no more than faint opalescence upon addn of Valser's reagent. Always test main filtrate by addn of ca 1 ml of the strychnine sulfate soln to assure complete pptn of Hg. If pptn was incomplete, repeat detn. Dry crucible 1 hr at 105°, cool in desiccator, and weigh. Calc. % Hg compound in sample on basis of mol. wt of 916.78 for ppt of strychnine.HI.HgI_2.

COMMENTS OF COLLABORATORS

Most collaborators agreed that the wording of the method should be changed from "add about 10 drops of liquid bromine" to "add a definite excess of liquid bromine." Miss Barry did additional work on the merbromin to show that the amount of bromine specified was not sufficient. When the specified amount of bromine was increased by two drops or more, consistent results were obtained. Mr. Olen E. Morgan also did additional work to show that a larger amount of bromine was required to digest the merbromin. He found that 15 drops of bromine were required to produce an excess. The results reported in Table 1 are those in which an excess of bromine was used, except the results reported by Collaborator B.

Some collaborators preferred a pear-shaped flask for digestion and porcelain chips or silicon carbide chips to prevent bumping. Two collaborators suggested that the directions for testing the filtrate for complete removal of strychnine from the precipitate should be changed from "no more than an opalescence" to "no more than a faint opalescence."

TABLE 1.—*Per cent mercury recovered by collaborators*

COLLABORATOR	SAMPLE 1 C_6H_5HgCl	SAMPLE 2 HgI_2	SAMPLE 3 NITROMERSOL	SAMPLE 4 HgO OINTMENT	SAMPLE 5 Hg_2Cl_2 MIXTURE	SAMPLE 6 MERBROMIN SOLUTION
A	99.5	99.7	99.5	99.0	99.6	89.5
	99.8	100.7	99.9		99.5	90.0
B	100.3	100.2	98.2	98.7	99.2	78.5[a]
	99.4	100.4	98.9	97.3	98.1	80.0[a]
C	100.7	100.7	99.8	100.0	99.3	89.0
	100.0	100.2	99.0	97.2	98.1	90.0
D	99.3	99.9	99.4	99.8	98.1	93.0
	99.4	100.4	99.2	100.6	99.2	93.0
E	99.7	101.2	98.1	98.6	97.9	88.5
	99.6	101.4	98.2	98.8	99.7	87.5
F	99.4	99.2	98.4	99.1	100.2	—
	99.0	101.2	98.0	98.5	99.2	—
G	99.3	101.8	99.3	100.0	98.4	87.0
	99.4	101.2	99.5	103.2	98.6	87.0
		101.3				
H	98.9	100.4	100.0	101.1	99.8	90.5
	99.3	100.3	100.0	97.5	99.1	90.0
I	96.9	100.3	93.7	99.7	97.1	88.5
	97.1	100.0	97.7	100.9	97.7	88.5
J	100.0	99.8	98.2	98.2	98.4	—
	99.9	100.0	98.7	98.7	98.2	—
					98.7	
Av.	99.3	100.5	98.7	99.1	98.8	88.2
Std. Dev.	±0.71	±0.65	±1.16	±1.30	±0.85	±3.12

[a] These results are considered invalid. For details see discussion.

STATISTICAL ANALYSIS OF RESULTS

Pooled variance for the 6 sets[1] 2.19
Variance for set No. 6 only 9.73
F value = 9.73/2.19 = 4.44 (Critical value at 1.0% level = 2.50)

Standard Deviation Calculated by Considering All Results as a Single Set:

(1) Entire group (117 D.F.) ±4.6%
(2) Sets 1, 2, 3, 4, and 5 only ±2.3%

These figures contain (1) inherent error of the method, (2) personal operator error, and (3) deviations associated with different type of samples.

[1] YOUDEN, W. J., *Statistical Methods for Chemists*, John Wiley and Sons, Inc., New York, p. 12.

Standard Deviation Calculated by Considering Results on Each Sample as a Separate Set and Pooling Results:

(1) Six sets (110 D.F.) ±1.5%
(2) Sets 1, 2, 3, 4, and 5 only ±1.0%

Probable error on sets 1, 2, 3, 4, and 5 (1% level): ±2.62%

These figures contain (1) inherent error of the method, and (2) personal error due to different operators but not the deviations associated with different types of samples.

DISCUSSION

The results for the merbromin are fairly consistent, except those reported by Collaborator B which are considered invalid on the basis of the collaborator's statement that the specified amount of bromine was insufficient to give an excess. An excess of bromine was added after the solution was acidified. When the solution is acidified a lumpy mass is usually formed, which removes some of the mercury from the field of reaction. In this instance the directions for the addition of an excess of bromine before acidifying were not followed; these directions were written into the method as a result of collaborative study. In spite of these considerations the results were included in the table and statistical computations.

The other results on merbromin seem low, because the mercury content of the merbromin was lower than the theoretical value, calculated on the basis of a molecular weight of 750.71. The Associate Referee assayed the dry powdered merbromin before preparing the solution and found 25.03 per cent and 24.94 per cent of mercury, corresponding to 93.68 per cent and 93.34 per cent merbromin. When the average of these results is used as a standard, the calculated recoveries on merbromin, excluding results reported by Collaborator B, range from 93.0 per cent to 99.5 per cent merbromin. An aqueous solution of merbromin is unstable, and one collaborator stated that a precipitate had formed in the sample. The formation of a precipitate may have caused some of the variations in the results.

Some of the results on pure mercurials are higher than desirable. This may be a result of inadequate washing of the precipitate, as was suggested by some of the collaborators. In order to clarify this point, the instructions for washing the precipitate and testing the filtrate were changed from "an opalescence" to "a faint opalescence."

Collaborator D obtained 121 per cent and 127 per cent recoveries of mercuric oxide in the ointment, which seemed outside the range of probable error. Another sample of the ointment was sent to this collaborator with a request to repeat the determinations. The repeat determinations gave 98.6 per cent and 98.8 per cent recoveries of mercuric oxide, which were the results included in Table 1.

CONCLUSIONS

Collaborative work has been done on pure mercury compounds, solutions, ointments, and tablet material, the usual types of preparations

found on the market. Better results for these preparations have been obtained by this method than by any other used by the Associate Referee. Other mercury preparations, such as contraceptive jellies, creams, and suppositories, should be studied. The sensitivity of the method should be based on the minimum concentration of mercury in the sample.

RECOMMENDATIONS

It is recommended*—

(1) That the method for mercury in pharmaceutical preparations be adopted as first action.

(2) That additional work be done on other organic mercurials of definite composition.

(3) That the method be studied for the lowest limit of concentration of mercury in the sample.

ACKNOWLEDGMENTS

The Associate Referee wishes to express his appreciation to James H. Cannon for the statistical analysis of the results, and to the following collaborators, all of the Food and Drug Administration, for their work and helpful suggestions: David J. Miller, Buffalo; Catherine G. Cunningham, Boston; Donald M. Taylor, Denver; Olen E. Morgan, Atlanta; F. E. Yarnall, Kansas City; Garland L. Reed, Cincinnati; T. J. Call, Chicago; Helen C. Barry, New Orleans; Mary A. McEniry and Matthew L. Dow, St. Louis.

* For report of Subcommittee B and action of the Association, see *This Journal*, 40, 25, 26 (1957)

REPORT ON ULTRAVIOLET SPECTROPHOTOMETRIC METHODS FOR DRUGS (PHENYLETHYLAMINES)

By F. A. Rotondaro (Food and Drug Administration, Department of Health, Education, and Welfare, Philadelphia 6, Pa.), *Associate Referee*

Last year's report (unpublished) brought out the fact that many widely used drugs of therapeutic value contain a phenylethyl moiety in their structure. The therapeutically important sympathomimetic drugs are derivatives of phenylethylamine, for example, amphetamine, ephedrine, the tropine alkaloids, benzyl penicillin, phenobarbital, methadone, meperidine, and many "antihistamine" drugs.

It was also pointed out that the ultraviolet absorption characteristics of all these drugs were essentially the same as that of the benzene nucleus

modified by the character of the alkyl chain. Kumler and associates (1) reported on the ultraviolet absorption of D-phenylcarbonyl compounds such as phenylacetone and methadone. Freeman (2) utilized the absorption characteristic of the carbonyl group of phenylacetone in the 295 mμ region to differentiate it from its amphetamine derivative. Ebstein and Van Meter (3) reported in some detail the absorption characteristics of amphetamine base in alcohol, chloroform, and ether and of the sulfate at various *p*H levels in water and in several alcohol-water mixtures.

To obtain adequate background material, a series of absorption curves were made to correlate the structure and absorption characteristics of benzene, toluene, ethylbenzene, *n*-propylbenzene, benzyl alcohol, β-phenylethyl alcohol, phenylacetic acid, α-hydroxyphenyl acetic (mandelic) acid, β-phenylethylamine, amphetamine, methamphetamine, ephedrine, phenylpropanolamine, mephentermine, N-β-dimethylphenylethylamine, and aniline. Significant differences were shown in the absorption characteristics of most of these compounds in alcohol and chloroform or in chloroform and 0.1*N* sulfuric acid.

The methods of assay for the sympathomimetic drugs that are given in the U.S.P. XV (4), the N.F. X (5), the 8th Edition of *Official Methods of Analysis* (6), and N.N.R. (7) generally separate the amine base from an alkaline solution with ether (or chloroform or by distillation) and determine the base by titration. The chief drawbacks of these methods, especially when they are applied to commercial drug products, are their lack of specificity and the difficulty of avoiding contaminants.

An appreciable measure of the specificity of spectrophotometric methods for these amines can be derived from the ratio of the absorbance at the maxima to the absorbances at the two adjacent minima. Table 1 summarizes the absorption characteristics of β-phenylethylamine and six sympathomimetic drugs in 0.1*N* sulfuric acid. More positive identification can be obtained by comparing their absorption curves in various solvents. For practical purposes, chloroform and sulfuric acid are sufficient (see Figs. 1, 2, and 3).

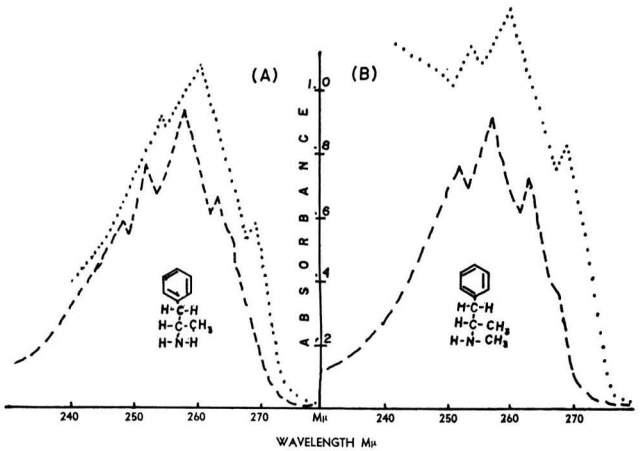
The quantitative determination is based on the absorbance of the amines in 0.1*N* sulfuric acid. Figure 4 shows the characteristic curve of the parent substance, β-phenylethylamine. Interference from impurities is materially reduced by using a simplified baseline method (8, 9) to measure three characteristic absorbances within the spectral range of 10 mμ from 252–262 mμ.

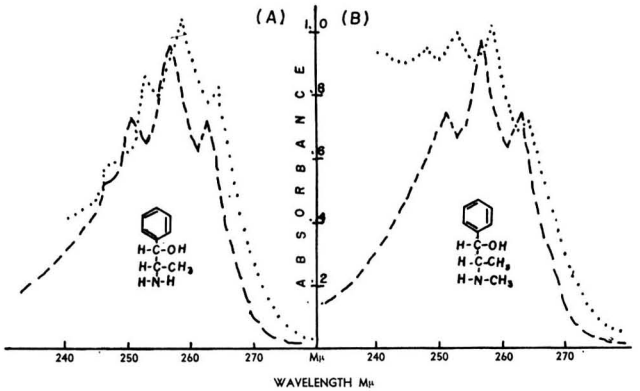
The amine base can be separated from the drug diluents by any of several modified shake-out or distillation methods. However, a very practical method, developed primarily for relatively small samples (5–10 mg), has been used for the past several years. This method avoids the necessity of centrifuging to break up troublesome emulsions formed when alkaline solutions are extracted with solvents such as chloroform. For all

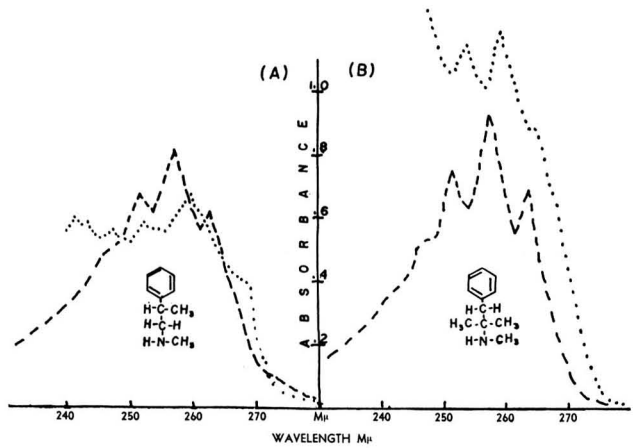
TABLE 1.—*Spectrophotometric data*[a] *for seven phenylethylamines*

BASE	MIN. 1	A	MAX.	A	MIN. 2	A	BASELINE ABSORBANCE	MAX./MIN. 1	MAX./MIN. 2
	mμ		mμ		mμ				
β-Phenylethylamine	254.0	1.140	257.5	1.500	260.5	1.010	0.425	1.31	1.48
Amphetamine	254.0	1.030	257.5	1.400	261.5	0.925	0.422	1.36	1.51
Methamphetamine	254.0	0.930	257.5	1.210	261.5	0.830	0.330	1.30	1.46
Mephentermine	254.0	0.790	257.5	1.130	261.0	0.690	0.430	1.43	1.64
Vonedrine	253.5	0.810	257.0	1.080	261.0	0.750	0.300	1.33	1.43
Ephedrine	253.5	0.810	256.5	1.170	260.5	0.775	0.342	1.45	1.51
Propadrine	253.0	0.880	256.5	1.260	260.5	0.830	0.405	1.43	1.52

[a] Solutions: 1 mg/ml 0.1*N* sulfuric acid. Cells: 10 mm, fused silica, matched. Slit width: 0.5 mm. Spectrophotometer used: Beckman Model DU (no photo-multiplier).







Reading from top to bottom, as follows:

FIG. 1.—A: Amphetamine; B: methamphetamine; both in chloroform (. . .) and in 0.1*N* sulfuric acid (– – –).

Fig. 2.—A: phenylpropanolamine; B: ephedrine; both in chloroform (. . .) and in 0.1*N* sulfuric acid (– – –).

Fig. 3.—A: vonedrine; B: mephentermine; both in chloroform (. . .) and in 0.1*N* sulfuric acid (– – –).

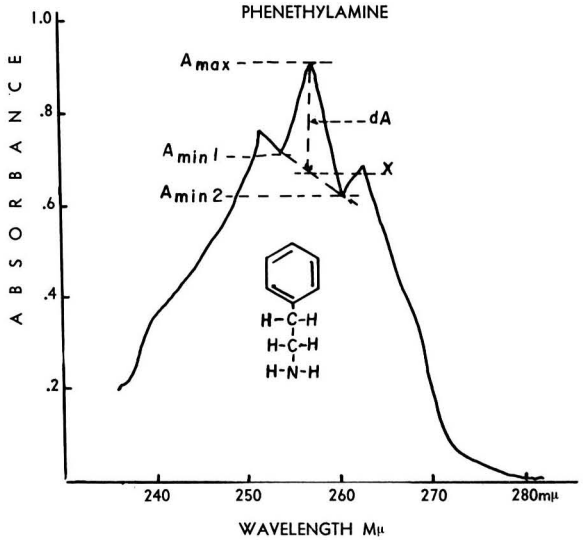


FIG. 4.—Spectrophotometric data for phenethylamine. $X = 0.5\ (A_{\min 1} + A_{\min 2})$ = average minima absorbance. $dA = A_{max} - 0.5(A_{\min 1} + A_{\min 2})$ = absorbance difference. Solvent: 0.1*N* sulfuric acid. Beckman Model DU spectrophotometer, 1 cm cell, 0.5 mm slit width between 250 and 270 mμ.

practical purposes, the amine base can be extracted almost completely with one measured portion of chloroform.

Phenylpropanolamine is an exception; only about 80 per cent of the amine is transferred to the chloroform. However, the transfer can be made precise enough for practical purposes. A measured aliquot of the chloroform extract is then withdrawn and washed with two small portions of 0.1*N* sulfuric acid. The concentration of the amine in the acid solution is determined by its absorbance.

Details of the method are as follows:

METHOD

APPARATUS

Spectrophotometer.—Capable of isolating bands of 1 mμ or less in region 250–270 mμ; equipped with 1 cm cells of quartz or fused Si (preferably matched pair).

PREPARATION OF STANDARD SOLUTION

Accurately weigh 500–700 mg of the phenylethylamine salt of known purity, transfer to 100 ml vol. flask, and dissolve in 0.1*N* H_2SO_4. Dil. to mark with the H_2SO_4 and mix well.

DETERMINATION

Weigh 20 or more units of sample (tablets or capsules) and obtain av. wt per unit. Grind 5–20 units to uniform 60–80 mesh powder.

Accurately weigh powd. sample contg 25–50 mg amine base and transfer to 40–50 ml glass-stoppered centrifuge tube. Add 5 ml 1*N* H_2SO_4, and swirl tube gently to aid

escape of liberated CO_2, if present. Test for acidity with litmus paper, adding more acid if necessary. Pipet in 20 ml $CHCl_3$ and 7 ml 1*N* NaOH, stopper securely, and shake 3–5 min. To second 40–50 ml centrifuge tube add 10 ml of the std soln and ca 0.5 g powd. Na_2SO_4. Swirl tube gently to dissolve salt, pipet in 20 ml $CHCl_3$ and 2 ml 1*N* NaOH, stopper securely, and shake 3–5 min.

Centrifuge tubes at 1500–1800 rpm 3–5 min. Withdraw 10 ml clear $CHCl_3$ layer by closing upper end of 10 ml pipet with index finger while lowering tip thru upper aq. layer. Wipe off any drops adhering to outer portion of pipet, and transfer 10 ml of the $CHCl_3$ layer to separator contg 10–15 ml 0.1*N* H_2SO_4. Stopper separator and shake thoroly, but not violently, 2–3 min. Let $CHCl_3$ layer sep. cleanly; then drain into second separator contg ca 10 ml 0.1*N* H_2SO_4. Shake, let sep., and discard $CHCl_3$ layer which may contain tablet lubricants and neutral products as flavoring materials, dyes, etc.

Wash 2 acid solns with 5 ml portion of fresh $CHCl_3$. Discard $CHCl_3$ wash. Filter 2 acid solns thru small wad of cotton wet with H_2O in neck of small funnel, collecting filtrate in 50 ml vol. flask. Rinse separators with several small portions of 0.1*N* H_2SO_4, passing rinse solns thru cotton filter into vol. flask. Dil. to mark with 0.1*N* H_2SO_4, stopper, and mix well.

Prep. *blank* acid soln by shaking 25 ml 0.1*N* H_2SO_4 with 3–5 ml $CHCl_3$. Allow to sep., and drain and discard $CHCl_3$. Filter acid soln thru cotton as above and dil. to 50 ml with 0.1*N* H_2SO_4.

Read absorbance of filtered solns in 1 cm cells at 0.5 mμ intervals in range of 252–255 mμ for first minima, 256–258 mμ for maxima, and 260–262 mμ for second minima. (It is essential to use same slit width for sample and std solns.)

CALCULATIONS

Calc. absorbance difference (ΔA) between absorbance at maxima and av. of 2 minima: $\Delta A = A_{max} - 0.5(A_{min\ 1} + A_{min\ 2})$.

Calc. absorptivity differential (Δa) produced by 1 g/l (1 mg/ml) of the std amine base or salt: $\Delta a_{std} = \Delta A_{std} \times 100/\text{wt std}$, where ΔA_{std} is absorbance difference for std soln; 100 is ml std soln measured; and wt std is mg std in aliquot measured.

Then, mg amine/unit of sample is: $(\Delta A_{sample} \times 50 \times 2 \times \text{av. wt of unit})/(\Delta a_{std} \times \text{wt sample})$, where 50 is vol. sample soln and 2 is aliquot factor. (All wts are expressed in mg.)

COLLABORATIVE STUDY

At the beginning of this year eight representative drug firms agreed to supply a sample of one or two phenylethylamines of their control standard quality and to participate in a collaborative study of the method. The following samples were received: Amphetamine sulfate, methamphetamine hydrochloride, mephentermine sulfate, ephedrine sulfate, phenylpropanolamine hydrochloride, phenylpropylmethylamine hydrochloride, and tablet granulation base containing no drugs.

All of the samples were found to comply with standard tests. Portions of each drug were mixed with tablet granulation base and ground to a fine powder to simulate powdered tablets. Most collaborators were sent two samples of a pure drug and two samples of the simulated powdered tablets containing the same drug mixed with the blank granulation. In addition, each collaborator was supplied with a portion of the blank granulation. Two collaborators were sent samples of the same lot of the commercial product.

TABLE 2.—*Collaborative results for amphetamine sulfate*

COLLABORATOR	STANDARD SAMPLE 21				UNKNOWN 21
	SLIT WIDTH	BASELINE ABSORBANCE II	BASELINE ABSORBANCE III	RECOVERY, PER CENT	FOUND, PER CENT
	mm				
5	0.125–0.150[a]	0.312 0.313	0.317 0.309	100.0	6.23 6.25
9	0.5	0.296 0.295	0.292 0.292	99.0	6.12 6.16
12	0.5	0.291 0.291	0.275 0.275	94.9	6.36 6.31
19	0.5	0.290 0.293	0.283 0.284	97.5	6.17 6.15
8	0.7	0.269 0.268	0.269 0.272	100.7	6.13 6.16
10	0.8	0.245 0.246	0.241 0.246	99.2	6.25 6.26
4	0.9	0.245 0.243	0.244 0.244	100.0	6.04 5.95
1	0.9	0.242 0.243	0.241 0.244	100.0	6.38 6.22
16	1.0	0.228 0.228	0.221 0.221	96.9	6.47 6.56
Average Theory				98.8	6.23 6.20

[a] Cary Recording Spectrophotometer used; all other used Beckman Model DU.

Samples were sent to the collaborators from the eight drug firms, to eight district laboratories of the Food and Drug Administration, and to two analysts in the Associate Referee's laboratory. A total of eighteen collaborators besides the Associate Referee tested the method on six pure drugs and six simulated commercial products.

Each collaborator was asked to do the following:

(1) Give general information on the type of spectrophotometer, slit width used, type of cells, etc.

(2) Prepare a standard stock solution from the pure samples and obtain absorbance readings in duplicate on dilutions of aliquots of the standard solution to obtain the points of reference, within 0.5 mμ, of the

TABLE 3.—*Collaborative results for methamphetamine hydrochloride*

COLLABORATOR	STANDARD SAMPLE 14				UNKNOWN 24
	SLIT WIDTH	BASELINE ABSORBANCE II	BASELINE ABSORBANCE III	RECOVERY, PER CENT	FOUND, PER CENT
	mm				
2	0.2	0.294	0.290	99.3	9.03
		0.297	0.297		9.23
6	0.3	0.302	0.297	97.7	9.00
		0.301	0.292		8.84
7	0.5	0.291	0.291	99.7	9.30
		0.291	0.289		9.32
9	0.5	0.293	0.289	99.1	9.30
		0.290	0.287		9.35
19	0.5	0.290	0.289	99.5	9.25
		0.288	0.287		9.15
12	0.5	0.272	0.277	102.6	9.43
		0.273	0.280		9.36
13	0.5	0.276	0.274	100.2	9.17
		0.277	0.280		9.17
8	0.7	0.261	0.267	101.5	8.97
		0.260	0.262		9.00
15	0.7	0.249	0.243	98.8	9.59
		0.247	0.247		9.40
1	0.9	0.239	0.237	99.9	8.85
		0.237	0.237		9.00
Average				99.8	9.19
Theory					9.25

maxima and the adjoining minima and to establish the absorbance value of the pure material.

(3) Obtain a measure of the accuracy of the method by adding the blank granulation to measured aliquots of the standard stock solution, to simulate commercial preparations; run these aliquots through the entire method, and use the absorbance obtained to calculate the drug content of the unknown.

(4) Test the method on unknowns made from the pure drug and the blank granulation, or on actual commercial products.

TABLE 4.—*Collaborative results for mephentermine sulfate*

COLLABORATOR	STANDARD SAMPLE 15				UNKNOWN 25
	SLIT WIDTH	BASELINE ABSORBANCE II	BASELINE ABSORBANCE III	RECOVERY, PER CENT	FOUND, PER CENT
	mm				
7	0.5	0.279	0.279	100.0	9.69
		0.279	0.279		9.62
12	0.5	0.276	0.276	99.1	9.80
		0.277	0.272		9.90
19	0.5	0.280	0.278	98.5	9.92
		0.280	0.274		9.85
11	0.7	0.248	0.248	100.2	9.75
		0.248	0.250		9.65
14	0.8	0.243	0.242	99.7	9.77
		0.240	0.240		9.80
16	1.0	0.210	0.213	101.7	9.88
		0.211	0.215		9.70
Average				99.9	9.78
Theory					9.75

RESULTS

Collaborative results are given in Tables 2–7. Figure 5 shows the curves of absorbance versus slit width for the maxima and the two adjacent minima of propadrine and vonedrine solutions (70 mg per 100 ml 1*N* sulfuric acid in 1 cm cells). The most reproducible results can be expected at slit widths of 0.4–0.7 mm.

A few of the collaborative results vary more than is desirable. However, in each case, the collaborator's comments indicated that the chief source of difficulty was either not using a constant slit width or variable aqueous-chloroform ratios in the extraction step. The ratio variation is especially critical for phenylpropanolamine because the amine base is appreciably soluble in water; consequently, the distribution coefficient is less favorable. A number of the collaborators were aware of this difficulty and suggested using saturated salt solutions, as in the U.S.P. methods for ephedrine and many other procedures. However, care must be taken to avoid salt super-saturation in extraction, since acid salts like sodium sulfate adsorb amine bases and the presence of undissolved salt could prevent the extraction of appreciable amounts of amine bases.

The volumes and concentrations of the acid and alkali indicated in the

TABLE 5.—*Collaborative results for N-β-methylphenylethylamine (vonedrine hydrochloride)*

COLLABORATOR	STANDARD SAMPLE 16				UNKNOWN 26
	SLIT WIDTH	BASELINE ABSORBANCE II	BASELINE ABSORBANCE III	RECOVERY, PER CENT	FOUND, PER CENT
	mm				
17	0.5	0.282	0.279	98.7	6.33
		0.282	0.278		6.30
19	0.5	0.280	0.280	99.9	6.30
		0.280	0.279		6.30
12	0.5	0.281	0.281	99.1	6.45
		0.281	0.276		6.35
18	0.5	0.274	0.274	100.2	6.45
		0.274	0.275		6.35
11	0.7	0.254	0.244	96.1	6.60
		0.254	0.244		6.64
10	0.8	0.245	0.240	97.2	6.40
		0.249	0.240		6.42
3	1.1	0.193	0.195	101.0	6.41
Average				98.9	6.42
Theory					6.40

method produce solutions uniform enough to yield fairly reproducible results. However, as several collaborators pointed out, the recoveries from manufactured tablets containing diluents are seldom as good as those from simulated samples, regardless of the procedure used. The present study confirmed this fact; the blank tablet diluent did not appreciably affect per cent recovery of the pure drugs.

Of the collaborators from industry, one considered the method satisfactory but preferred a distillation procedure to separate the amine; four made no comments that their results were in very good agreement with theory; three had some difficulty with the slit width adjustments; and one had trouble with emulsions.

Of the ten collaborators from the Food and Drug Administration, one considered the method rapid; another, workable; a third, convenient; and a fourth, good for small samples. Four others reported very good results and made various suggestions about technique; several had some difficulty in adjusting slit width, but their results were generally in good agreement.

TABLE 6.—*Collaborative results for ephedrine sulfate*

COLLABORATOR	STANDARD SAMPLE 12				UNKNOWN 22
	SLIT WIDTH	BASELINE ABSORBANCE II	BASELINE ABSORBANCE III	RECOVERY, PER CENT	FOUND, PER CENT
	mm				
5	0.14[a]	0.297 0.293	0.287 0.286	97.2	6.27 6.66
2	0.20	0.284 0.284	0.284 0.278	98.8	6.23 6.26
19	0.5	0.283 0.283	0.275 0.278	97.8	6.33 6.23
12	0.5	0.274 0.273	0.267 0.264	96.9	6.44 6.45
6	0.5	0.268 0.268	0.259 0.260	96.6	6.47 6.66
14	0.8	0.233 0.230	0.227 0.224	97.4	6.36 6.00
4	0.9	0.218	0.215	98.6	5.85
3	1.1	0.178	0.170	95.5	6.48
Average Theory				97.6	6.34 6.35

[a] Cary Recording Spectrophotometer used; all others used Beckman Model DU.

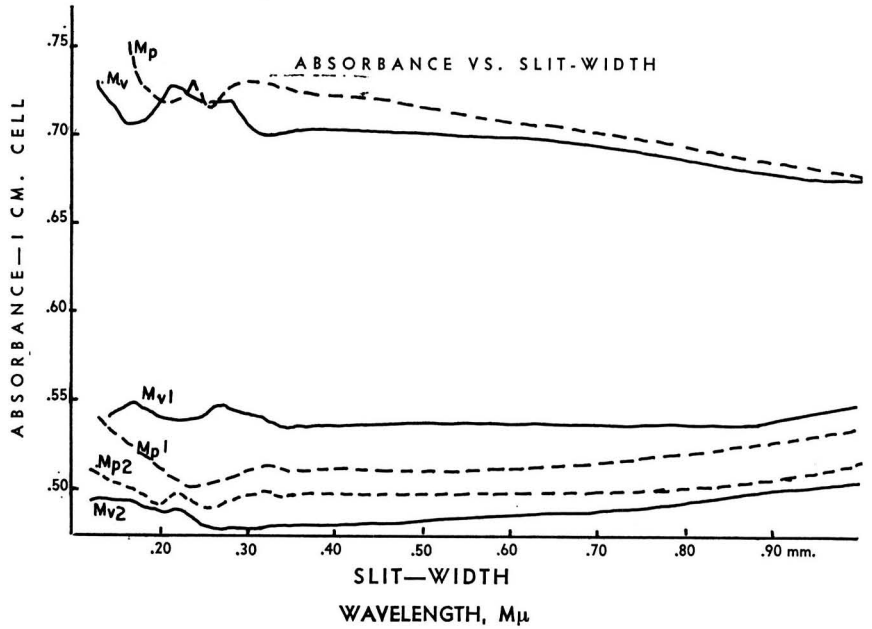


TABLE 7.—*Collaborative results for phenylpropanolamine (propadrine hydrochloride)*

COLLABORATOR	STANDARD SAMPLE 17				UNKNOWN 27
	SLIT WIDTH	BASELINE ABSORBANCE II	BASELINE ABSORBANCE III	RECOVERY, PER CENT	FOUND, PER CENT
	mm				
17	0.5	0.298	0.246	81.9	24.8
		0.300	0.244		25.1
12	0.5	0.296	0.243	80.0	23.4
		0.304	0.238		23.9
18	0.5	0.295	0.242	81.8	24.5
		0.294	0.240		24.7
19	0.5	0.300	0.248	83.0	24.7
		0.296	0.246		24.6
13	0.5	0.290	0.234	80.3	24.9
		0.292	0.234		24.7
15	0.7	0.263	0.219	82.8	24.4
		0.266	0.221		24.7
4	0.9	0.248	0.203	81.3	22.1
		0.249	0.201		
Average				81.6	24.4
Theory					25.0[a]

[a] Questionable.

SUMMARY AND RECOMMENDATIONS

The excellent duplication of the differential absorbances reported by the collaborators are noteworthy (see Tables 2–7, column headed "baseline absorbance II"). These values are for the standard stock solutions prepared in Part 2 of the collaborative work. The equivalent results reported in the next column ("baseline absorbance III") are the absorbances of the same stock solutions run by the proposed method under Part 3.

Table 8 summarizes the results given in Tables 2–7. It can be concluded that on the basis of 90 determinations by 19 collaborators for the six

FIG. 5.—Plots of absorbance versus slit width for the maxima and the two adjacent minima of solutions of propadrine and vonedrine (70 mg base/100 ml 0.1*N* sulfuric acid. Switch position: 0.1 for slit widths below 0.5 mm; 1.0 for slit widths above 0.5 mm. Curves M_p, M_{p1}, and M_{p2}: Propadrine, $A_{max\ 256.5}$, $A_{min\ 1,\ 253.0}$, and $A_{min\ 2,\ 260.5}$, respectively. Curves M_v, M_{v1}, and M_{v2}: Vonedrine, $A_{max\ 257.0}$, $A_{min\ 1,\ 253.5}$, and $A_{min\ 2,\ 261.0}$, respectively.

TABLE 8.—*Summary of collaborative results*

	NO. DETERMINATIONS	STANDARD			UNKNOWN		
		AV. RECOVERY[a]	STD DEV.[b]	SLIT WIDTH	THEORY[c]	AV. FOUND	STD DEV.[b]
		per cent		*mm*	*per cent*	*per cent*	
Amphetamine	18	98.8	±2.21	0.14–1.0	6.20	6.23	±0.142
	12			0.14–0.70		6.20	±0.074
Methamphetamine	20	99.8	±1.31	0.20–0.90	9.25	9.19	±0.181
	18			0.20–0.70		9.21	±0.157
Mephentermine	12	99.9	±1.04	0.5 –1.0	9.75	9.78	±0.099
	8			0.5 –0.70		9.78	±0.109
Vonedrine	13	98.9	±1.27	0.5 –1.1	6.40	6.42	±0.105
	10			0.5 –0.7		6.42	±0.120
Ephedrine	14	97.6	±3.5	0.14–1.10	6.35	6.34	±0.225
	10			0.14–0.70		6.40	±0.164
Propadrine	13	81.6	±1.12	0.5 –0.90	24.30	24.40	±0.790
	12			0.5 –0.70		24.45	±0.480

[a] Determined for aliquots of the standard stock solution with added tablet diluent to simulate compressed tablets.

[b] Std Dev. $= \sqrt{\frac{\Sigma d^2}{n}}$.

[c] Calculated from amounts of pure drugs mixed with diluents.

derivatives, the over-all precision of the method is 100.0±2.1 per cent (one standard deviation unit) or 100.0±4.2 per cent at a 95 per cent confidence level. However, on the basis of 70 determinations by 13 collaborators who used slit widths of 0.7 mm or less, the precision is 100.0±1.7 per cent (one standard deviation unit) or 100.00±3.4 per cent (two standard deviation units) at a 95 per cent confidence level.

It is recommended* that the method be adopted as first action, and that the study be extended to oxygenated phenylethylamines.

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George O. Sharp and E. D. Carlhuff, The Wm. S. Merrell Co., Cincinnati 15, Ohio

* For report of Subcommittee B and action of the Association, see *This Journal*, 40, 25, 26 (1957).

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REPORT ON INFRARED SPECTROPHOTOMETRIC METHODS FOR DRUGS

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During the last several years the infrared spectra of a number of drugs have been studied in order to develop spectrophotometric methods for their estimation.[1] A method for atropine sulfate in tablets gave good results when submitted to a collaborative study.[1] Methods for nitroglycerine in tablets and N-lauroyl sarcosinate in tooth paste proved satisfactory. An extensive collaborative study, planned for this year, was abandoned because of the lack of enough collaborators. Accordingly, this year the Associate Referee continued to study the spectra of additional pharmaceuticals.

[1] CAROL, J., *This Journal*, 37, 692 (1954); 38, 640 (1955); 39, 632 (1956).

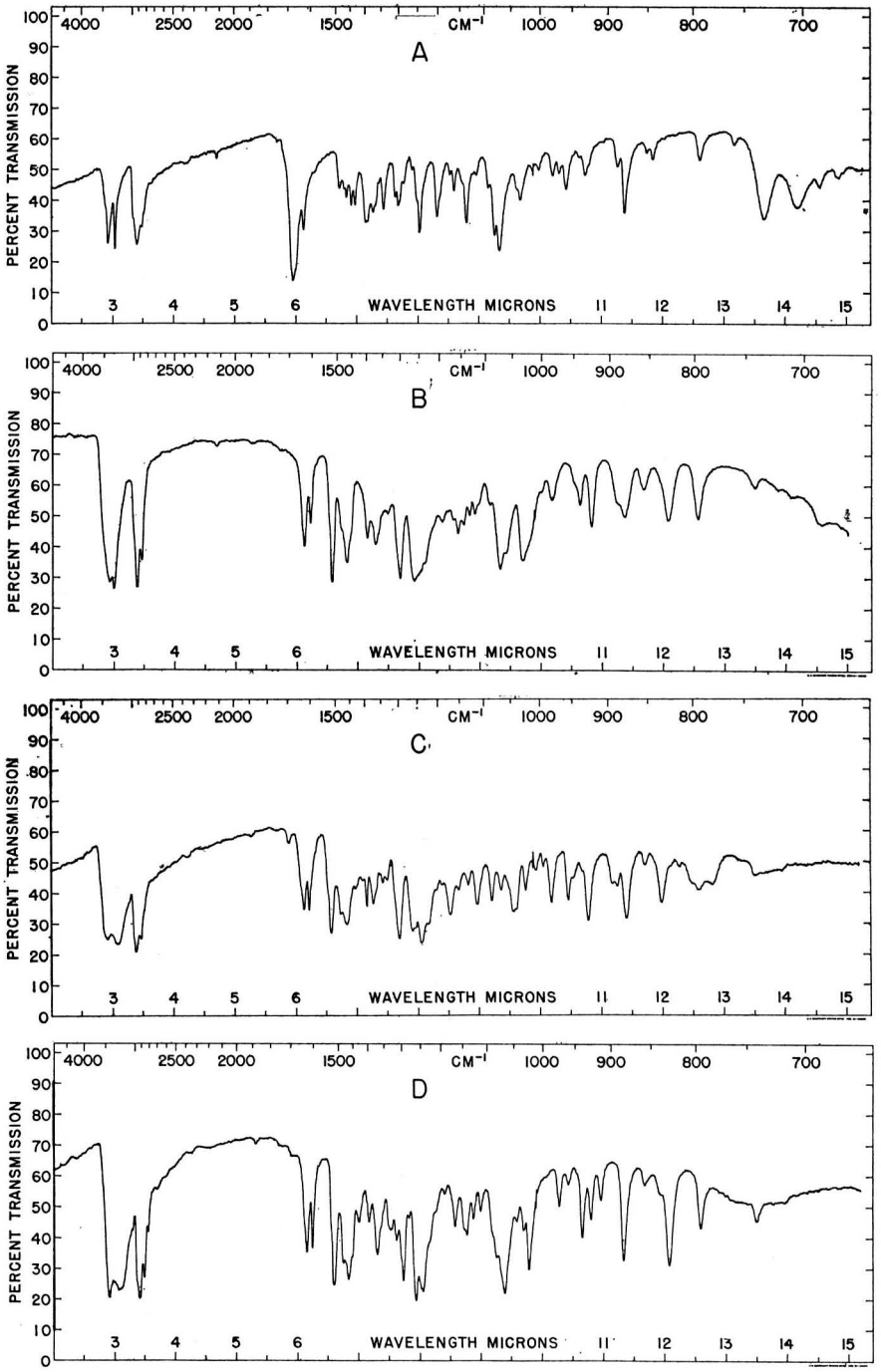

FIG. 1.—The absorption spectra of **A**: ethinyl testosterone; **B**: ethinyl estradiol; **C**: estradiol-17α; and **D**: estradiol-17β.

TABLE 1.—*Infrared absorbance bands for the spectrophotometric determination of various steroids*

COMPOUND	SELECTED WAVELENGTH, μ
Estradiol-17β, U.S.P. Reference Standard	9.5, 11.45
Estradiol-17α, m.p. 223–224°	11.55
Estrone, U.S.P. Reference Standard	12.21
Equilin, m.p. 235–237°	12.35
Equilenin, m.p. 256–257°	11.80
Ethinyl estradiol, U.S.P.	6.70
Ethinyl testosterone (Ethistrone, U.S.P.)	9.46

Quantitative infrared procedures for the estrogenic ketosteroids, estrone, equilin, and equilenin,[2] and the estrogenic diol, estradiol-17β,[3] have been reported. Because these compounds are insoluble in carbon disulfide or carbon tetrachloride, they must be converted to soluble esters for the usual liquid-holding cell procedure of analysis. Conversion to the ester (benzene sulfonyl) requires an additional step in the analysis and is rather time-consuming. A more direct spectrophotometric analysis is made possible by the pressed potassium bromide disk technique. Good quantitative analyses of a number of substances have been achieved by this procedure.[4]

The absorbance spectrum of each of the steroids cited and of two closely related ones—ethinyl estradiol and ethinyl testosterone—was recorded from 2μ to 15μ (a Perkin-Elmer infrared spectrophotometer, Model 21, was employed) by using pressed potassium bromide disks (see footnote 1) containing 2 mg steroids and 200 mg potassium bromide. Ethinyl estradiol and ethinyl testosterone were included in this study because there are no adequate procedures for their estimation by conventional methods. A study of these spectra (Figs. 1–2 and Table 1) shows, for each compound, one or more absorbance bands suitable for quantitative measurements.

The possibility of estimating estradiol-17β in the presence of its physiologically inactive epimer, estradiol-17α, is especially interesting, as is the determination of the three estrogenic ketosteroids in mixtures.

A series of pressed disks containing varying amounts of ethinyl testosterone (ethistrone, U.S.P.) per 200 mg potassium bromide was prepared. The $A_{B9.50\mu}$ was determined for each disk. A tabulation of these data (Table 2) shows close adherence to Beer's law. Thus a single standard may be used in the proposed general procedure.

METHOD

APPARATUS AND REAGENTS

(a) *Recording spectrophotometer.*—Suitable for measuring absorbance in the infrared spectrum.

[2] CAROL, J., MOLITOR, J. C., and HAENNI, E. O., *J. Am. Pharm. Assoc., Sci. Ed.*, **37**, 173 (1948)
[3] CAROL, J., *ibid.*, **39**, 425 (1950).
[4] KIRKLAND, J. J., *Anal. Chem.*, **27**, 1537 (1955).

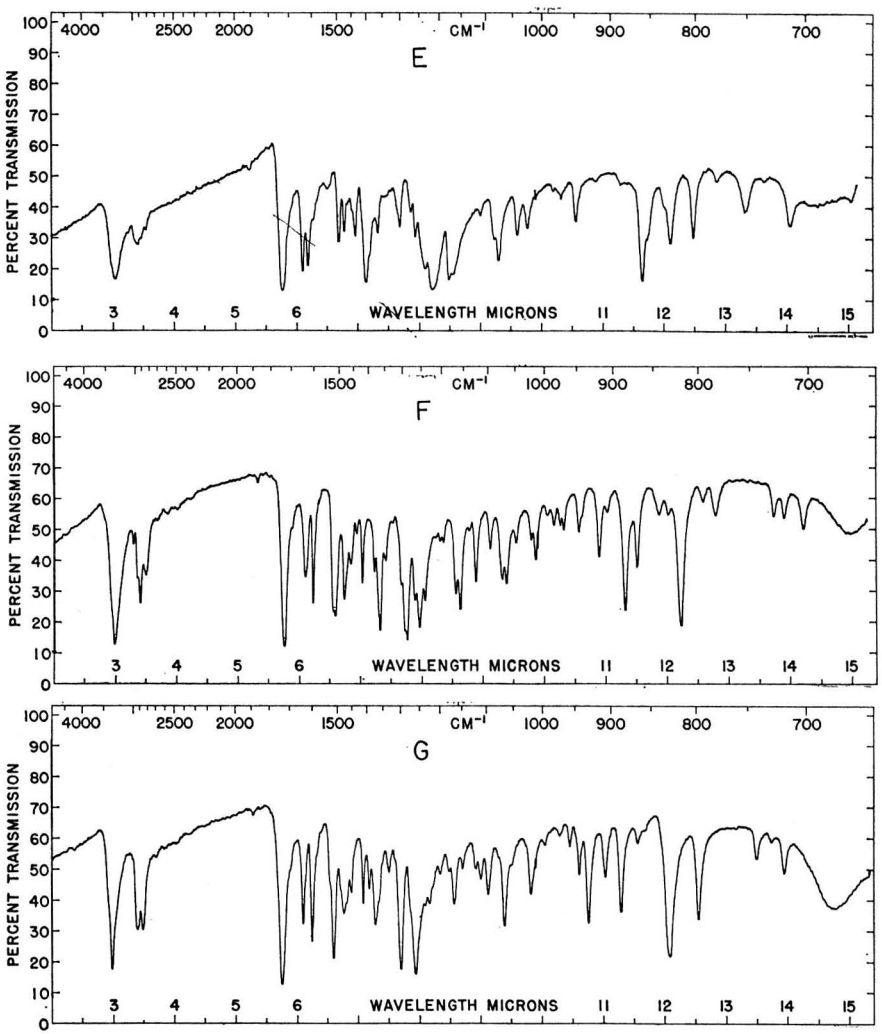

FIG. 2.—The absorption spectra of **E**: equilenin; **F**: equilin; and **G**: estrone.

TABLE 2.—*Beer's law check for disks of ethinyl testosterone*

WT SAMPLE/200 MG KBr	$A_{B9.46\mu}$
mg	
0.50	0.152
1.00	0.309
1.50	0.468
2.00	0.610
2.50	0.753

TABLE 3.—*Determination of ethinyl testosterone in tablets*

SAMPLE	ETHINYL TESTOSTERONE	
	DECLARED	FOUND
	mg	*mg*
1	5.0	5.05
2	25.0	24.9

(b) *Hydraulic press.*—Capable of exerting a pressure of at least 10 tons.

(c) *Evacuable die.*—Designed to press disks 12 mm in diameter (see footnote 4).

(d) *Potassium bromide.*—Suitable for the production of transparent disks (such as that furnished by the Harshaw Chemical Co., Cleveland, Ohio).

(e) *Ethinyl testosterone.*—Ethistrone, U.S.P.

DETERMINATION

Transfer a weighed sample of powdered tablet containing 20–25 mg ethinyl testosterone to a 125 ml separator containing 5–10 ml H_2O. Extract with 3 successive 25 ml portions of $CHCl_3$. Filter each extract through a pledget of cotton into a small tared beaker. Evaporate to dryness on a steam bath with the aid of a current of air, cool, and weigh. Transfer 10 mg residue, carefully weighed, to a small agate mortar, add exactly 1.0000 g KBr, and grind thoroughly. Transfer exactly 200 mg of this mixture to the die, assemble, evacuate to at least 1 mm Hg, and apply pressure of at least 10 tons for 5 min. In a similar fashion, prepare a standard disk, using 10 mg ethinyl-estradiol. Determine the $A_{B\ 9.50\mu}$ of the sample and standard disks.

RESULTS AND RECOMMENDATION

Two commercial samples of ethinyl testosterone in tablets were analyzed by the procedure described. The results shown in Table 3 indicate that the procedure is satisfactory.

It is recommended* that the infrared analysis of all the drugs reported on during 1953, 1954, and 1956 be submitted to collaborative study and that typical dosage forms of each drug be used.

No reports were given on alkali metals, microscopic tests, norepinephrine (Arterenol) in solutions of epinephrine, or organic iodides and separation of halogens.

* For report of Subcommittee B and action of the Association, see *This Journal*, 40, 25, 26 (1957).

REPORT ON NUTRITIONAL ADJUNCTS

By O. L. KLINE (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D.C.), *Referee*

During the past year, important progress has been made in the program of the section on nutritional adjuncts.

Methods for the determination of four different antibiotics in feed supplements have been proved reliable by collaborative study, and the Referee concurs in the recommendation* of the Associate Referee that these methods be adopted as first action. We can look forward to the application of these microbiological methods to finished feeds, and to a possible application of chemical methods to feed supplements.

The Associate Referee on Methods for Amino Acids has developed a plan of extensive collaborative study involving eight amino acids. It is hoped that this study can be completed during the coming year.

Methods for determining vitamin A in mixed feeds present important problems. The use of saponification and chromatography in vitamin A assay is complicated at best, but the complex composition of feeds and the presence of the vitamin in a stabilized form increase the difficulties. The Associate Referee must not only improve the critical steps of the assay, but, through collaborative work, must also help others to develop skill in chromatographic separations.

A change in the magnesium oxide adsorbent available commercially has created an additional problem. Sea Sorb 43, which is presently available, may have to be altered by heat treatment to standardize its adsorptive and oxidative properties. The Referee concurs in the recommendation that this subject be studied further.

Similar problems are involved in standardizing a method for vitamin A in margarine. Although no method could be recommended for adoption this year, the work is continuing.

The report on carotene contains a significant finding, namely, that the adsorbent Sea Sorb 43 is equivalent to the commonly used magnesium oxide reagent 2642. Aso, by a well-coordinated check sample program, analysts in this field are continually checking and improving the precision of their results by the method of assay. The recommendation to continue the program is approved.

In continued effort to apply the fluorometric method for thiamine to cereals and bakery products, the Associate Referee has found several minor modifications of the method necessary to increase precision. He has recommended further study, and the Referee concurs.

The Associate Referee on Pantothenic Acid, in attacking the complex problem of applying the dual enzyme hydrolysis to foods, has been im-

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 22, 23 (1957).

peded by the difficulty of procuring satisfactory enzyme reagents. If each analyst had to prepare his own reagents, the method would be too unwieldy for routine use. Collaborative study is now in progress, and we look forward to a final report next year.

The Associate Referees have recommended further study of a chemical method for vitamin B_6 and of microbiological methods for both free and bound folic acid. Both recommendations are approved.

Finally, the Referee approves the recommendation of the Associate Referee on Microbiological Methods that certain technical modifications be studied collaboratively. Particular commendation is due this Associate Referee. He has successfully completed the extensive, painstaking task of editorially revising the several microbiological methods into a unified form that will not only save space in *Official Methods of Analysis* but will also emphasize the uniformity of the methods. The minor modifications necessary to accomplish this are the subject of his proposed study.

Again, the Referee wishes to thank the analysts who have participated in the collaborative work, and to recognize the efforts of the Associate Referees that have contributed to the success of this year's program.

REPORT ON THIAMINE IN ENRICHED CEREAL AND BAKERY PRODUCTS

By Lewis H. McRoberts (Food and Drug Administration, Department of Health, Education, and Welfare, San Francisco 2, Calif.), *Associate Referee*

The Associate Referee has previously reported investigations of methods and collaborative studies (1–3) that led to the adoption of a rapid acid hydrolysis thiochrome procedure for thiamine in enriched flour, **38.29–38.32** (4).

The present report describes experiments designed to show whether the more involved method, **38.22–38.28** (4), should be changed. This method, used to determine thiamine in bakery products, includes acid hydrolysis, enzyme digestion, and base-exchange separation. In the study of this method, experiments were conducted to clarify the following points:

(1) The necessity of carrying out the enzyme digestion in the presence of the residual bread solids after the initial acid hydrolysis.

(2) The amount of thiamine lost when sample solutions or standard solutions are purified by the base-exchange separation.

(3) The use of water-saturated isobutyl alcohol as a solvent for thiochrome, instead of anhydrous isobutyl alcohol.

(4) The use of anhydrous sodium sulfate, sodium chloride, or potassium

chloride, or other reagents, to insure that the separated isobutyl alcohol-thiochrome solutions remain clear before the photofluorometer readings, and the reasons for using sodium or potassium chlorides.

(5) The effect of daylight on solutions of thiochrome in isobutyl alcohol.

In the Eighth Edition of *Official Methods of Analysis* (4), no reference is made to the determination of vitamins in cereal or bakery products, other than the rapid fluorometric method for thiamine in enriched flour, **38.29–38.32**. References to the general methods for vitamins and adequate directions for preparing fresh bread samples should appear in the chapter on cereal foods, Chapter 13.

The usual procedure is to air-dry the bread as in **13.71(a)** except to dry in the absence of light (necessary when riboflavin is to be determined) and to determine the air-dry factor, or ratio of the air-dry weight to the original fresh weight, so that the results can be calculated on the fresh basis.

A weighed amount of air-dried enriched bread (6–12 grams), calculated to contain 20–40 mmg of thiamine, is then used for the general thiamine procedure, **38.22–38.28**. In this procedure, the sample is hydrolyzed by acid, the residual bread solids and the solution are diluted to volume, and the resulting mixture is filtered or centrifuged. It is assumed that the natural or combined thiamine and the added thiamine are completely dissolved in the acid solution. An aliquot of this clarified solution is then digested with enzyme, purified by base exchange, and oxidized to thiochrome; the thiochrome is then transferred from aqueous solution to isobutyl alcohol solution. The intensity of the fluorescence of the isobutyl alcohol solutions is measured by a photofluorometer and thiamine is calculated by comparing the readings with those obtained from standard thiamine that has been carried through the same procedure.

ENZYME DIGESTION

Enzyme digestion is necessary in the analysis of bakery products for thiamine because of the presence of cocarboxylase, the pyrophosphoric acid ester of thiamine. Although the combined form of thiamine is oxidized to a fluorescent thiochrome, the pyrophosphoric acid group prevents extraction by isobutyl alcohol; unless this acid group is removed, low results are obtained.

Part of the combined thiamine is doubtless contributed by the yeast used in the baking; there is also evidence that the yeast causes part of the thiamine that is added for enrichment to be converted to cocarboxylase (5). After the acid hydrolysis, the cocarboxylase or the phosphoric acid ester of thiamine that is produced by the acid treatment should be in solution, since both of these compounds are freely soluble in water. This assumption provides a reasonable basis for the present procedure, namely,

the solution is diluted to volume after acid hydrolysis and an aliquot of the liquid portion is taken, provided the liquid can be satisfactorily separated from the residual bread solids. Method **38.22–38.28** does not contain any directions for separating this liquid portion.

The Associate Referee has found that after the bread is hydrolyzed by acid, it is difficult to obtain a clear solution either by filtering or by centrifuging. When this method is used for many products other than bread, there may be definite reasons for diluting to volume after the acid hydrolysis. However, in the analysis of bread the Associate Referee prefers to adjust the *p*H to 4.5 after the acid hydrolysis and then proceed directly to the enzyme digestion in the presence of the residual bread solids (6). This enzyme digestion helps to clarify the mixture further so that a perfectly clear liquid is readily obtained by filtering or centrifuging. The separation is adequate, one dilution step is eliminated, and the analyst is assured that the enzyme is in contact with the bread solids.

Comparative analyses made on two samples of bread (Table 1) provide evidence that equivalent results can be obtained by the suggested modification. For the analysis of bread, an alternative method of preparing the sample solution has been described and recommended for collaborative study (see *Recommendations*).

TABLE 1.—*Comparison of official and modified filtration steps in determining thiamine in enriched bread*

SAMPLE	THIAMINE, MG/LB, AIR-DRY BASIS	
	OFFICIAL FILTRATION (AFTER ACID HYDROLYSIS)	MODIFIED FILTRATION (AFTER ENZYME DIGESTION)
Bread No. 1	2.09	2.13
Bread No. 2	1.99	1.94
Bread No. 2	1.99	1.96

BASE-EXCHANGE PURIFICATION

In the collaborative studies in which the rapid flour procedure was compared with the then official base-exchange method (7) results by the base-exchange method showed a definite trend of about 7 per cent higher. In later comparisons of direct and procedural standards, made in this laboratory and at two other laboratories of the Food and Drug Administration, it became apparent that losses in the procedural standard could account for this difference.

Harold E. Theper of the St. Louis District found that the standard thiamine was not completely eluted from the column by the prescribed 15 ml of the acid potassium chloride reagent, and that further elution with 15 ml more of this reagent raised the reading about 5 per cent. He also investigated the addition of gelatin (8) as a protective substance. In the

analyses of enriched flour, with these two modifications he was able to obtain results by the base-exchange procedure that agreed closely with those by the rapid flour procedure. He did not report any experiments with bread. His suggestion to increase the amount of the acid potassium chloride eluting reagent was adopted and is now included in the method.

Beulah M. Moses of the Philadelphia District studied the effect of the *p*H of the procedural standard just before the base-exchange step. She found that the *p*H was also a definite factor that could account for loss in this standard, and concluded that the *p*H should be adjusted to 4.5–5.0 (9). At present, the method contains no direction to adjust the *p*H at this step; the *p*H should have been adjusted to 4.0–4.5 for the enzyme digestion and would probably be lowered when the solution is diluted to volume with 0.1*N* sulfuric acid, as prescribed in the method.

The Associate Referee conducted the following experiments to check further the effect of *p*H on standard solutions and on bread sample solutions that were passed through the base-exchange column:

A *p*H meter was used to adjust the solution to the various acidities listed. Throughout all of the experiments the base-exchange column was eluted as in the purification step, **38.25**: "Elute thiamine from the base-exchange silicate by passing thru column five 4.0–4.5 ml portions of almost boiling acid-KCl soln. . . ." Enough purified base-exchange silicate ("Thiochrome DeCalso," Fisher Scientific Co., Pittsburgh, Pa.) was used to fill the constricted portion of the tube described in **38.22(f)** (about 12 cm high). In most of these experiments the *p*H range was 2.0–4.5. The *p*H of 2.0 would not be expected to occur in normal use of the method, but it serves to show the effect of excess acidity. If the solution for enzyme digestion was adjusted to the acid side of the range of bromcresol indicator and later diluted to volume with 0.1*N* sulfuric acid, the *p*H might become 3.0. The *p*H of 4.5 was used to check the findings of Moses (9) that the range of 4.5–5.0 was the best to insure recovery of thiamine from the base-exchange column. If 150 ml of 0.1*N* sulfuric acid is used in the beginning, about 11.0 ml of the 2*N* sodium acetate must be added to adjust the *p*H to 4.5. The solution becomes strongly buffered, and at this point, adding more sodium acetate makes very little change. Adding 50 ml of water causes no change. Direct comparison standards that were not passed through base exchange were prepared in the acid potassium chloride reagent used to elute the thiamine for the column.

The results of these experiments are tabulated in Tables 2 and 3. The general conclusion is that both procedural standard and sample solutions should be adjusted to about *p*H 4.5 just before the base-exchange separation. No thiamine could be detected in the wash solutions from the base-exchange column when this control was maintained. Thiamine is definitely lost when the *p*H of the solution added to the column is below the range 4.0–4.5. The loss from the procedural standard seems to be due to

TABLE 2.—*Recovery of thiamine from pure solution of 0.2 mmg/ml in 0.1N H_2SO_4 by base exchange (pH meter control)*

	pH	NET PHOTOFLUOROMETER READINGS[a]				ELUTION LOSS	
		ELUATE BASIS		DIRECT STD BASIS		READING	PER CENT
		READING	AV.	READING	AV.		
I. Elution with 0.1*N* HCl–25% KCl							
Original solution	1.5	72.0 72.0	72.0	77.5 77.5	77.5	5.5	7.1
Adjusted solution	2.5	74.5 72.5	73.5	—	—	4.0	5.2
	3.5	75.0 74.5	74.8	—	—	2.7	3.5
	4.5	76.5 76.5	76.5	—	—	1.0	1.3
	5.5	72.0	72.0	—	—	5.5	7.1
II. Elution with Neutral 25% KCl[b]							
Original solution	1.5		74.0		87.5	13.5	15.4
Adjusted solution	3.3		78.0		—	9.5	10.9
	4.5		79.0		—	8.5	9.7

[a] Reading corrected for blanks at oxidation step.
[b] The readings are not directly comparable from experiment I to II because of different photofluorometer settings.

incomplete elution from the column; with the sample solution, part of the thiamine washes through when the acidity is too high.

For the past year both the procedural and the direct standard have been compared in the analysis of bread. The results of these comparisons are presented in Table 4. The average loss of thiamine from procedural standards is about the same as the loss from pure thiamine solution that is carried through only the base-exchange step. One experiment was conducted with pure thiamine solution in which neutral 25 per cent potassium chloride was used to elute the solution from the base-exchange column. Thiamine losses were much greater than when the prescribed 0.1*N* acid-25 per cent potassium chloride was used (Table 2).

While the use of a procedural standard might compensate for losses of thiamine, this investigation confirms the previous finding that there is little loss from the standard or sample solution when the *p*H is adjusted to 4.0–4.5 just before the base-exchange separation. This is also the range

TABLE 3.—*Thiamine from bread sample solutions after acid hydrolysis, enzyme digestion, and base-exchange separation: comparison of pH adjustment previous to base-exchange (pH meter control)*

DESCRIPTION OF SAMPLE AND STANDARD	pH	PHOTOFLUOROMETER READINGS[a]			
		ELUATE	WASH SOLUTION	TOTAL	PER CENT LOSS
Bread No. 1	2.0	73.0	4.8	77.8	6.6
Bread No. 1	3.0	81.5	3.2	84.7	4.4
Bread No. 1	4.5	85.5	1.6	87.1	2.2
Procedural Std 1	4.5	72.5	0.0	72.5	0.0
Direct Std 1	—	—	—	73.0	—
Bread No. 2	2.0	70.0	4.8	74.8	6.6
Bread No. 2	3.0	78.5	0.8	79.3	1.1
Bread No. 2	4.5	85.5	0.0	85.5	0.0
Procedural Std 2	4.5	72.5	0.0	72.5	0.0
Direct Std 2	—	—	—	73.0	—
Bread No. 3	2.0	79.0	9.6	88.6	10.4
Bread No. 3	3.0	86.0	3.2	89.2	3.5
Bread No. 3	4.5	87.5	0.0	87.5	0.0
Bread No. 4	2.0	79.0	4.8	83.8	5.2
Bread No. 4	3.0	85.0	1.6	86.6	1.7
Bread No. 4	4.5	86.0	0.0	86.0	0.0
Procedural Std 3 and 4	2.0	86.5	0.0	86.5	0.0
Procedural Std 3 and 4	3.0	89.0	0.0	89.0	0.0
Procedural Std 3 and 4	4.5	90.0	0.0	90.0	0.0
Direct Std 3 and 4	—	—	—	92.0	—

[a] Readings corrected for blanks.

specified for the previous step, the enzyme digestion, **38.24**. However, the analyst is directed to use bromcresol indicator to adjust the *p*H. It is believed that the method should specify a *p*H meter control to standardize the color.

The following color changes were noted with bromcresol indicator when 0.1*N* sulfuric acid was neutralized with 2*N* sodium acetate: *p*H 2.0, orange; *p*H 3.0, orange-yellow; *p*H 3.5, yellow; *p*H 4.0, green; *p*H 4.5, blue-green; *p*H 5.0, blue.

ISOBUTYL ALCOHOL

(1) *Comparison of Water-Saturated and Anhydrous Isobutyl Alcohol.*—The routine operation in this laboratory has been to recover all of the isobutyl alcohol from the thiamine determination by washing with water and then distilling. Usually the product has a lower fluorescent blank than that of new redistilled alcohol. This procedure would doubtless be continued if the purification step could be shortened.

TABLE 4.—*Comparison of photofluorometric readings of procedural and direct thiamine standards*[a] *(pH adjusted at 4.5 before base-exchange separation)*

THIOCHROME READINGS FROM 1 MMG THIAMINE							
DIRECT STANDARD			PROCEDURAL STANDARD				
OXIDIZED SOLUTION	BLANK	NET READING[b]	OXIDIZED SOLUTION	BLANK	NET READING	LOSS	
						READING	PER CENT
70.5	4.0	66.5	70.5	5.0	65.5	1.0	1.5
66.5	3.0	63.5	66.5	3.0	63.5	0.0	0.0
48.0	2.0	46.0	48.0	2.0	46.0	0.0	0.0
52.0	2.0	50.0	51.0	3.0	48.0	2.0	4.0
59.0	3.0	56.0	58.0	4.0	54.0	2.0	3.6
58.0	4.0	54.0	56.0	3.0	53.0	1.0	1.9
65.0	4.0	61.0	64.0	4.0	60.0	1.0	1.6
60.0	4.0	56.0	59.0	3.0	56.0	0.0	0.0
74.0	4.0	70.0	73.0	4.0	69.0	1.0	1.4
97.0	5.0	92.0	95.0	5.0	90.0	2.0	2.2
						Av.	1.6

[a] Method **38.22–38.28**, modified.
[b] Corrected for blank.

The present method specifies that the isobutyl alcohol reagent, **38.22**(**1**), be redistilled in all-glass apparatus, but it has been assumed that the reference is to anhydrous isobutyl alcohol, the boiling point of which is in the range 105–108°C. To recover the anhydrous form from the alcohol in the determination, the first half of the distillate must be discarded or returned to the still. However, practically all of the distillate could be used if water-saturated alcohol were equally applicable. Duplicate analyses for thiamine were made on one sample of bread with anhydrous isobutyl alcohol and with water-saturated isobutyl alcohol. With three separate sample weights, the results were 1.46, 1.47, and 1.49 mg per lb with the anhydrous alcohol, and 1.49, 1.45, and 1.47 mg per lb with the water-saturated alcohol.

(2) *Prevention of Clouding in Isobutyl Alcohol Solutions of Thiochrome.*—After thiamine is oxidized to thiochrome by the addition of the alkaline ferricyanide solution, thiochrome is extracted from each of the oxidizing bottles with a constant volume of isobutyl alcohol. The resulting water-saturated alcohol may become cloudy before the intensity of the thiochrome fluorescence is measured in the photofluorometer. Various means have been used to prevent this clouding. Part of the water can be removed from the separated alcohol with anhydrous sodium sulfate, or additional isobutyl alcohol may be added to insure that water does not come out of solution. Both of these steps are time-consuming; constant amounts of either reagent must be added to insure that the same volume change

takes place in all of the sample tubes and standard tubes. The same result can be achieved by adding enough sodium chloride or potassium chloride to saturate the aqueous phase before the alcohol is separated. If anhydrous isobutyl alcohol is used and the conditions of volumes in the oxidation step are observed (4), the salt effect will reduce the amount of water transfer to the alcohol by one-half. The reverse is true in using isobutyl alcohol that has previously been saturated with water; the decrease in the alcohol volume is doubled in the presence of added salt.

In either case enough salt should be added to insure a slight excess over the amount required to saturate the aqueous phase. About 2.5 grams of either sodium chloride or potassium chloride will saturate the aqueous phase under the conditions described in either the official method, **38.22–38.28**, or the rapid method, **38.29–38.32**. In the base-exchange procedure (4), the elution reagent provides about half of this amount; in some instances this has not been enough to prevent clouding. Therefore the method should provide for the addition of enough sodium chloride or potassium chloride to saturate the aqueous phase.

LIGHT EFFECT

DeMerre and Seibold (10) have published the results of an extensive investigation of the effects of various forms of light on thiochrome solutions. Their conclusions were as follows:

"(1) Direct sunlight produces total destruction of thiochrome solutions in a very short time. Natural daylight also produces total destruction. As anticipated, the rate of destruction is slower than in the case of direct sunlight, but not substantially.

"(2) Dim daylight in intensities not higher than 10 foot candles is not detrimental, for a reasonable length of time, to thiochrome solutions. This brightness is sufficient for working purposes and thiochrome assays may be made under these conditions.

"(4) Ultraviolet light acts similarly to sunlight. . . . "

The following experiments were conducted by the Associate Referee to determine the extent of thiochrome losses when isobutyl alcohol thiochrome solutions are exposed to light in the glassware currently used in this laboratory:

Equal amounts of thiamine (1 mmg in 5 ml of 0.1*N* sulfuric acid) were oxidized as in the official method, **38.22–38.28**, except that 2.5 grams of sodium or potassium chloride was added as a clarifying agent. In Experiment I, the oxidized solutions were exposed to direct north daylight (in the absence of direct sunlight) in the original 50 ml glass-stoppered Pyrex shakeout bottles previous to the separation of the isobutyl alcohol. In Experiment II the separated isobutyl alcohol solutions were exposed to this same light in the test tube cuvettes that were used to obtain the photofluorometer readings.

TABLE 5.—*Effect of exposure to daylight on thiochrome solutions in isobutyl alcohol (from 1 mmg thiamine)*[a]

TIME (MINUTES)	EXPOSURE I: IN PYREX BOTTLES OVER NaOH SOLUTION				EXPOSURE II: IN PYREX TEST TUBES			
	SERIES 1		SERIES 2		SERIES 3		SERIES 4	
	READING	% LOSS, AV.	READING	% LOSS, AV.	READING	% LOSS, AV.	READING	% LOSS, AV.
0[b]	96	—	98	—	94	—	96	—
0[b]	96	—	99	—	94	—	96	—
10	86		88		84		82	
		10.9		10.2		10.7		14.6
10	85		89		84		82	
20	75		78		73		72	
		22.9		20.3		22.4		25.0
20	73		79		73		72	
30	65		72		64		62	
		31.8		25.9		31.9		34.9
30	66		74		64		63	

[a] Series 1 and 3: Oxidized in presence of NaCl to saturate; Series 2 and 4: Oxidized in presence of KCl to saturate.
[b] Blackout controls.

The results of these experiments are tabulated in Table 5. Definite losses of thiochrome are shown to take place on undue exposure to direct daylight in periods of 10, 20, and 30 minutes. The light loss was not increased by letting the isobutyl alcohol-thiochrome solutions stand in contact with the alkaline aqueous phase. Although the conditions of the experiment may not apply to ordinary operating conditions during analyses, i.e., the intensity of daylight should be reduced in transmission through window glass, it is believed that the method should include some warning about this definite possibility of loss during and after the oxidation of thiamine to thiochrome.

In this laboratory the same precautions are taken to exclude daylight at this point as during the entire analysis for riboflavin. After the readings under Series 1 and Series 2 were recorded, all of the solutions in the cuvettes were exposed for 30 minutes to the usual operating light source from overhead electric lights, with no appreciable changes from the original readings.

RECOMMENDATIONS

It is recommended*—

(1) That Chapter 13, "Cereal Foods," in *Official Methods of Analysis*,

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 22, 23 (1957).

be extended to include descriptions of sample preparation and references to Chapter 38, "Nutritional Adjuncts," for methods for vitamins in flour, bread, macaroni, and similar enriched cereal and bakery products.

(2) That the following changes be made in the methods for fluorometric determination of thiamine, **38.22–38.32**:

(**a**) The following parenthetical statement should be inserted at the beginning of the method: "(During final oxidation of thiamine, **38.26** or **38.32**, protect solns from light to prevent destruction of thiochrome.)"

(**b**) The description of isobutyl alcohol, **38.22**(**1**), should be changed to read: "*Isobutyl alcohol.*—Redist. in all-glass app. Redistd product may be used as anhyd. or H_2O-satd."

(**c**) The first line of procedure for oxidation of thiamine to thiochrome, **38.26**, should be changed to read: "To each of 4 or more ca 40 ml tubes (or reaction vessels) add ca 1.5 g NaCl or KCl and 5 ml of the Std Soln."

(**d**) The first line of the oxidation procedure, **38.32**, should be changed to read: "Proceed as under **38.26**, except add ca 2.5 g NaCl or KCl to each tube (or reaction vessel) before addn of the 5 ml Std Soln, **38.30**, or 5 ml assay soln, **38.31**."

(**e**) The last line of the procedure for enzyme hydrolysis, **38.24**, should be changed to read: "Cool, dil. to 100 ml with H_2O, and filter thru paper known not to adsorb thiamine."

(3) That the following alternative procedures for the extraction, **38.23**, and enzyme hydrolysis, **38.24**, be studied collaboratively during the coming year as a method for determination of thiamine in bakery products and other enriched cereal products:

After acid hydrolysis, **38.23**(**a**), cool soln to room temp. and adjust *p*H to ca 4.5 by addn of 2*N* Na acetate soln, precisely with *p*H meter control if available, or approximately with bromcresol green indicator and spot plate; end point should be definitely on blue side of green-blue change. Use constant amount of hydrolyzing acid and stdze amount of 2*N* Na acetate used by *p*H meter control. Add 5 ml of the enzyme soln, **38.22**(**d**), mix, and incubate 3 hr at 45–50°. Stir mixt. of solids and liquid at 15–30 min. intervals. Cool, dil. to desired vol. with H_2O, and filter thru paper known not to adsorb thiamine. Check *p*H of filtrate (should be close to 4.5) for subsequent base exchange purification, **38.25**.

(4) That the title of this subject be changed from "Thiamine in Enriched Cereal Products" to "Thiamine in Enriched Cereal and Bakery Products" and that work be continued on the development of methods for thiamine in these products.

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REPORT ON VITAMIN B_6 (CHEMICAL)

By WALLACE L. HALL (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D.C.), *Associate Referee*

In a paper entitled "A New Chemical Method for the Determination of Vitamin B_6" (*This Journal*, **35**, 479 (1952)) Sweeney and Hall described a method in which the vitamin is demethylated by heating an acid solution containing selenium as catalyst, and adding cyanogen bromide and an aromatic amine to the demethylated vitamin to produce a colored solution that can be measured spectrophotometrically.

During the course of that study, but not reported at the time, it was observed that the selenium-catalyst oxidation of pyridoxine gave rise to intensely fluorescent solutions similar to the solution discussed by Huff and Perlzweig (*J. Biol. Chem.*, **155**, 345 (1944)); yet in our procedure there was a threefold to fourfold increase in sensitivity.

Recently Fujita and coworkers (*J. Vitaminol. (Osaka)*, **1**, 267 (1955)), in a series of four papers, successfully determined pyridoxine, pyridoxal, and pyridoxamine by fluorometry. We have started to study the use of fluorometry, a possible chemical method.

It is recommended* that chemical methods for vitamin B_6, particularly fluorometric methodology, be further studied.

* For report of Subcommittee A and action of the Association, see *This Journal*, **40**, 22, 23 (1957).

REPORT ON PANTOTHENIC ACID

ASSAY FOR TOTAL PANTOTHENIC ACID: 1956 COLLABORATIVE STUDY

By EDWARD W. TOEPFER (Human Nutrition Research Branch, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Md.), *Associate Referee*

Three dried samples—alfalfa leaf meal, whole egg, and brewer's yeast—were sent to 10 collaborating laboratories for assay. A double enzyme

TABLE 1.—*1956 A.O.A.C. collaborative study on the microbiological assay for total pantothenic acid in food*

SAMPLE	COLLABORATOR	TOTAL PANTOTHENIC ACID, MMG/G								FREE PANTOTHENIC ACID, MMG/G	COLLABORATOR'S PROCEDURE, MMG/G	
		ASSAY MEDIUM I				ASSAY MEDIUM II						
		INTESTINAL PHOSPHATASE-PIGEON LIVER		INTESTINAL PHOSPHATASE-HOG KIDNEY		INTESTINAL PHOSPHATASE-PIGEON LIVER		INTESTINAL PHOSPHATASE-HOG KIDNEY				
		FIRST ASSAY	SECOND ASSAY	FIRST ASSAY	SECOND ASSAY	FIRST ASSAY	SECOND ASSAY	FIRST ASSAY	SECOND ASSAY		FIRST ASSAY	SECOND ASSAY
Alfalfa leaf meal	1	35.8	39.3	41.5	—	44.1	42.6	39.3	41.6		40.7	—
	2	34.0	32.5	30.6	31.0	32.7	32.6	33.8	34.0		36.0	30.7
	3	42.5	40.8	42.7	42.0	44.2	41.3	43.0	41.6	31.1	—	—
	4	41.5	43.0	44.2	43.0	42.1	42.1	44.2	43.8		45.1	44.2
	5	26.4	—	36.6	31.9	23.8	—	37.7	31.9		23.0	22.2
											37.7	
Whole egg powder	1	72.8	64.2	—	—	88.9	91.1	78.3	76.8		—	—
	2	48.0	58.0	—	—	55.2	—	—	—		54.3	54.0
	3	91.8	89.5	—	—	—	—	—	—	53.6	—	—
	4	94.7	—	—	—	—	—	91.6	—		96.0	95.2
	5	46.0	—	92.0	53.5	44.2	—	86.5	51.5		49.6	55.1
Dried brewer's yeast	1	181	199	186	188	192	191	173	176		—	—
	2	141	—	—	—	111	119	—	—		—	—
	3	—	—	—	—	—	—	194	192	59.6	—	—
	4	193	—	—	—	—	—	190	—		196.2	196.0
	5	105	—	149	100	105	—	149	107		112.3	123.3

system was used for the preparation of sample extracts, and *L. plantarum* was used for the microbiological method.

The double enzyme systems were the pigeon liver extract treated twice with Dowex, prepared in each of the collaborating laboratories, used with intestinal phosphatase; and hog kidney extract,[1] commercially treated with resin and lyophilized, used with intestinal phosphatase.

A number of assays were made in the Associate Referee's laboratory to establish the levels for the use of the hog kidney preparation. This program caused a delay in the distribution of the samples and materials to the collaborators.

The study gave information on the assay medium and on the enzymes used in sample extraction (see Table 1). On the basis of results, the Associate Referee recommends* that the collaborative study be continued.

[1] E. Dickinson, Pentex Inc., Kankakee, Ill., supplied the hog kidney preparation used in this study.
* For report of Subcommittee A and action of the Association, see *This Journal*, **40**, 22, 23 (1957).

REPORT ON FOLIC ACID (MICROBIOLOGICAL METHOD)

By HENRY W. LOY, JR. (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D.C.), *Associate Referee*

Many workers have found that the present method for folic acid,[1] which was designed for the determination of total folic acid, cannot be satisfactorily applied to various food products. Folic acid is added to food supplements and to pharmaceutical and veterinary preparations. A microbiological method that will measure added or free folic acid is desirable, at least until the bound forms of folic acid can be determined accurately. After a method has been established for the determination of free folic acid, the bound forms of folic acid activity can be studied, and some of the newer enzyme preparations that have become available since the present method was written can be investigated.

If the enzyme study is successful, the sample solution containing total folic acid could be substituted in the method applicable to free folic acid, in a similar manner to the present program on pantothenic acid.

The proposed revision of microbiological methods for nutritional adjuncts[2] includes both the titrimetic and turbidimetric methods for the assay of free folic acid. Except for necessary modifications, the proposed methods are basically similar to the present method for total folic acid; they contain little that is new. A brief collaborative study will be sufficient to establish their acceptability.

[1] *Official Methods of Analysis*, 8th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1955, p. 830.
[2] *This Journal*, **40**, 856 (1957).

RECOMMENDATIONS

It is recommended*—

(1) That the microbiological assay for free folic acid by both the titrimetric and turbidimetric methods be given further collaborative study during the coming year.

(2) That a microbiological method for bound folic acid be studied, and that some of the newer enzyme preparations that have become available since the present method was adopted be used.

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 22, 23 (1957).

REPORT ON PROPOSED REVISION OF MICROBIOLOGICAL METHODS FOR *OFFICIAL METHODS OF ANALYSIS*

By HENRY W. LOY, JR. (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D.C.), *Associate Referee*

At the suggestion of the Chairman of the Committee on Revision of *Official Methods of Analysis*, an attempt was made to revise into a more concise form the microbiological methods for the B vitamins that appear in Chapter 38, "Nutritional Adjuncts."

In the Eighth Edition of *Official Methods of Analysis*[1] the titrimetric methods for riboflavin, nicotinic acid, folic acid, and pantothenic acid, and the turbidimetric method for vitamin B_{12} appear separately. Since many points in the methods for the five vitamins are similar, a large part of the revision[2] is designed to conserve space, and chiefly involves editorial changes.

Turbidimetric methods have come into wide use during the past several years. The turbidimetric method for each vitamin has been included in the revision where applicable, and has been written in a manner similar to the present method for vitamin B_{12}. For low-potency materials in which extraneous turbidity or color is present in the assay solution in an amount that would interfere with turbidimetric measurements, the titrimetric method is preferred. In preparing the proposed revision, technical changes were made only where necessary for uniformity.

A recommendation for adoption of both titrimetric and turbidimetric methods will be made at a later date. At this time the revision is not final. Several of the modifications that are included and a few others that may be made later will need further collaborative study before all of the revised methods can be adopted.

[1] *Official Methods of Analysis*, 8th Ed., Association of Official Agricultural Chemists, Washington, D. C., 1955.

[2] *This Journal*, 40, 855 (1957).

The proposed revision has been submitted to a number of laboratories that have had wide experience in using microbiological methods. The few comments that have been received are favorable to the proposal. After further comments have been received, a plan for collaborative study of technical modifications will be submitted to the collaborators. It is hoped that this study can be completed during the coming year.

It is recommended* that the proposed revision of microbiological methods, particularly the technical modifications, receive further collaborative study during the coming year.

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The Associate Referee wishes to express appreciation to the following collaborators and their organizations for cooperation in this study:

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E. W. Toepfer, Bureau of Human Nutrition, U.S.D.A., Beltsville, Md.

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 22, 23 (1957)

REPORT ON ANTIBIOTICS IN ANIMAL FEEDS

By WILLIAM A. RANDALL, *Former Associate Referee*,* and JOHN M. BURTON (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D.C.)

The collaborative assay for the determination of antibiotics in animal feed supplements has now been completed. The project was started late in 1953 by the appointment of an Associate Referee on Antibiotics in Animal Feeds, at the recommendation of O. L. Kline, the Referee. Progress reports have been published in *This Journal*, **38**, 686 (1955); **39**, 124 (1956).

Commercial samples of currently used feed supplements to be used in the collaborative assay were obtained through the cooperation of three firms: Lederle Laboratories Division of the American Cyanamid Co., Inc., who supplied the chlortetracycline supplements; Merck and Co., Inc., who supplied one of the penicillin supplements and the bacitracin

* Deceased.

supplement; and Chas. Pfizer and Co., Inc., who supplied the oxytetracycline supplement and the other penicillin sample.

Methods for the extraction of the antibiotics from the feed were obtained from various sources and tested by microbiological assay procedures developed in the laboratories of the Division of Antibiotics, Food and Drug Administration. When satisfactory extraction procedures and assay methods were developed, they were compiled into a manual entitled *Tentative Methods for the Determination of Antibiotics in Animal Feeds, October 1955*. These mimeographed methods, accompanied by an explanatory letter, were sent to 56 animal feed testing laboratories and manufacturers of antibiotics. Sixteen of the 56 laboratories agreed to participate in the collaborative assay, but six later withdrew for one reason or another.

The ten final collaborators were Commercial Solvents Corporation, Food Research Laboratories, Inc., Lederle Laboratories Division of the American Cyanamid Company, Wyeth Laboratories, Inc., Merck and Co., Inc., Eastern States Farmers' Exchange, Inc., Chas. Pfizer and Co., Inc., Wyoming State Department of Agriculture (penicillin only), Ralston Purina Company, and S. B. Penick Company (bacitracin only). The Division of Antibiotics also participated, making a total of 11.

The feed samples sent to the participants for assay contained the following: Procaine penicillin, 4 g[1] of drug per pound; procaine penicillin, 4.2 g per pound; zinc bacitracin, 6.545 g[2] per pound; chlortetracycline, 1.8 g[3] per pound; chlortetracycline, 10 g per pound; and oxytetracycline, 5.0 g[4] per pound. In addition, cultures of the test organisms and the standard antibiotics were sent to the collaborators.

Each collaborator was aked to test five aliquots on different days to check on the homogeneity of the feed supplement and the variability of the assay by the following methods:

Microbiological Methods

(U.S.P. Reference Standards are obtainable from U.S.P. Reference Standards, 46 Park Avenue, New York 16, New York)

CULTURE MEDIA

(a) *Agar medium A.*—Dissolve 6.0 g peptone, 4.0 g pancreatic digest of casein, 3.0 g yeast ext., 1.5 g beef ext., 1.0 g anhyd. dextrose, and 15 g agar in H_2O, and dil. to 1 l. Adjust with 1*N* NaOH or HCl (1+9) so that after sterilization for 30 min. at 121° *p*H is 6.5–6.6. (Difco Penassay Seed Agar and BBL Seed Agar have been found satisfactory.)

(b) *Agar medium B.*—Dissolve 6.0 g peptone, 3.0 g yeast ext., 1.5 g beef ext., 1.0 g anhyd. dextrose, and 15 g agar in H_2O, and dil. to 1 l. Adjust with 1*N* NaOH or HCl (1+9) so that after sterilization for 30 min. at 121° *p*H is 6.5–6.6. (Difco Yeast Beef Agar has been found satisfactory.)

(c) *Agar medium C.*—Dissolve 6.0 g peptone, 3.0 g yeast ext., 1.5 g beef ext.,

[1] 1 g procaine penicillin = 1,009,000 units of activity.
[2] 1 g bacitracin = 42,000 units of activity.
[3] 1 g chlortetracycline = 1,000,000 mmg of chlortetracycline hydrochloride activity.
[4] 1 g oxytetracycline = 1,000,000 mmg of oxytetracycline base.

and 15 g agar in H_2O, and dil. to 1 l. Adjust with 1*N* NaOH or HCl (1+9) so that after sterilization for 30 min. at 121° *p*H is 6.5–6.6 (Difco Penassay Base Agar and BBL Base Agar have been found satisfactory.)

(d) *Agar medium D.*—Use agar medium C adjusted with 1*N* NaOH or HCl (1+9) so that final pH is 5.6–5.7.

(e) *Broth medium.*—Dissolve 5.0 g peptone, 1.5 g yeast ext., 1.5 g beef ext., 3.5 g NaCl, 1.0 g anhyd. dextrose, 3.68 g anhyd. K_2HPO_4, and 1.32 g anhyd. KH_2PO_4 in H_2O, and dil. to 1 l. Adjust with 1*N* NaOH or HCl (1+9) so that after sterilization for 30 min. at 121° pH is 6.95–7.05. (Difco Penassay Broth and BBL Antibiotic Assay Broth have been found satisfactory.)

REAGENTS

(a) *Phosphate buffer.*—*p*H 6. Dissolve 8.0 g anhyd. KH_2PO_4 and 2.0 g anhyd-K_2HPO_4 in H_2O and dil. to 1 l.

(b) *Phosphate buffer.*—*p*H 4.5. Dissolve 13.6 g anhyd. KH_2PO_4 in H_2O and dil. to 1 l.

(c) *Pyridine-buffer soln.*—Mix 1 vol. pyridine and 4 vol. *p*H 6.0 buffer.

(d) *Acid-acetone.*—Mix 1 vol. 4*N* HCl, 13 vol. acetone, and 6 vol. H_2O.

(e) *Acid-methanol.*—Mix 1 vol. HCl and 50 vol. MeOH.

APPARATUS

(Jars of high speed blenders, if used in place of mortars and pestles, must be cleaned with great care, after disassembling, to eliminate all traces of antibiotics. All equipment must be thoroly cleaned after use and heat-treated, if possible, 2 hr at 200°.)

(a) *Cylinders (cups).*—Polished stainless steel cylinders, 8 ± 0.1 mm o.d., 6 ± 0.1 mm i.d., and 10 ± 0.1 mm high (obtainable from S & L Metal Products Corp., 25 Lafayette St., Brooklyn 1, N.Y.).

(b) *Petri dishes (plates).*—20×100 mm with porcelain covers glazed on outside. (BBL Brewer cover lids with filter pad inserts have also been found satisfactory for absorbing H_2O of syneresis.)

STOCK CULTURES OF TEST ORGANISMS

Cultures are obtainable from American Type Culture Collection (A.T.C.C.), 2029 M Street, N.W., Washington 6, D. C. For appropriate test organism designated below, prep. slant culture on 1 or more tubes of agar medium A. Incubate 18–24 hr at indicated temp. between 26° and 35°, held constant to ±0.5°, and finally store in dark at ca 10°. Do not use if more than 2 weeks old.

(a) *Sarcina lutea.*—A.T.C.C. No. 9341. Incubate stock culture at 26°. Use for penicillin assay.

(b) *Micrococcus flavus.*—A.T.C.C. No. 10240. Incubate stock culture at 32–35°. Use for bacitracin assay.

(c) *Bacillus cereus var. mycoides.*—A.T.C.C. No. 11778. Incubate stock culture at 30°. Use for chlortetracycline and oxytetracycline assays.

PREPARATION OF STANDARD CURVE

Prep. std curve simultaneously with assay soln. Prep. concns of the U.S.P. Reference Standard (described under each antibiotic). Use indicated concn as reference concn.

Prep. plates with appropriate base agar layer and appropriate seed agar layer (described under each antibiotic). Place 6 cylinders on each plate at ca 60° intervals on 2.8 cm radius. Fill 3 alternate cylinders with reference concn and other 3 cylin-

ders with one of other concns of std. Use 3 plates for each concn required for std curve, except reference concn (total of 18 plates). Incubate plates 18–24 hr at appropriate temp., and read diams of zones of inhibition by means of mm ruler, calipers, or calibrated projection device. In each set of 3 plates average the 9 readings of the reference concn and the 9 readings of concn being tested. Av. of all 54 readings of reference concn from 18 plates is correction point for curve. Correct av. value obtained for each concn to figure it would be if reference concn reading on that set of 3 plates were same as correction point. For example, if in correcting second concn of std curve, av. of 54 readings of reference concn is 20.0 mm, and av. of 9 readings of reference concn of this set of 3 plates is 19.8 mm, correction is +0.2 mm. If the av. reading of second concn on same 3 plates is 17.0 mm, corrected value is 17.2 mm. Plot corrected values, including correction point, on semilog graph paper, using logarithmic scale for concn and arithmetic scale for av. zone diams. Reference point for detg potency of assay soln is indicated zone size of reference concn on best straight line drawn thru plotted values.

DETERMINATION

Use 3 plates for each assay soln. On each plate fill 3 alternate cylinders with reference concn and fill other 3 cylinders with assay soln. Incubate plates 18–24 hr at appropriate temp. and read diam. of zones of inhibition. Average the 9 readings of reference concn and the 9 readings of assay soln. If assay soln gives larger average than reference concn, add difference between them to reference point on std curve. If assay soln gives smaller value than reference concn, subtract difference between them from reference point on std curve. Using corrected value of assay soln, det. quantity of antibiotic from std curve.

PENICILLIN

STANDARD SOLUTIONS

(a) *Stock soln.*—Weigh accurately, in atmosphere of 50% relative humidity or less, ca 10 mg U.S.P. Penicillin Reference Standard. Dissolve in sufficient *p*H 6 buffer to give concn of exactly 100 units/ml. Store in dark at ca 10° no longer than 2 days. Stock soln may be frozen for 2 months in small aliquots at ca −20°. Each aliquot should be enough for 1 day's use only.

(b) *Standard curve.*—Dil. appropriate aliquots of stock soln, (a), with enough *p*H 6 buffer to obtain concns of 0.01, 0.02, 0.03, 0.05, 0.10, 0.20, and 0.30 units/ml. Reference concn is 0.05 units/ml.

PLATES

(a) *Base layer.*—Add 10 ml melted agar medium A to sterile Petri dishes, distribute evenly, and allow to harden on *perfectly level surface.*

(b) *Seed layer.*—Prep. inoculum for plates by one of following methods:

(1) *Broth culture.*—Wash growth from stock culture of *S. lutea* with ca 3 ml broth medium, and transfer liquid to 100 ml broth medium. Incubate 48 hr at ca 26° with continuous mechanical agitation. (This 48 hr culture is inoculum and it may be stored at ca 10° no longer than 2 weeks.) Before actual assay, det. by prepn of trial plates the optimum concn (usually 2–5%) of inoculum to be added to agar medium B to obtain zones of inhibition of adequate size and sharpness.

(2) *Roux bottle culture.*—Wash growth from 24 hr slant culture with ca 3 ml broth medium, and transfer liquid to surface of 300 ml agar medium A in Roux bottle. Spread suspension evenly over entire surface with aid of sterile glass beads. Incubate 24 hr at 26° and wash growth from agar surface with ca 15 ml broth medium. Using photoelectric colorimeter and 18 mm diam. test tube as absorption cell, det. transmittance of 1:10 diln of this bulk suspension at 650 mμ, and, if

necessary, adjust by diln so that 1:10 diln gives 10% transmittance. Use adjusted bulk suspension (not 1:10 diln) in prepg seed layer. (Bulk suspension may be stored at ca 10° several months.) Before actual assay, det. by prepn of trial plates the optimum concn (usually 0.3–0.5%) of inoculum to be added to agar medium B to obtain zones of inhibition of adequate size and sharpness.

Add appropriate amount of organism suspension to agar medium B which has been melted and cooled to 48°. Mix thoroly and add 4.0 ml to each plate contg base layer. Distribute agar evenly by tilting plates from side to side with circular motion and allow to harden. *Use plates same day prepd.*

ASSAY SOLUTION

(a) *Feed supplement concentrates.*—If supplement contains more than 100 g penicillin/lb, use 1 g sample; for lower concns, use 3 g sample. Add 25 ml aq. acetone (1+1) or pH 6 buffer (lower concns only), shake 2 min., let settle, and decant supernatant soln into 100 ml vol. flask. Repeat washing, shaking, and decanting with 25 and 50 ml portions aq. acetone (1+1) or pH 6 buffer. Combine decantates, dil. to 100 ml, and mix. Centrifuge 15 ml combined decantates ca 15 min. at 2000 rpm. Dil. aliquot of clear soln with enough pH 6 buffer to obtain estimated concn of 0.05 units/ml.

(b) *Mixed feeds.*—To 10 g sample add 35 ml pH 6 buffer or aq. acetone (1+1), shake 2 min., let settle, and decant supernatant soln into 100 ml vol. flask. Repeat washing, shaking, and decanting with 35 and 30 ml portions *p*H 6 buffer or aq. acetone (1+1). Combine decantates, dil. to 100 ml, and mix. Centrifuge 15 ml combined decantates ca 15 min. at 2000 rpm. Dil. aliquot of clear soln with enough *p*H 6 buffer to obtain estimated concn of 0.05 units/ml.

Place measured quantity of sample in 100 ml graduated cylinder and proceed as below. Designate final measured vol. obtained as assay soln.

ASSAY

Using penicillin std curve, assay soln, and plates, proceed as under PREPARATION OF STANDARD CURVE and DETERMINATION.

BACITRACIN

STANDARD SOLUTIONS

(a) *Stock soln.*—Dry ca 40 mg U.S.P. Bacitracin Reference Standard 3 hrs at 60° in vacuum oven at 5 mm pressure or less. Det. accurate dry wt and dissolve in enough *p*H 6 buffer to give concn of exactly 100 units/ml. Store in dark at ca 10° no longer than 5 days.

(b) *Standard curve.*—Dil. appropriate aliquots of stock soln, (a), with enough *p*H 6 buffer to obtain concns of 0.02, 0.03, 0.06, 0.10, 0.20, 0.40, and 0.80 units/ml. Reference concn is 0.20 units/ml.

PLATES

(a) *Base layer.*—Add 10 ml melted agar medium C to sterile Petri dishes, distribute evenly, and allow to harden on *perfectly level surface.*

(b) *Seed layer.*— Wash growth from stock culture of *M. flavus* with ca 3 ml broth medium and transfer liquid to surface of 300 ml agar medium A in Roux bottle. Spread suspension evenly over entire surface with aid of sterile glass beads and incubate 18 hr at 32–35°. Wash growth from agar surface with ca 25 ml 0.9% NaCl soln. Using photoelectric colorimeter and 18 mm diam. test tube as absorption cell, det. transmittance of 1:50 diln of this bulk suspension at 650 mμ, and, if necessary, adjust by diln so that 1:50 diln gives 75% transmittance. (Adjusted bulk suspension, not 1:50 diln, is used in prepg seed layer.) Store adjusted bulk suspension at

ca 10°. Before actual assay, det. by prepn of trial plates the optimum concn (usually 0.3–0.5%) of inoculum to be added to agar medium A to obtain zones of inhibition of adequate size and sharpness. For actual assay add appropriate amount of inoculum to agar medium A which has been melted and cooled to 48°. Mix thoroly and add 4.0 ml to each of plates contg base layer. Distribute agar evenly by tilting plates from side to side with circular motion, and allow to harden. *Use plates same day prepd.*

ASSAY SOLUTION

Using mortar and pestle or high-speed blender, grind 3 g feed supplement concentrate or 10 g mixed feed with 50 ml pyridine-buffer soln and transfer mixt. to 100 ml centrifuge tube. Wash mortar and pestle or jar with 50 ml pyridine-buffer soln and combine washings with ext. in centrifuge tube. Shake well 5 min., and centrifuge ca 15 min. at 2000 rpm. Dil. aliquot of clear soln with enough *p*H 6 buffer to obtain estimated concn of 0.20 units/ml. Designate soln obtained as assay soln.

ASSAY

Using bacitracin std curve, assay soln, and plates, proceed as under PREPARATION OF STANDARD CURVE and DETERMINATION.

CHLORTETRACYCLINE HYDROCHLORIDE

STANDARD SOLUTIONS

(a) *Stock soln.*—Weigh accurately ca 40 mg U.S.P. Chlortetracycline Hydrochloride Reference Standard. Dissolve in enough 0.01*N* HCl to give concn of exactly 1000 mmg/ml. Store in dark at ca 10° no longer than 5 days.

(b) *Standard curve.*—Dil. appropriate aliquots of stock soln, (a), with enough pH 4.5 buffer to obtain concns of 0.005, 0.01, 0.02, 0.04, 0.06, 0.08, and 0.10 mmg per ml. Reference concn is 0.04 mmg/ml.

PLATES

(a) *Base layer.*—Add 6.0 ml melted agar medium D to sterile Petri dishes, distribute evenly, and allow to harden on *perfectly level surface.*

(b) *Seed layer.*—Wash growth from stock culture of *B. cereus* var. *mycoides* with ca 3 ml sterile H_2O, transfer to surface of 300 ml agar medium A, and incubate 7 days at 30°. Wash growth from agar surface with ca 25 ml H_2O and heat suspension 30 min. at 65°. Centrifuge and decant. Wash residual spores 3 times with sterile H_2O, centrifuging and decanting each time. Discard H_2O washings. Heat residual spores 30 min. at 65° and resuspend in sterile H_2O. Keep this stock suspension at ca 10°. Before actual assay, det. by prepn of trial plates optimum concn (usually 0.03–0.10%) of inoculum to be added to agar medium D to obtain zones of inhibition with as little as 0.01 mmg chlortetracycline hydrochloride/ml. For actual assay add appropriate amount of inoculum to agar medium D which has been melted and cooled to 48°. Mix thoroly and add 4.0 ml to each of plates contg base layer. Distribute agar evenly by tilting plates from side to side with circular motion, and allow to harden. *Use plates same day prepd.*

ASSAY SOLUTION

Using mortar and pestle or high-speed blender, grind 2 g feed supplement concentrate or 10 g mixed feed with 50 ml acid-acetone soln and transfer mixt. to 100 ml centrifuge tube. Wash mortar and pestle or blender jar with 50 ml acid-acetone and combine washings with ext. in centrifuge tube. Shake well 5 min. Centrifuge ca 15 min. at 2000 rpm. Remove 10 ml clear soln and adjust to *p*H 4.5 with 1*N* NaOH. Dil. adjusted soln with enough *p*H 4.5 buffer to obtain estimated concn of 0.04 mmg/ml. Designate soln so obtained as assay soln.

ASSAY

Using chlortetracycline hydrochloride std curve, assay soln, and plates, proceed as under PREPARATION OF STANDARD CURVE and DETERMINATION.

OXYTETRACYCLINE

STANDARD SOLUTIONS

(a) *Stock soln.*—Weigh accurately ca 40 mg U.S.P. Oxytetracycline Reference Standard. Dissolve in enough 0.1*N* HCl to give concn of exactly 1000 mmg/ml. Store in dark at ca 10° no longer than 5 days.

(b) *Standard curve.*—Dil. appropriate aliquots of stock soln, (a), with enough *p*H 4.5 buffer to obtain concns of 0.025, 0.05, 0.10, 0.20, 0.40, 0.60, and 0.80 mmg per ml. Reference concn is 0.20 mmg/ml.

ASSAY SOLUTION

Proceed as under CHLORTETRACYCLINE ASSAY SOLUTION except that where reference is made to acid-acetone, replace by acid-methanol, and where reference is made to 0.04 mmg/ml, replace by 0.20 mmg/ml.

ASSAY

Using oxytetracycline std curve and assay soln, and chlortetracycline plates, proceed as under PREPARATION OF STANDARD CURVE and DETERMINATION.

RESULTS AND RECOMMENDATIONS

Table 1 shows the number of times each collaborating laboratory assayed a given feed sample. A statistical analysis was made of the data to evaluate the reliability of the assay procedure.

The individual results of each laboratory were averaged to obtain an average potency for each laboratory for each antibiotic feed assayed. The average potencies for each laboratory were then averaged to obtain a combined average potency for all laboratories for each antibiotic feed.

TABLE 1.—*Number of assays submitted by the collaborating groups*

COLLABORATING GROUP	M1001A (4.0 G PROC. PEN./LB)	M881 (4.2 G PROC. PEN./LB)	M881A (6.545 G BACI./LB)	M993 (1.8 G CHLORTET./LB)	M993A (10 G CHLORTET./LB)	M1001 (5.0 G OXYTET./LB)
Commercial Solvents Corp.	15	15	15	15	15	15
Eastern States Farmers Exchange	10	10	5	5	5	5
F.D.A.	20	18	15	18	19	10
Food Research Labs.	4	4	4	3	3	4
Lederle	6	6	10	6	6	10
Merck and Co.	9	9	10	14	17	18
S. B. Penick	—	—	1	—	—	—
Pfizer	5	5	5	5	5	5
Ralston Purina[a]	1	1	1	1	1	1
Wyeth	13	15	15	15	15	15
State of Wyoming	1	1	—	—	—	—

[a] Company submitted one average figure based on an unknown number of assays.

TABLE 2.—*Statistical data for collaborative assays of antibiotics in feeds*

FEED SUPPLEMENT LOT. NO.	ANTIBIOTIC	PER CENT POTENCY		95% CONFIDENCE LIMITS		NUMBER OF LABORATORIES INCLUDED	NUMBER OF LABORATORIES DISCARDED
		THEORY	ASSAY	LOWER	UPPER		
M1001A	Proc. Pen.	100	106.9	83.8	130.0	9	1
M881	Proc. Pen.	100	99.6	84.0	115.2	9	1
M881A	Zinc Bac.	100	99.0	84.9	113.1	10	0
M993	Chlortetracycline	100	95.7	82.1	109.3	7	2
M993A	Chlortetracycline	100	101.2	85.6	116.8	9	0
M1001	Oxytetracycline	100	119.0	100.4	137.6	8	1

The 95 per cent confidence limits were then calculated. However, if an inspection of the results indicated that the average potency of a particular laboratory deviated too widely from those of the other laboratories, it was not included in calculating the combined average potency and 95 per cent confidence limits. If the suspected aberrant potency did not fall within the 95 per cent confidence limits calculated in this manner, it was discarded.

Table 2 shows the combined average potency for each antibiotic feed, the 95 per cent confidence limits, the number of laboratories included in the final average, and the number, if any, of laboratory averages that had to be discarded. For example, for antibiotic feed M881, containing procaine penicillin, a combined average potency of 99.6 per cent was obtained with 95 per cent confidence limits of 84.0–115.2 per cent, calculated by using the average potency of 9 laboratories. The average potency of the tenth laboratory was discarded because the average potency value did not fall between 85–115.2 per cent. The results reported by 11 laboratories for the collaborative study of 6 samples of feed supplements containing either procaine penicillin, zinc bacitracin, chlortetracycline, or oxytetracycline were considered to agree well enough to support the following recommendation:

It is recommended* that the methods studied collaboratively for the determination of the antibiotics procaine penicillin, zinc bacitracin, chlortetracycline, and oxytetracycline in feed supplements be adopted as first action.

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 22, 23 (1957).

REPORT ON CAROTENE

By F. W. QUACKENBUSH (Department of Biochemistry, Purdue University, Lafayette, Ind.), *Associate Referee*

During the year we were informed that the magnesia, Westvaco 2642, that we have been using in the official analysis of carotene might not continue to be available; the company producing it had no other market for the product and it had therefore become a special non-profit item.

After several other samples of magnesia from different sources were tested, it appeared that another Westvaco product, sold under the tradename "Sea Sorb 43", most nearly resembled the standard magnesia in its adsorptive qualities.

All participants in the work on alfalfa meal check samples were requested to compare Sea Sorb 43 with No. 2642 in analyzing the February and March samples. Nearly all responded. Their results are summarized as follows:

	Sample No. 76 (94 collaborators)		*Sample No. 77 (87 collaborators)*	
	Av.	*Std. Dev.*	*Av.*	*Std. Dev.*
Magnesia, No. 2642	112.6	8.3	92.7	5.6
Magnesia, Sea Sorb 43	111.1	9.0	93.5	5.9

It was concluded that Sea Sorb 43 gave essentially the same results as the standard magnesia 2642 specified in the procedure.

It is recommended* that the carotene procedure be modified to provide for the use of Sea Sorb 43 as an alternative adsorbent with No. 2642, and that the work be continued.

* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 22, 23 (1957).

REPORT ON VITAMIN A IN MIXED FEEDS

By D. B. PARRISH (Kansas Agricultural Experiment Station, Manhattan, Kan.) *Associate Referee*

Development of a suitable method for determining Vitamin A in mixed feeds has been complicated by introduction of various stabilized vitamin A products, use of low levels of vitamin A, changes in the composition of commercial feeds, and addition of DPPD (diphenylphenylene diamine).

Only methods involving an alkali digestion (1) have appeared suitable for analysis of feeds containing vitamin A stabilized in a gelatin matrix. Many analysts, however, have felt that these methods are too cumbersome for routine work and that their use probably would be limited. Recently

a method was published which is shorter, does not require saponification, and is reported to be applicable to samples containing stabilized vitamin A and also DPPD (2). It is possible that, with modifications, this procedure will be suitable for use on all types of feed samples. DPPD causes complications in the analysis when alkali digestion is employed (1), but its use has been discontinued recently (3).

TABLE 1.—*Composition of feed samples*

INGREDIENT[a]	QUANTITY
	per cent
Corn, white-yellow mixed	48
Soybean oilmeal, 44%	26
Wheat middlings	10
Oats	3
Fish meal	2
Brewer's yeast	1
Non-fat milk solids	1
Mineral premix[b]	4
Vitamin-antibiotic premix[c]	1
Alfalfa meal, 20%	2
Lard	2
Vitamin A	
Sample 1, 7.55 Units/g[d]	
Sample 2, 7.27 Units/g[e]	
Sample 3, 7.03 Units/g[f]	

[a] All ingredients except corn, soybean oil meal, and oats first were combined in appropriate premixes.
[b] Salt, calcium carbonate, bone meal, and manganese sulfate.
[c] B-vitamins, choline, vitamin D, vitamin K, Terramycin concentrate, and wheat middlings.
[d] Source: Nopcay-5000 (Nopco Chemical Company).
[e] Source: Pfizer A-10-p (Chas. Pfizer and Co.).
[f] Source: 3.30 units from Peter Hand-5000, which contains Rovimix (Peter Hand Foundation). 3.73 units from Permadry-10,000 (Stabilized Vitamins, Inc.,).

Collaborative work for 1956 was planned to compare results by three methods: I, a saponification method (1); II, the new rapid extraction method (2); and III, the first action method (4). Some analysts have been confused as to the types of samples for which the latter method could be used, and it was hoped results of these comparisons would help to clarify this problem. Thirty-two laboratories agreed to participate in the 1956 study. Twenty-one submitted reports. Some laboratories, however, were able to complete only a part of the work outlined.

Three samples and a blank free of vitamin A were sent to collaborators. All samples had the same composition, except for vitamin A (Table 1), which was added at 3000 units per pound, based on the manufacturer's guarantee. The stated contents of vitamin A in the feeds are based on analyses of the supplements used. Since the trend is to the use of fat in feeds, 2 per cent fat was added to all feeds for this study. Feedstuffs were

ground so that 90 per cent passed a 30-mesh sieve and 10 per cent passed a 100-mesh sieve[1]. All ingredients except corn, oats, and soybean meal were combined in premixes. Feed was mixed carefully, first by machine and then by hand. It is difficult to prepare feeds of uniform vitamin A content when low levels of vitamin A are added, but analyses indicated that satisfactory uniformity was attained in these samples.

DIRECTIONS TO COLLABORATORS

Samples were sent in small polyethylene bags and collaborators were asked to refrigerate them immediately on receipt. Within 4 days, samples were to be mixed by rolling on non-adsorbent paper, divided into 4 parts, transferred to small, tightly closed bottles, and placed in cold storage. Purpose of the subsamples was to avoid possible losses of vitamin A from repeated opening, mixing, sampling, etc., each time analyses were performed. Large samples were supplied so that collaborators could practice the unfamiliar methods. All results following the practice trials were to be reported.

Collaborators were asked to check adsorbent characteristics and standardize all columns according to directions in Method I (1). Use of chromatographic tubes of 12 mm inside diameter was suggested. Attention was called to the fact that the quantity of solvent mixture for elution of vitamin A from the column could not be specified exactly because it depended in part on composition of the feed. For example, added fat speeds elutions. The amount of solvent for elutions is determined by techniques such as observation of pigment bands, painting extruded chromatographic columns with antimony trichloride reagent, and inspections under ultraviolet light.

Collaborators were invited to do additional research on methods and techniques and to report results. If tests indicated that Method III was unsuited for one or more samples, use of that method could be omitted. Collaborators were asked to determine vitamin A on the blank and also to determine losses of vitamin A on analysis. A 20 g sample, instead of 10 g, was specified in Method III. Since DPPD is not used in commercial feeds at present, all reference to it in Method I was to be disregarded.

Method II, as given below, was modified slightly from the original (2).

METHOD II

SPECIAL APPARATUS

(a) *Extraction apparatus.*—Glass-stoppered centrifuge bottle, glass-stoppered Erlenmyer flask, glass-stoppered mixing cylinder, or separatory funnel, 250 ml capacity.

(b) *Suction device.*—Use device similar to **6.12** (4).

SPECIAL REAGENTS

(a) *Methanol.*—Absolute methanol.

[1] Data from W. D. Lewis, Chas. Pfizer and Co., Inc.

(b) *Surface active agent.*—Dissolve 12 g Triton X-100 (Rohm and Haas Co., Philadelphia, Pa.) and 28 g sodium tripolyphosphate in 800 ml water and dilute to 1000 ml with methanol.

(c) *Mixed solvent.*—Hexane (Skellysolve B) and diethyl ether (15+85), v/v.

DETERMINATION

To 40 ml surfactant and 10 ml methanol in an extraction apparatus add 20 g feed. Mix thoroughly. Add 60 ml mixed solvent and extract by shaking vigorously for 15 min. (mechanical shaker is useful). Let solvent layers separate on standing, or centrifuge at low speed. Draw off clear supernatant layer, using suction device. Evaporate to dryness an aliquot containing 40–50 mmg vitamin A (or as large an aliquot as possible on low-potency samples), using vacuum device (1). Dissolve residue completely in 15–20 ml hexane and pour on prepared column (1). Rinse container twice with small portions hexane and continue as under *Chromatography* in Method III (4).

RESULTS AND DISCUSSION

On all three samples carotene averaged 4.3 mmg/g by Method I, and 4.5 to 5.0 mmg/g by the other two methods (Tables 2, 3, and 4). Many analysts observed that the pigments appeared to separate poorly and elute too rapidly when using Methods II and III. Some ascribed this result to presence of added fat in the samples or to overloading of the small-size columns, and also in Method III to the presence in the extract of traces of methanol or "Triton" which acted as eluting agents. Saponification of the samples, in Method I, removed a part of the materials in the feed responsible for rapid elution and allowed better separations of pigments, which resulted in lower carotene values.

Sample 1 contained a wax-stabilized vitamin A which should be extracted satisfactorily by each of the three methods. Average vitamin A contents found by all methods are less than the quantities added (Table 2). The lowest values are by Method I. Different investigators reported losses of vitamin A on analysis of 8–40 per cent by Method I and 2–26 per cent by Methods II and III. Some analysts corrected results by subtracting the reading on the vitamin A-free blank, some by correcting for carotenoids in the extract which produced blue colors with antimony trichloride, and others by using a combination of corrections. The higher average values obtained by Methods II and III are somewhat misleading since several investigators did not correct for yellow pigments in the vitamin A extract; these pigments could account for up to 20 per cent of the apparent vitamin A reading.

Average quantity of vitamin A found in Sample 2 (Table 3) by Method I was less than that added. No problems seemed to have arisen in using Method I on Sample 2, except those discussed for Sample 1. Average content of vitamin A in Sample 2 by Method II was less than that by Method I, but more than that by Method III. Gade and Kadlec[2] reported that heating the sample for one hour at 40°C. in the presence of the mixed sol-

[2] Private communication.

TABLE 2.—*Results on Sample 1*

COLLABORATOR	METHOD I			METHOD II			METHOD III		
	CAROTENE	VIT. A	VIT. A CORRECTED[a]	CAROTENE	VIT. A	VIT. A CORRECTED	CAROTENE	VIT. A	VIT. A CORRECTED
	Mmg/g	*Units/g*	*Units/g*	*Mmg/g*	*Units/g*	*Units/g*	*Mmg/g*	*Units/g*	*Units/g*
1	4.9	5.6	4.7	3.8	6.8	6.0	4.7	8.6	7.8
2	4.6	5.7	7.5	—	5.0	5.9	—	6.7	8.0
3	3.5	7.1	6.7	5.1	6.6	6.2	4.5	8.0	7.5
4	4.3	6.6	7.4	—	—	—	4.3	7.0	7.7
5	4.1	6.6	—	4.9	9.3	12.4	4.4	5.9	7.9
6	4.2	6.5	6.8	3.6	7.2	8.0	4.3	6.0	8.2
7	4.0	6.6	5.8	2.9	9.4	8.6	4.7	8.0	7.3
8	3.6	6.7	6.8	—	—	—	—	9.6[b]	7.3
9	2.6	5.1	6.7	3.9	5.3	6.7	3.7	6.0	6.9
10	—	6.2	7.5	—	5.6	6.7	—	7.2	8.7
11	4.3	5.6	6.7	5.1	6.0	7.1	4.6	6.5	7.0
12	4.1	2.1	2.8	5.0	4.0	17.8[b]	4.3	3.5[b]	10.5[b]
13	—	6.8	—	5.6	7.7	—	—	—	—
14	5.3	6.1	5.9	—	—	—	—	—	—
15	7.9[b]	13.8[b]	10.6	6.6	10.8[b]	7.0	7.4[b]	7.0	5.1
16	—	5.2	—	—	6.2	—	—	6.1	—
17	2.9	4.7	2.8	—	4.8	3.8	—	5.9	3.3[b]
18	4.1	7.1	7.7	4.8	6.2	6.5	4.5	6.0	6.1
19	—	0.6[b]	0.2[b]	—	2.4	2.1	—	7.3	6.3
20	—	—	—	—	—	—	4.9	6.7	—
21	4.4	7.9	6.6	5.9	7.9	5.9	4.7	8.4	7.4
Av.	4.3	6.1	6.1	4.8	6.5	7.4	4.7	6.9	7.2
Std. Dev.	1.17	2.48	2.36	1.05	2.08	3.64	0.87	1.33	1.53
Av. adjusted[b]	4.1	6.0	6.4	4.8	6.3	6.6	4.5	6.9	7.2

[a] Corrections based on loss of vitamin A on analysis, reading on blank, correction for carotenoids in extract, or combinations of these.
[b] Values more than two times standard deviation eliminated from adjusted average.

TABLE 3.—*Results on Sample 2*

COLLABORATOR	METHOD I			METHOD II			METHOD III		
	CAROTENE	VIT. A	VIT. A CORRECTED[a]	CAROTENE	VIT. A	VIT. A CORRECTED	CAROTENE	VIT. A	VIT. A CORRECTED
	Mmg/g	*Units/g*	*Units/g*	*Mmg/g*	*Units/g*	*Units/g*	*Mmg/g*	*Units/g*	*Units/g*
1	4.8	5.2	4.9	3.6	3.6	3.0	4.6	2.6	2.4
2	4.6	6.0	6.8	—	5.3	6.2	—	0	0
3	3.9	7.1	6.8	5.2	7.6	7.1	4.3	0.5	0
4	4.5	8.1	9.1	—	—	—	4.5	0	—
5	4.5	7.0	—	3.9	2.9	3.9	3.9	2.0	2.7
6	4.2	6.6	6.9	3.6	—	—	4.3	—	—
7	3.9	7.2	6.4	4.2	9.2	8.4	—	—	—
8	3.4	6.6	6.6	—	—	—	—	—	—
9	3.0	6.1	8.0	2.9	6.0	7.5	3.8	0.2	0.2
10	—	8.4	10.1	—	5.0	6.0	—	0.6	0.7
11	4.3	4.8	5.8	5.0	7.6	8.9	3.9	0.2	0.2
12	4.0	0[b]	—	4.9	0	—	4.5	0	—
13	—	7.5	—	5.5	7.1	—	—	—	—
14	5.1	8.0	7.7	—	—	—	—	—	—
15	7.5[b]	14.4[b]	11.2	8.7[b]	8.4	4.5	6.8[b]	1.8	(−0.6)
16	—	4.4	—	—	1.1	—	—	0.8	—
17	2.9	5.1	3.1	—	5.6	4.6	—	2.5	0
18	4.2	5.7	6.2	4.4	5.2[c]	5.5[c]	4.7	0.9	0.9
19	—	0.7	0.3[b]	—	2.8	2.6	—	—	0
20	—	—	—	—	—	—	—	—	—
21	4.6	8.8	7.1	5.7	4.0	1.9	4.8	1.7	0.5
Av.	4.3	6.4	6.7	4.8	5.1	5.4	4.6	—	—
Std. Dev.	1.03	2.95	2.56	1.49	2.56	2.19	0.82	—	—
Av. adjusted[b]	4.1	6.3	7.1	4.4	5.1	5.4	4.3	—	—

[a] Corrections based on loss of vitamin A on analysis, reading on blank, correction for carotenoids in extract, or combination of these.
[b] Values more than two times standard deviation eliminated from adjusted average.
[c] A modified extraction used.

TABLE 4.—*Results on Sample 3*

COLLABORATOR	METHOD I			METHOD II			METHOD III		
	CAROTENE	VIT. A	VIT. A CORRECTED[a]	CAROTENE	VIT. A	VIT. A CORRECTED	CAROTENE	VIT. A	VIT. A CORRECTED
	Mmg/g	*Units/g*	*Units/g*	*Mmg/g*	*Units/g*	*Units/g*	*Mmg/g*	*Units/g*	*Units/g*
1	3.5	4.4	3.3	3.6	3.5	2.4	4.4	5.1	4.3
2	4.6	4.3	5.2	—	5.5	6.5	—	—	4.4
3	3.6	6.2	5.9	5.3	7.7	7.3	4.4	3.9	3.4
4	4.3	5.4	6.2	—	—	—	4.6	4.5	5.0
5	4.7	6.4	—	4.7	11.1[b]	14.8[b]	3.6	4.3	5.7
6	4.2	6.2	6.5	3.5	6.2	6.9	4.2	3.9	5.0
7	3.9	5.4	4.6	4.6	7.5	6.7	4.9	6.5	5.7
8	3.5	6.2	6.3	—	—	—	—	6.3	4.6
9	3.6	4.9	6.5	3.3	5.4	6.8	3.9	3.1	3.5
10	—	5.9	7.1	—	6.1	7.4	—	3.3	3.9
11	4.4	4.7	5.7	5.2	6.6	7.9	4.5	3.7	4.2
12	4.3	5.8	7.7	4.8	2.9	12.6	4.2	2.0	6.0
13	—	6.9	—	5.4	6.4	—	—	—	—
14	5.1	8.7	8.3	—	—	—	—	—	—
15	7.4[b]	11.4[b]	8.2	9.0[b]	7.8	3.9	5.9[b]	6.9	4.6
16	—	—	—	—	4.1	—	—	3.6	—
17	2.7	6.7	4.7	—	4.8	3.8	—	3.7	1.3[b]
18	4.3	5.6	6.1	4.4	4.2	4.4	4.5	3.7	3.8
19	—	0.5[b]	0.2[b]	—	2.7	2.5	—	3.2	2.2
20	—	—	—	—	—	—	—	—	—
21	4.5	6.8	5.4	5.7	6.8	5.8	4.8	5.3	3.8
Av.	4.3	5.9	5.8	5.0	5.8	6.6	4.5	4.3	4.2
Std. Dev.	1.01	2.08	1.92	1.48	2.11	2.39	0.57	1.32	1.21
Av. adjusted[b]	4.1	5.9	6.1	4.6	5.5	6.1	4.4	4.3	4.4

[a] Corrections based on loss of vitamin A on analysis, reading on blank, correction for carotenoids in extract, or combination of these.
[b] Values more than two times standard deviation eliminated from adjusted average.

vent and surfactant markedly improved extractions of Sample 2 by Method II. It is possible that a part of the variation among laboratories resulted from temperature differences during extractions. As with Sample 1, some of the higher values obtained by Method II on Sample 2 may have resulted from the fact that all analysts did not correct for carotene in the extract on which vitamin A was determined.

As a result of data obtained, most investigators realized that Sample 2 probably contained a stabilized vitamin A unextractable by Method III. Since results by Method III are meaningless, they are not averaged in Table 3.

Similar to findings for Samples 1 and 2, the average vitamin A found for Sample 3 (Table 4) by Method I is less than that added. Sample 3 contained a part of the vitamin A in the form of a gelatin-stabilized product; therefore it would not be expected that Method III would be suitable, and only a part of the vitamin A would be extracted. Presumably vitamin A added as "Perma-Dry" was extracted rather completely, but a part of the gelatin-stabilized vitamin A was not extracted. The gelatin-stabilized vitamin A of Sample 3 has a somewhat different composition from that of Sample 2, which possibly makes it easier to extract. Again a part of the apparent vitamin A in Sample 3 by Methods II and III probably resulted from presence of carotene in the extract.

The recoveries of vitamin A, the standard deviations (Tables 2, 3, and 4), and the coefficients of variation, which may be calculated from them, indicate that improvement is needed before wide use can be made of vitamin A analyses by any of these methods for feed control purposes.

To present the data for replicate determinations on each sample by each method from each laboratory would require too much space, but an estimate of precision is of interest. Data were rated as satisfactory, fair, and poor on the basis of replication of data for each sample by each method. In general, a rating of satisfactory indicated maximum variation of 10 per cent, and fair 10–20 per cent, although some small deviations were allowed on certain samples. On this basis 10 laboratories were rated satisfactory on carotene analyses, 7 fair, and 4 poor. Seven were rated satisfactory on vitamin A analyses, 10 fair, and 4 poor. Precision appeared to be primarily a function of the laboratory, as none rated satisfactory on carotene analyses and poor on vitamin A analyses or vice versa.

Method I appeared to be the best of the three tried, but several analysts objected to the time required and some did not like to use the smaller columns. With the volume of extract for chromatography called for in Method I, vitamin A was found in the eluate before all the extract had been added to the column.

Many investigators reported that they were unable to separate carotene and vitamin A satisfactorily by Methods II and III with the 12 mm diameter columns, and several were unable to do so with the 22 mm diam-

eter columns. Materials in the feed samples sent to collaborators, particularly the added fat, made the smaller columns unsuitable unless the extract to be chromatographed was concentrated to 10–15 ml. As originally developed, Method III is unsatisfactory when samples contain added fat, since elution is so rapid that vitamin A and carotene cannot be separated. (In fact, a 0.3 per cent fat-in-hexane solution showed promise as an eluting agent.)

Results and comments from several investigators indicated that the magnesia now being used is less effective than that formerly obtained. Some analysts preferred to use the magnesia routinely employed in their laboratory and did not standardize it as suggested in the directions. Since it is difficult to separate carotene and vitamin A on weakened magnesia, some investigators suggest that these substances should be eluted together and a proper correction made.

Data from limited studies in 3 laboratories indicated that results with Sea Sorb 43[3] in chromatography of vitamin A were approximately the same as those obtained with magnesia 2641.

As vitamin A ester was eluted from the magnesia columns, two fluorescing bands were observed, one below carotene and the other above. This was not observed by the author when alumina columns were used. Since the upper fluorescing band eluted similar to vitamin A alcohol and gave a blue color with antimony trichloride, it suggested that a part of the ester was being decomposed to form the alcohol during chromatography. The vitamin A alcohol formed would be lost during the elutions in Methods II and III.

It was noted that, with Method I, some analysts recovered only 60 per cent of the added vitamin A. Reports of vitamin A studies in the literature often show higher recoveries. The present feed samples contain only relatively small quantities of vitamin A, and the ratios of pigments and other fat-soluble substances to vitamin A is higher than in most other supplemented products. These factors no doubt increase the difficulties of designing methods to obtain good vitamin A recoveries from feed samples.

Some analysts have used a microscopic technique to identify the type of vitamin A product added to feed samples. However, at the vitamin A levels used in these feeds, no one who reported was willing to make an identification in this manner.

Information was obtained on the degree of experience of the laboratories and analysts doing the collaborative studies. All laboratories have had more than 3 years' experience in analyzing vitamin A in feeds and concentrates. About one-third said that their experience with feeds had been intermittent.

Method I or similar methods involving saponification seem to be recognized as applicable to feeds containing the greatest variety of vitamin A

[3] Westvaco Mineral Products Corp., Newark, Calif.

products and additives. But DPPD, recently discontinued as a feed supplement, interferes in methods such as Method I that use saponification. Several analysts do not feel that Method I will be used widely because it is long and cumbersome, and allows many opportunities for loss of vitamin A. Method III does not appear suitable for analyzing samples containing added fats. This should be of concern to all doing control work, since fats are being used in increasing quantities in mixed feeds. The principles of Method II offer a new approach; it has the advantages of speed and simplicity. It is hoped that the limited success with Method II will stimulate more work on such methods to determine whether they can be perfected to replace the longer procedure.

The following modifications and simplifications of Method II have given good results on some feed samples:

Place 20–40 g feed in a 300–500 ml glass-stoppered boiling flask. Add 0.5 g Tide detergent (The Procter and Gamble Co., Cincinnati, Ohio), 0.5 g sodium tripolyphosphate, and 50–100 ml 70% methanol at 60°C. Agitate 5 min. to keep feed in suspension. Add 100–200 ml Skellysolve B and shake vigorously 10 min. Let settle 5 min., pour 25 ml supernatant solution into a 35–50 ml glass-stoppered centrifuge tube, add 2 g activated magnesia, and shake briefly. Add 2 g activated alumina; again shake briefly. Centrifuge at moderate speed 3 min., transfer 10 ml of the clear supernatant solution to a colorimetric absorption tube, and determine carotenoids at 440 mμ. Connect absorption tube to vacuum source and evaporate solvent by agitating tube in H_2O at 60–65°C. Cool tube and add 1 ml $CHCl_3$ to dissolve residue completely. Determine vitamin A by Carr-Price reaction at 620 mμ. Correct for blue color of $SbCl_3$ and carotenoids by use of previously determined factor or curve established by using α-β carotene and $SbCl_3$. (Sample size and quantities of reagents are selected so that the final reading at 620 mμ is in the proper range.)

A longer alternate procedure which allows for improved precision and a carotene determination on the feed sample is as follows: Pass 50 ml supernatant solution from extraction through a 7 cm×22 mm 1:1 MgO-Supercel column. Elute with 10% acetone in Skellysolve B until all carotene is eluted. Concentrate under vacuum, add 1 ml $CHCl_3$, and proceed as above. (Samples containing large amounts of fat may have to be washed once with water before chromatography and dried with Na_2SO_4 to remove traces of methanol. Fat plus methanol sometimes causes poor separation of pigments. Normally the trace of methanol causes rapid but satisfactory elution.)

SAMPLING

When feeds containing low levels of stabilized vitamin A are analyzed, sampling is of special importance. Stabilized vitamin A products are prepared in potencies of 250,000 to 500,000 units per gram in the form of small granules or beadlets. One product on the market averages 2 units per beadlet and another 5 units. In preparing the supplement finally used in feed mixing, the beadlets are not subdivided, but are merely diluted out with carrier. In feeds that contain 2000 units of vitamin A per lb, like these products, there would be only an average of 2.2 and 0.9 beadlets, respectively, per gram of feed; and at 1000 units per lb, only one-half as many. Thus good sampling and proper sample preparation are essential.

The small number of vitamin A-containing particles per gram of mixed feeds has led several analysts to advocate use of at least 40 g samples on feeds of low vitamin A potency. Increased precision has followed use of the larger samples.

RECOMMENDATIONS

It is recommended*—

(1) That increased efforts and collaborative work be directed toward evaluating the shorter, non-saponification methods for vitamin A in mixed feeds.

(2) That special study be given to correction factors or other means for overcoming effects of substances that interfere in the Carr-Price reaction for vitamin A.

ACKNOWLEDGMENT

The Associate Referee acknowledges the cooperation of the following collaborators (listing is alphabetical). The asterisk after the names of several collaborators signifies that they reported work in addition to that requested.

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* For report of Subcommittee A and action of the Association, see *This Journal*, 40, 22, 23 (1957).

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REPORT ON THE DETERMINATION OF VITAMIN A IN MARGARINE

By KENNETH MORGAREIDGE (Food Research Laboratories, Inc., 48-14 Thirty-Third St., Long Island City, N.Y.), *Associate Referee*

This report on the determination of vitamin A in margarine covers a two-year period, since no report was presented at the 1955 meeting of this Association. During this interval, the Associate Referee has further studied the tandem-column method for the chromatographic purification of vitamin A fractions obtained from margarine-unsaponifiable material (1, 2). Concurrently, the single column chromatographic method of Rosner and Kan (3) was studied collaboratively under the auspices of the Vitamin A Sub-Committee of the National Margarine Manufacturers Association. The Associate Referee is pleased to acknowledge the cooperation of this group. Additional observations on the single column procedure were also made by the Associate Referee.

EXPERIMENTAL

Application of the double-column, or tandem-column, method of chromatography to vitamin A extracts from margarine has been referred to in previous reports (4, 5). To eliminate collection of multiple eluate fractions, the method originally described (1) was modified by the use of a weak source of long wavelength ultraviolet light to excite fluorescence of the vitamin A band so that it could be collected in a single fraction by visual observation of the column.

During routine use of this procedure in these laboratories for the assay of commercial margarines, data were accumulated on the reproducibility of the method by replicating the assay on aliquots of a single sample of

TABLE 1.—*Reproducibility study on a single sample (double column procedure)*

ASSAY DATE	VITAMIN A (UNITS×10^{-3}/LB)	ABSORBANCE RATIOS	
		310/325 Mμ	334/325 Mμ
5-28-56	16.5	0.856	0.866
5-31-56	16.1	0.861	0.861
	16.4	0.858	0.862
6- 1-56	17.2	0.860	0.865
	16.7	0.860	0.860
6- 5-56	16.4	0.852	0.850
	16.8	0.879	0.855
	16.2	0.852	0.860
6-21-56	?	0.894[a]	0.889[a]
6-22-56	16.4	0.874	0.876
	17.0	0.868	0.862
6-25-56	17.1	0.870	0.858
Average:	16.6 ± 0.35	0.863	0.861
Coefficient of Variation: 2.1 per cent			
Morton-Stubbs factor		0.968	

[a] Omitted from average.

market margarine colored with aniline dyes. The results obtained over a period of one month are shown in Table 1.

The data illustrate three main points of interest. First, it is seen that the method is capable of excellent precision in a single laboratory: the coefficient of variation obtained in 11 trials was 2.1 per cent of the mean value. Second, when the chromatographic step does fail, abnormal absorbance ratios are obtained and the assay is considered unacceptable, e.g., in the run reported on June 21, 1956, in which the ratios exceeded the limit arbitrarily assigned for acceptability (0.857 ± 0.02) because the column had not been prepared properly.

The third point worthy of comment concerns the average values of the absorbance ratios. If a Morton-Stubbs type of correction is calculated, the corrected vitamin A content of the sample would be approximately 97 per cent of the observed potency. At the present time, this amount of correction is not considered significant for margarine.

A limited number of trials were conducted in which the single and double column procedures were compared on the same samples of margarine. The results are shown in Table 2. The aluminas for these columns were prepared in the laboratory of the Associate Referee in accordance with published instructions (2, 3). It was somewhat more difficult to obtain satisfactory performance of the chromatographic step when the single column of alkaline alumina was used than when tandem columns were employed. It will be noted that in only 1 out of 5 trials were the absorb-

TABLE 2.—*Comparison of single vs. double columns (spectrophotometric data)*

SAMPLE	SINGLE COLUMN[a]			DOUBLE COLUMNS[b]		
	(UNITS×10^{-3})/LB	R_{310}	R_{334}	(UNITS×10^{-3})/LB	R_{310}	R_{334}
A	clogged column			18.5	0.856	0.874
B	16.8	0.927	0.859	14.9	0.866	0.854
C	18.5	0.841	0.851	19.9	0.857	0.857
D	8.8	0.894	0.882	9.9	0.863	0.877
E	13.7	0.888	0.871	15.2	0.864	0.852

[a] Alkaline alumina prepared in the author's laboratory. Elution obtained with graded concentrations of ether in petroleum ether up to 36 per cent.
[b] After Boldingh and Drost, modified by use of weak ultraviolet light for tracing vitamin A band.

ance ratios obtained from the single column satisfactory and in reasonably good agreement with the double column results found. It has been our general experience, as illustrated by the limited series of trials shown here, that dependence on a single column of alkaline alumina places a greater demand on the skill and experience of the analyst for the manipulation of the chromatographic steps.

COLLABORATIVE STUDIES

During the course of the NAMM Check Sample Study for 1955–1956, a number of the cooperating laboratories agreed to try the single column chromatographic procedure with the assistance of Dr. Lawrence Rosner. The samples were also assayed by the antimony trichloride blue color method. For comparison, 7 of the 9 samples were also assayed by the double column procedure in the Associate Referee's laboratory. The data reported are summarized in Table 3.

The samples were taken from commercial lots of margarine, and, with the exception of those for March and April, 1956, were colored with aniline dyes. The two exceptions contained carotene both as a colorant and as a partial source of vitamin A activity.

It is of interest that the average values reported by the single column method generally agree quite well with those obtained by double chromatography. As expected, there is a significant tendency for the chromatographic-spectrophotometric values to be lower than those found by the colorimetric assay.

The greater variation of between-laboratory chromatographic data is not surprising; the number of collaborators was small and many of them had not had extensive previous experience with chromatography.

DISCUSSION AND RECOMMENDATION

Adequate standardization of methods for preparing aluminas for vita-

TABLE 3.—*Collaborative studies on vitamin A in margarine*[a]

SAMPLE DATE	VITAMIN A (UNITS×10^{-3})/LB		
	CHROMATOGRAPHIC METHOD		$SbCl_3$ COLORIMETRIC METHOD
	SINGLE COLUMN	DOUBLE COLUMNS	
Feb. '55	18.4±2.1 (6)	18.3	22.2±0.8 (11)
Mar. '55	15.3±0.8 (8)	—	17.3±0.9 (10)
Apr. '55	14.2±2.9 (5)	—	15.2±0.6 (9)
Dec. '55	16.4±2.4 (5)	18.5	18.9±1.4 (7)
Jan. '56	14.7±1.2 (5)	15.0	16.1±0.7 (7)
Feb. '56	16.0±2.2 (6)	16.7	19.0±1.4 (10)
Mar. '56	11.8±1.8 (5)	12.2	12.9±1.0 (8)
Apr. '56	12.6±1.7 (8)	12.9	13.4±1.3 (8)
May '56	16.5±2.7 (8)	15.1	17.7±1.4 (10)
Average Coefficient of Variation	13.1%		7.5%

[a] Figures in parentheses indicate number of laboratories reporting in NAMM Check Sample Study. Results for double columns obtained in Associate Referee's Laboratory only.

min A chromatography still presents a problem. To try to eliminate this variable in the collaborative study, alkaline alumina from a single lot prepared in one laboratory was distributed to all of the collaborators. Unfortunately, the particular batch employed exhibited a background fluorescence that tended to obscure the fluorescence of the vitamin A band. Some of the collaborators reported that they could eliminate this difficulty by reheating the alumina to 750°C. Steps have been taken to solve this problem.

The continued cooperation of the Vitamin A Sub-Committee of the NAMM in further collaborative studies has been assured, and plans have been made for more extensive trials during the coming year.

It is recommended* that studies be continued to develop a satisfactory chromatographic-spectrophotometric assay procedure for vitamin A in margarine.

ACKNOWLEDGMENTS

The collaboration of Joseph M. Icken, Food Research Laboratories, who conducted much of the assay work, is gratefully acknowledged.

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* For report of Subcommittee A and action of the Association, see *This Journal*, **40**, 22, 23 (1957).

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(5) MORGAREIDGE, K., *ibid.*, **38**, 688 (1955).

No reports were given on microbiological method for vitamin B_{12} or vitamin D.

REPORT ON WATERS, MINERAL AND SALT

By R. A. OSBORN (Division of Food, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D.C.), *Referee*

It is recommended* that studies of methods for the examination of waters be continued.

No reports were given on Agricultural Liming Materials.

No reports were given on Baking Powder and Baking Chemicals.

No reports were given on Caustic Poisons.

No reports were given on Cosmetics.

No reports were given on Emission Spectrography.

No reports were given on Enzymes.

No reports were given on Gelatine, Dessert Preparations, and Mixes.

No reports were given on Radioactivity.

No reports were given on Standard Solutions.

* For report of Subcommittee D and action of the Association, see *This Journal*, 40, 39 (1957).

ANNOUNCEMENTS

DAIRY PRODUCTS:

Catherine G. Cuningham, Food and Drug Administration, Boston 10, Mass., has been appointed Associate Referee on Frozen Desserts.

Sammie J. Merritt, Louisiana Department of Health, New Orleans 7, La., has been appointed Associate Referee on Reconstituted Milk in Fluid Market Milk.

DECOMPOSITION AND FILTH IN FOODS (CHEMICAL INDICES):

W. J. Dyer, Fisheries Research Board of Canada, Halifax, Nova Scotia, Canada, has been appointed Associate Referee on Trimethylamine.

Mrs. Jean A. Gaul, Food and Drug Administration, New Orleans 16, La., has been appointed Associate Referee on Animal Fecal Matter.

OILS, FATS, AND WAXES:

H. W. Conroy, Food and Drug Administration, Kansas City 6, Mo., has been appointed Associate Referee on Antioxidants.

MISCELLANEOUS DRUGS:

Theodore E. Byers, Food and Drug Administration, Cincinnati 2, Ohio, has been appointed Associate Referee on Bismuth.

CONTRIBUTED PAPERS

THE RELIABILITY OF COLLABORATIVE TESTING FOR A.O.A.C. METHODS*

By Morris D. Finkner (Biometrical Services, Agricultural Research Service, U. S. Department of Agriculture, Plant Industry Station, Beltsville, Md.)

The results of scientific research must be evaluated in some way to determine their usefulness. Collaborative testing has become a standard procedure for evaluating proposed A.O.A.C. methods. Although this procedure is considered essential to the work of the Association, it has certain limitations, the most important of which are:

(1) Because collaborators are usually asked to cooperate on a voluntary basis, the investigator cannot always secure the desired number of qualified collaborators (the term "collaborator" in this paper refers to a laboratory that cooperates in a testing program).

(2) The investigator has very little control over the test procedures, once the collaborators have been supplied with the test material.

(3) Collaborators do not always realize the importance of their role in the over-all testing program.

Because of these and other limitations, A.O.A.C. Referees have become concerned about the reliability of collaborative testing.

FUNDAMENTAL REQUIREMENTS

Several fundamental requirements must be fulfilled before any testing procedure can be considered reliable. First, conditions of the test must be representative of a specified population: the collaborators must be a sample representative of a population of laboratories about which inferences are to be drawn. For example, if a method is developed for use under conditions of constant temperature and humidity, it is totally inappropriate to include in the sample a laboratory which does not have constant temperature and humidity facilities. Such a laboratory would not be representative of the intended population.

Second, the testing procedure must be unbiased. Absence of bias is a measure of accuracy. In many cases it is impossible to detect the presence of bias; therefore certain precautions must be taken to diminish the probability of encountering a statistical bias. The most important precaution is randomization. The element of randomization must prevail throughout the entire structure of collaborative testing.

It is also important that the collaborators carefully follow the instruc-

* Presented at the 70th Annual Meeting of the Association of Official Agricultural Chemists, Oct. 15–17, 1956, at Washington, D. C.

tions of the procedure. Every detail is important and even the slightest deviation from the proposed procedure may bias the results. The collaborators must assume this responsibility to try to establish the accuracy of the collaborative test.

Third, the results of the test must be reproducible. This attribute is a measure of precision. If a collaborative test results in a high degree of precision it is considered to be reproducible: the random variability is small enough so that similar results can be expected if the test is repeated. If the degree of precision is not as favorable as it should be, however, the causes of the variability should be identified and measures taken to reduce it.

The fourth requirement involves the scope of the test. The materials for which the method has been designed must be included in the test. If the method was developed for only one specific material, the test should be limited to this material. However, if the method is to be used for a number of different materials, all of them must be included in the test.

In addition to these requirements, the test procedure must be practical. Funds and facilities are seldom available for an unlimited testing program. It may be necessary at times to accept less than ideal testing procedures to facilitate the testing program; the fundamental requirements may be only partially fulfilled. It should be emphasized, however, that for each compromise in fulfilling the requirements, the inferences that can be drawn will be limited to some extent. If practical considerations have required too many compromises, the accompanying limitations may completely negate the test.

THE ANALYSIS OF VARIANCE

If the collaborative testing program has been properly planned, the resulting information may be analyzed by a statistical technique commonly known as the analysis of variance. This technique aids materially in interpreting the results by providing a convenient method for reduction of the data.

The pattern of the analysis depends largely upon the statistical model under consideration. The model reflects the properties of the design, a portion of which may be under the control of the investigator; these are called the fixed effects. It also reflects some uncontrolled factors called random variables.

If the experimental and sampling errors in the model can be assumed to be non-correlated and are of about the same magnitude, the analysis of variance will give unbiased minimum variance estimates of the effects. In addition, if the errors are normally distributed, the F and "t" test can be used to test for the significance of responses, and confidence intervals for means can be computed.

The statistical model that uses several sources of variation can take one

of two forms: (1) Every variable, except the general mean, is a random variable; or (2) there is a mixture of random variables and fixed effects. Model 1 is called a random model, Model 2 a mixed model. In either case, the random sources of variation can be isolated as components of variance that have additive properties; the total variance of an observation is equal to the sum of the corresponding components of variance. It is also convenient to estimate components of variance from the analysis of variance; in this estimation, if no tests of significance are desired, the assumption specifying normality can be relaxed.

In collaborative testing, two general sources of variability can easily be discerned: the variability between laboratories, generally the largest source of variability and unfortunately one that cannot be controlled by the investigator; and the within-laboratory source of variability, which is, to a certain degree, under the control of the investigator, and which may be composed of a number of different components of differing degrees of magnitude and importance. All of these components can be estimated, provided a proper sampling design has been employed.

HIERARCHICAL OR NESTED CLASSIFICATION

A sampling technique ordinarily used in sample surveys but equally applicable to collaborative testing is the hierarchical or nested classification. This sampling scheme may be used with either the random or the mixed statistical model. It will be described first in connection with the mixed model.

Mixed Model.—Suppose a chemical method has been submitted for collaborative testing. A number of laboratories are selected for this program; they are the only laboratories interested in using this particular method. In the statistical sense, therefore, the laboratories are considered as fixed effects. The average within-laboratory variability constitutes the random or error variability. Several possible sources of variability may contribute to this error, and each source should be included in the sampling design. It might be suspected, for example, that variability exists among different analysts, among days, and among determinations made on the same day. Therefore the statistical model is:

$$Y_{ijkm} = \mu + \alpha_i + \beta_{ij} + \gamma_{ijk} + \delta_{ijkm} \qquad (1)$$

where μ represents the general mean, α_i the ith laboratory, β_{ij} the jth analysts in the ith laboratory, γ_{ijk} the kth day for the (ij) analysts, and δ_{ijkm} the mth determination on the (ijk) day. The experiment, then, consists of r laboratories, p analysts per laboratory, s days per analysts, and t determinations per day. All are random variables except μ and α_i. The analysis of variance for this particular model is found in Table 1.

The analysis of variance can be used to test the significance of certain hypotheses by comparing appropriate mean squares of the analysis. The hypothesis under test is always the null hypothesis (Ho:) with respect to

TABLE 1.—*Analysis of variance for the hierarchical or nested classification*

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARES	EXPECTED MEAN SQUARES
Total	$rpst-1$		
Laboratories	$r-1$	M_1	$\sigma_t^2+t\sigma_s^2+st\sigma_p^2+pst\theta_r$
Analysts within laboratories	$r(p-1)$	M_2	$\sigma_t^2+t\sigma_s^2+st\sigma_p^2$
Days within analysts and laboratories	$rp(s-1)$	M_3	$\sigma_t^2+t\sigma_s^2$
Determinations within analysts, days, and laboratories	$rps(t-1)$	M_4	σ_t^2

the source of variation of interest. The proper error term to be used in the F ratio for testing a null hypothesis can be determined by examining the expected mean squares.

For example, testing for a significant day-to-day variation is actually testing the null hypothesis that $\sigma_s^2=0$. The proper error term for this test is M_4, since σ_t^2 is a common term in both M_3 and M_4. Therefore the F ratio equals $(\sigma_t^2+t\sigma_s^2)/\sigma_s^2$. If the F ratio equals 1, it can be concluded that $\sigma_s^2=0$, and the null hypothesis is accepted.

However, since the mean squares are computed from the sample data, the components of variance are only estimates of the population parameters and the significance of the test is based on a specified probability. The value of F for days is determined, therefore, by $F=M_3/M_4$ with $rp(s-1)$ and $rps(t-1)$ degrees of freedom.

Other tests of significance from this analysis are shown in Table 2. Since laboratories are fixed effects, inferences regarding laboratories are limited to those under test.

The confidence interval or band for the general mean (μ) can also be computed from this analysis. The parameter μ estimated by $\bar{y}$ on a single determination basis is:

$$\bar{y} = \frac{\Sigma Y_{ijkm}}{rpst}. \tag{2}$$

The population variance of the mean ($\sigma_{\bar{y}}^2$), based on the average variability within laboratories, is:

$$\sigma_{\bar{y}}^2 = \frac{\sigma_p^2}{p} + \frac{\sigma_s^2}{ps} + \frac{\sigma_t^2}{pst}. \tag{3}$$

TABLE 2.—*Tests of significance for the sources of variation indicated in Table 1*

NULL HYPOTHESIS	F RATIO	DEGREES OF FREEDOM
$\sigma_s^2=0$	M_3/M_4	$rp(s-1)$ and $rps(t-1)$
$\sigma_p^2=0$	M_2/M_3	$r(p-1)$ and $rp(s-1)$
$\theta_r=0$	M_1/M_2	$(r-1)$ and $r(p-1)$

The statistical problem involved is to obtain values for the components of variance. These can be estimated from the analysis of variance in the following manner:

$$\sigma_t^2 \doteq V_t = M_4 \tag{4}$$

$$\sigma_s^2 \doteq V_s = \frac{M_3 - M_4}{t} \tag{5}$$

$$\sigma_p^2 \doteq V_p = \frac{M_2 - M_3}{st}. \tag{6}$$

The population variance of the mean $\sigma_{\bar{y}}^2$ is estimated by $V_{\bar{y}}$ and is equal to:

$$V_{\bar{y}} = \frac{V_p}{p} + \frac{V_s}{ps} + \frac{V_t}{pst} \tag{7}$$

with $r(p-1)$ degrees of freedom. Therefore the confidence interval for μ is:

$$\bar{y} \pm \sqrt{V_{\bar{y}}}\,(t) \tag{8}$$

where t is the value taken from the t-table for a specified probability level with $r(p-1)$ degrees of freedom. The width of the confidence interval is a measure of precision and an indication of the reliability of the test. If the confidence interval is too wide for general acceptability, the variance of the mean may be reduced in either of two ways: (1) by improving certain techniques or (2) by increasing the sampling intensities.

Random Model.—Suppose the conditions of this test had been altered slightly, e.g., the chemical method had been designed for a rather large population of laboratories. The laboratories selected as collaborators would then constitute a random sample of this population. The model

$$Y_{ijkm} = \mu + \alpha_i + \beta_{ij} + \gamma_{ijk} + \delta_{ijkm}$$

is exactly the same as before except that all effects but μ are random variables. Therefore σ_r^2 is a random element and is added to the component of variance model. The mechanics of the analysis of variance, the testing of significance, and estimation of variance components are exactly the same as before. The laboratory component, σ_r^2, is estimated by V_r and is computed by referring to Table 1:

$$V_r = \frac{M_1 - M_2}{pst}. \tag{9}$$

The variance of the mean is now:

$$\sigma_{\bar{y}}^2 = \frac{\sigma_r^2}{r} + \frac{\sigma_p^2}{rp} + \frac{\sigma_s^2}{rps} + \frac{\sigma_t^2}{rpst} \tag{10}$$

and is estimated from the analysis of variance for an individual laboratory as:

$$V_{\bar{y}} = V_r = \frac{V_p}{p} + \frac{V_s}{ps} + \frac{V_t}{pst} \tag{11}$$

with $(r-1)$ degrees of freedom. The confidence interval for μ is:

$$\bar{y} \pm \sqrt{V_{\bar{y}}}\,(t) \tag{12}$$

The interpretation of results in the case of the random model is broadened to include inferences about a population of laboratories. The confidence interval, therefore, is wider than for the mixed model in which the only laboratories considered were those included in the test. If the confidence interval is considered too wide for general acceptance of the method, the sources of variation should be carefully examined before the method is discarded. If the major source of variability is due to the within-laboratory components, the contribution of these components to the variance of the mean may be reduced in either of the two ways discussed previously. If the variability among laboratories is the major source of variation, the method under test cannot be accepted generally for all laboratories in the population.

Numerical Example.—As a numerical example to illustrate the hierarchical or nested sampling design, suppose a method for determining total gossypol in cotton seed meal has been subjected to collaborative testing. The design consisted of 4 laboratories, 2 analysts within each laboratory, 2 testing days for each analyst, and duplicate determinations on each day. Therefore, in the notation used above, $r=4$, and $p=s=t=2$. The analysis of variance for the collaborative test is presented in Table 3.

The analysis of variance indicated a significant variation between laboratories and between days within analysts and laboratories. The variation between analysts within laboratories was not significant. An estimate of the mean percentage of gossypol was 0.798 per cent.

If the model is mixed, which specifies laboratories as fixed effects, the estimates of the variance components are:

$$V_t = 0.000162; \qquad V_s = 0.000319; \qquad V_p = 0.000019$$

Therefore, from (7), the estimated variance of the mean percentage of gossypol is:

$$V_{\bar{y}} = \frac{0.000019}{(2)} + \frac{0.000319}{(2)(2)} + \frac{0.000162}{(2)(2)(2)} \tag{13}$$

$$V_{\bar{y}} = 0.000109$$

TABLE 3.—*Analysis of variance of numerical example illustrating the hierarchical or nested classification* ($r=4$, $p=s=t=2$)

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARES	F
Total	31		
Laboratories	3	$M_1=0.008233$	9.41[a]
Analysts within laboratories	4	$M_2=0.000875$	1.09
Days within analysts and laboratories	8	$M_3=0.000800$	4.94[a]
Determinations within days, analysts, and laboratories	16	$M_4=0.000162$	

[a] Denotes significance at the 0.01 level of probability.

The standard error of the mean, $\sqrt{V_{\bar{y}}}$, is 0.0105 and the value of t for 4 degrees of freedom and a 95 per cent probability level is 2.776. Hence the 95 per cent confidence limits for μ are:

$$\begin{aligned} 0.798 - 0.029 < \mu < 0.798 + 0.029 \\ 0.769 < \mu < 0.827. \end{aligned} \tag{14}$$

Assuming the completely random model, the variation between laboratories is a random element and V_r is estimated to be 0.000920. This component of variance must be included in the estimate of the variance of the mean which is computed from (11) as:

$$\begin{aligned} V_{\bar{y}} &= 0.000920 + \frac{0.000019}{2} + \frac{0.000319}{4} + \frac{0.000162}{8} \\ V_{\bar{y}} &= 0.00102. \end{aligned} \tag{15}$$

The standard error $\sqrt{V_{\bar{y}}}$ is 0.032 and the 95 per cent confidence interval (3 d.f.) is:

$$\begin{aligned} 0.798 - 0.102 < \mu < 0.798 + 0.102 \\ 0.696 < \mu < 0.900. \end{aligned} \tag{16}$$

The confidence interval resulting from the random model is much wider than that from the mixed model: the relatively large component of variance associated with laboratories must be included in the random model to account for the broader interpretation which includes inferences about an entire population of laboratories.

TWO-WAY OR CROSS CLASSIFICATION

Collaborative testing of a method designed for two or more specific but different materials requires the two-way or cross classification. Since all materials are submitted to each collaborator for testing, material means can be computed by summing across laboratories. Likewise, laboratories can be identified according to the materials, and laboratory means can be obtained by summing across materials. The sampling scheme within laboratories and materials is exactly the same as for the nested classification. This design may be used for either the random or mixed model. Two cases of the mixed model will be described.

Mixed Model: Laboratories Fixed.—Suppose the same conditions prevail as described for nested classification except that the chemical method under test is designed for several different materials. In this case, materials and material by laboratory interaction are fixed effects and are included in the statistical model. The model is:

$$Y_{hijkm} = \mu + \tau_h + \alpha_i + (\tau\alpha)_{hi} + \beta_{hij} + \gamma_{hijk} + \delta_{hijkm} \tag{17}$$

where μ is the general mean, τ_h is the effect of the hth material, α_i the ith laboratory, and $(\tau\alpha)_{hi}$ is the interaction of the hth material and the ith laboratory, all fixed effects. The random variables—β_{hij}, γ_{hijk}, δ_{hijkm}—represent analysts, days, and determinations, respectively. The analysis

TABLE 4.—*Analysis of variance for the two-way or cross classification (laboratories fixed)*

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARES	EXPECTED MEAN SQUARES
Total	$crpst-1$		
Materials	$c-1$	M_1	$\sigma_t^2+t\sigma_s^2+st\sigma_p^2+rpst\theta_c$
Laboratories	$r-1$	M_2	$\sigma_t^2+t\sigma_s^2+st\sigma_p^2+cpst\theta_r$
Materials × laboratories	$(c-1)(r-1)$	M_3	$\sigma_t^2+t\sigma_s^2+st\sigma_p^2+pst\theta_{cr}$
Analysts within laboratories	$cr(p-1)$	M_4	$\sigma_t^2+t\sigma_s^2+st\sigma_p^2$
Days within analysts and laboratories	$crp(s-1)$	M_5	$\sigma_t^2+t\sigma_s^2$
Determinations within days, analysts, and laboratories	$crps(t-1)$	M_6	σ_t^2

of variance for this model is in Table 4, and the tests of significance available from this analysis are indicated in Table 5.

The population variance of a material mean is:

$$\sigma_{\bar{m}}^2 = \frac{\sigma_p^2}{rp} + \frac{\sigma_s^2}{rps} + \frac{\sigma_t^2}{rpst}. \tag{18}$$

The sample estimate of this variance for an individual laboratory is:

$$V_{\bar{m}} = \frac{V_p}{p} + \frac{V_s}{ps} + \frac{V_t}{pst}. \tag{19}$$

The components of variance are computed from the analysis of variance as before and confidence intervals can be established for each material mean.

Mixed Model: Laboratories Random.—The second case of the use of the mixed model applies to a situation in which collaborators are a random sample of a population of laboratories and inferences are intended to apply to the population. The model

$$Y_{hijkm} = \mu + \tau_h + \alpha_i + (\tau\alpha)_{hi} + \beta_{hij} + \gamma_{hijk} + \delta_{hijkm} \tag{20}$$

is the same as before except that α_i and $(\tau\alpha)_{hi}$ are now random variables. Since laboratories and the material by laboratory interaction are random

TABLE 5.—*Tests of significance for sources of variation indicated in Table 4*

NULL HYPOTHESIS	F RATIO	DEGREES OF FREEDOM
$\sigma_s^2=0$	M_5/M_6	$crp(s-1)$ and $crps(t-1)$
$\sigma_p^2=0$	M_4/M_5	$cr(p-1)$ and $crp(s-1)$
$\theta_{cr}=0$	M_3/M_4	$(c-1)(r-1)$ and $crp(p-1)$
$\theta_r=0$	M_2/M_4	$(r-1)$ and $cr(p-1)$
$\theta_c=0$	M_1/M_4	$(c-1)$ and $cr(p-1)$

TABLE 6.—*Analysis of variance for the two-way or cross classification (laboratories random)*

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARES	EXPECTED MEAN SQUARES
Total	$crpst-1$		
Material	$c-1$	M_1	$\sigma_t^2+t\sigma_s^2+st\sigma_p^2+pst\sigma_{cr}^2+rpst\theta_c$
Laboratories	$r-1$	M_2	$\sigma_t^2+t\sigma_s^2+st\sigma_p^2+cpst\sigma_r^2$
Materials × laboratories	$(c-1)(r-1)$	M_3	$\sigma_t^2+t\sigma_s^2+st\sigma_p^2+pst\sigma_{cr}^2$
Analysts within laboratories	$cr(p-1)$	M_4	$\sigma_t^2+t\sigma_s^2+st\sigma_p^2$
Days within analysts and laboratories	$crp(s-1)$	M_5	$\sigma_t^2+t\sigma_s^2$
Determinations within days, analysts, and laboratories	$crps(t-1)$	M_6	σ_t^2

variables, some changes must be made in the expected mean squares. These changes are presented in the analysis of variance in Table 6.

Tests of significance are essentially the same as before, except for testing the material means, in which the error term is the material by laboratory interaction ($Ho:\theta_c=0$; $F=M_1/M_3$). This test is of little importance, however, since the materials were selected because they were known to be different.

The population variance of a given material mean is:

$$\sigma_{\bar{m}}^2 = \frac{\sigma_r^2}{r}+\frac{\sigma_{cr}^2}{r}+\frac{\sigma_p^2}{rp}+\frac{\sigma_s^2}{rps}+\frac{\sigma_t^2}{rpst}. \tag{21}$$

The estimate of this variance for an individual laboratory is:

$$V_{\bar{m}} = V_r + V_{cr} + \frac{V_p}{p}+\frac{V_s}{ps}+\frac{V_t}{pst}. \tag{22}$$

The degrees of freedom associated with this variance are not known exactly and consequently the probability associated with the computed confidence interval can be only approximated.

Numerical Example.—A numerical example can be used to illustrate the two-way or cross classification scheme. Suppose the method for determining gossypol must be satisfactory for four different lots of cottonseed meal known to vary in gossypol content. The 4 different meals are sent to each of 4 collaborators. The sampling structure within laboratories is 2 analysts, 2 days per analyst, and 2 determinations per day. Then $c=r=4$ and $p=s=t=2$, making a total of 128 individual determinations for the entire test.

The analysis of variance assuming laboratories as fixed effects is presented in Table 7.

Although the F value for meals is significant ($F=0.6595/0.00184$), it

TABLE 7.—*Analysis of variance of numerical example illustrating two-way or cross classification* ($c=r=4$, $p=s=t=2$)

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARES	F
Total	127		
Meals	3	$M_1=0.6595$	
Laboratories	3	$M_2=0.0396$	29.55[a]
Meals × laboratories	9	$M_3=0.00184$	1.37
Analysts within laboratories	16	$M_4=0.00134$	0.98
Days within analysts and laboratories	32	$M_5=0.00137$	7.61[a]
Determinations within days, analysts, and laboratories	64	$M_6=0.00018$	

[a] Denotes significance at the 0.01 probability level.

is of little importance in this case, since meals of different gossypol content were deliberately used. The day-to-day variability is significantly greater than the variability between duplicate determinations. Variability between analysts and the meal by laboratory interaction are non-significant. Differences among laboratories are significant.

Components of variance determined from the analysis of variance are $V_t=0.00018$, $V_s=0.00060$, and $V_p=0$. The estimated variance of a mean, from (19), is:

$$V_{\bar{m}} = \frac{0}{2} + \frac{0.00060}{4} + \frac{0.00018}{8} \tag{23}$$

$$V_{\bar{m}} = 0.00017.$$

The standard error is 0.013 and the 95 per cent confidence interval (9 d.f.) is ± 0.030.

By using these same data for the case in which the components of variance associated with laboratories and the laboratory by meal interaction are random, V_r and V_{cr} are estimated to be 0.00120 and 0.00006, respectively. The analysis of variance and the important tests of significance remain unchanged. The interpretation, however, is now based on a population of laboratories. Because of this, the variance of a given meal mean is larger than before by the amount equal to the laboratory and laboratory-by-meal contribution. Hence, from (22),

$$V_{\bar{m}} = 0.00120 + 0.00006 + \frac{0}{2} + \frac{0.00060}{4} + \frac{0.00018}{8} \tag{24}$$

$$V_{\bar{m}} = 0.00143.$$

The standard error is 0.038 and the approximate confidence interval is ± 0.12. The confidence interval is approximate because the degrees of freedom are not exactly known. It is obvious, however, that variability among laboratories contributes most to the variance of the mean; under the broader interpretation, this necessarily widens the confidence interval.

SUMMARY

Collaborative testing is an important phase of developmental research. It is also a very difficult phase because the investigator's control is limited at many important stages. Consequently, the success of a collaborative test depends largely upon the conscientious efforts of the collaborating personnel.

Several fundamental requirements relate to the reliability of testing procedures. These requirements are concerned specifically with such factors as precision, accuracy, and scope.

Two general sampling designs, the hierarchical or nested classification and the two-way or cross classification, are illustrated by numerical examples. The statistical model which specifies the laboratories as random variables permits broader interpretations than the model which specifies the laboratories as fixed effects because inferences apply to a population of laboratories. Because of the inductive nature of these inferences with respect to a population of laboratories, the variability among the laboratories must be included in determining the precision which in turn reflects the reliability of the test.

ACKNOWLEDGMENT

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The chief source of reference was *Statistical Theory in Research,* by R. L. Anderson and T. A. Bancroft, McGraw-Hill Book Co., Inc., N. Y. (1952).

A CHEMICAL METHOD FOR THE DETERMINATION OF HISTAMINE IN CANNED TUNA FISH*

By O. S. Sager and William Horwitz (Division of Food, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.)

The presence of histamine has been shown to be associated with decomposition in fish. Geiger (1), in 1944, working with sardines, mackerel, and tuna, proposed the use of histamine content as one criterion for the relative freshness of fish. He stated: "Changes in the histamine content during the first 24 hours after catching of the fish seem to be more marked than changes in odor, appearance, the *p*H, free acids, or volatile bases." Geiger also found that precooking and canning did not change the hista-

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mine content significantly. In this work, histamine and related substances were determined by biological assay, in which an isolated segment of a guinea pig ileum was used. Fish containing more than 10 mg "histamine-like" substances per 100 g tissue were no longer regarded as "fresh."

However, Dyer and Mounsey (2), working with cod, showed that significant amounts of higher amines, like histamine, were not formed until the fish reached a stage of decomposition considerably more advanced than the stage at which the fish would be classified organoleptically as undesirable—in fact, a stage so advanced that food poisoning was possible.

Williams (3) reviewed the literature pertaining to the formation of histamine in tuna and other mackerel-like fishes and, using the Geiger bioassay technique for histamine on three experimental packs and several miscellaneous packs of tuna, showed that canned tuna containing amounts of histamine higher than 10 mg/100 g fish were class 3 fish or worse (see below) when packed (3, 4).

Hillig (5) used a similar bioassay on numerous authentic samples of skipjack, yellowfin, albacore, and bluefin tuna which he prepared and packed from fish in various stages of decomposition. His studies showed that little or no histamine is found in class 0, class 1, and class 2 fish but that appreciable amounts of histamine are found in class 3 and class 4 fish. Because of the repeated reference in this paper to these various classes of fish, the organoleptic classification system used by Hillig (5) is repeated as follows:

Class 0: No perceptible odor in flesh.

Class 1: An odor which while slightly "off" is only superficial in character, not deep-seated and not repugnant (often described as "fishy").

Class 2: A barely perceptible but deep-seated odor that is somewhat repugnant and connotes decomposition (often described as "stale").

Class 3: An odor similar to that of class 2 but having enough intensity to be readily noticeable; distinctly more repugnant than class 2 (often described as "taint").

Class 4: An odor of greater intensity, and decidedly more repugnant than class 3 (often described as "putrid").

Although the biological assay for histamine has been exhaustively studied and reported in the literature (see Code and McIntire for review (6)) and satisfactory results have been reported, it has been found that for the routine accurate determination of histamine in fish, the biological assay method has several disadvantages. Guinea pigs (as well as skilled technicians to dissect them) are not available in many laboratories; not all guinea pigs yield ileum segments suitable for use; nonreproducible response to a fixed standard or sample may occur not only because of random variations in the activity of the ileum segment but also because of variations in the manner of adding the solutions to the test chamber. To avoid variations in technique, a carefully designed apparatus of good

quality must be used, the samples must be added with extreme care, and for accurate results a statistical pattern of some sort should be followed, such as using numerous replicates and several dilutions of the same sample. Thus, although the biological method is excellent for qualitative work and rapid sorting, an accurate chemical method is needed for the determination of histamine in fish.

Basically, the method developed is an adaptation and modification of a method for histamine previously developed by chemists of the Food and Drug Administration (7) for the determination of histamine in streptomycin.

DISCUSSION OF THE METHOD

The chemical method for histamine in tuna fish, described in detail below, may be divided into five parts: (1) extraction of histamine from fish; (2) purification of the extracted histamine by adsorption on, and elution from, an ion-exchange column; (3) coupling of the purified histamine with a diazonium compound; (4) purification of the azo dye formed; and (5) photometric reading of the color formed.

(1) *Extraction of Histamine.*—Alcohols are excellent solvents for histamine. In this method, histamine is extracted from the coarsely ground tuna fish sample by grinding with methyl alcohol in a Waring Blendor. The vigorous cutting action assures complete contact of the solvent with the sample and extraction of the histamine. The methyl alcohol precipitates proteins, limits solution of inorganic ions which might later be selectively adsorbed on the cotton acid succinate (CAS) ion exchange column and thus interfere in the purification, allows very rapid gravity filtration of the extract, and yields a clear filtrate.

(2) *Purification of Histamine.*—An important advance in improving earlier methods for histamine was made by Kantor, Levine, and Fischbach (7) when they developed a method for determining histamine in streptomycin. In previous methods in which histamine was extracted with butanol from strongly alkaline solution, efficient quantitative extractions were difficult to obtain because the extraction coefficient was relatively poor. In addition, these workers showed that, in the alkaline solution, histamine may react with aldehyde in the solution to yield a nonextractable Schiff base. To circumvent this loss they added benzaldehyde, which reacts with histamine at such a rapid rate as to exclude the reaction between histamine and nonaromatic aldehydes in the original solution. The resultant benzylidene histamine is readily extracted by a benzene-butanol mixture. Use of this solvent rather than butanol alone prevents the carryover of water-soluble inorganic ions that might interfere with the adsorption of histamine on the CAS column (7).

When the benzene-butanol phase is passed over the CAS, the Schiff base, stable only in the highly alkaline conditions under which it was

formed, is decomposed by the acid CAS, the histamine is adsorbed on the carboxyl groups on the column, and the benzaldehyde passes through and is discarded. Cotton acid succinate was prepared by McIntire, *et al.* (8) specifically to purify the histamine extracts. After washing, histamine is displaced from the column with dilute sulfuric acid which simultaneously regenerates the acid form of the CAS, in which form it is ready for re-use. With care, the column can be re-used in this manner indefinitely.

(3) *Coupling of Histamine.*—The superiority of diazotized *p*-nitroaniline as a substitute for sulfanilic acid in the Pauly reaction was first shown by Gebauer-Fulnegg (9). In attempting to obtain a standard curve for this reaction on a series of histamine standards in this laboratory, we found that duplicate determinations would not give uniformly reproducible values and that the curves obtained did not closely follow Beer's law, especially at the higher concentrations. The cause of this variation in the results was traced to the use of 20 per cent sodium carbonate solution to neutralize the sulfuric acid in the eluate, so that it was difficult to obtain uniform *p*H in the test.

The *p*H conditions greatly affected the amount and type of color produced. Because of the strong alkalinity and high density of the sodium carbonate solution, uniform neutralization could not be produced in the reaction tube as the sodium carbonate solution was added, and the type of coupling which occurred immediately was different from that occurring at a later instant as the *p*H changed and as the rate of adding sodium carbonate solution was changed.

To eliminate this difficulty, the neutralization procedure was modified as follows: A buffer solution of sodium carbonate and sodium metaborate was first added to produce only partial neutralization; then solid borax, in excess of the amount needed to saturate, was added in one portion and the solution was immediately mixed to saturation. The combination of these three compounds yielded a strongly buffered solution which prevented localized areas of high alkalinity and uniformly yielded a *p*H of about 8.6. The need for constant *p*H conditions in this reaction to produce a standard curve following Beer's law was also noted by Schmidt and Gruhn (10), who used a boric acid-sodium carbonate buffer at *p*H 11 to produce a *p*H of 9.8 in their test solution.

Figure 1 shows typical standard curves obtained with the method. The figure shows the curve obtained on aqueous standards, **A**, on standards passed through CAS, **C**, and on standards obtained by adding untreated standard to "good" fish-methanol filtrate containing no histamine and then passing the filtrate through CAS, **B**. The figure illustrates: (1) the adherence to Beer's law, (2) the loss of a slight amount of histamine in the purification process, and (3) the complete recovery of histamine added to fish filtrate.

(4) *Purification of the Histamine-Azo Dye.*—The method outlined by

Rosenthal and Tabor (11) is used. The red azo dye formed is extracted with methyl isobutyl ketone which not only leaves many impurities behind in the alkaline aqueous phase but also stabilizes the color. The ketone extract is then washed with a barbital buffer at *p*H 7.7, to convert the extracted interfering red colors to yellow. For example, the red color formed by ammonia is converted to yellow by this process. The red color due to histamine is merely changed to another shade of red.

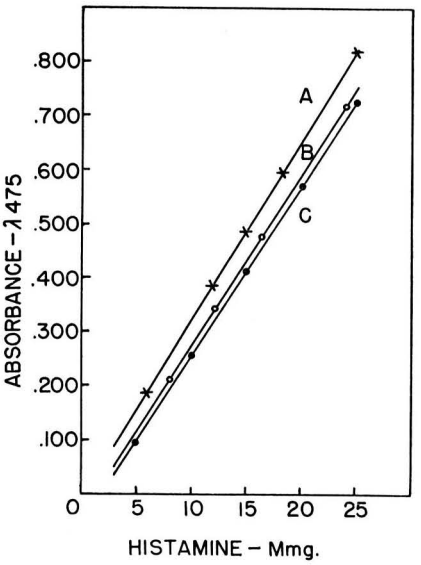

FIG. 1.—Comparison of histamine standard curves. **A**: Standards in aqueous solution. **B**: Standards in methyl alcohol fish filtrate passed through CAS. **C**: Standards in methyl alcohol solution passed through CAS.

(5) *Photometric Reading.*—Figure 2 presents spectrophotometric curves which illustrate how the *p*H affects the histamine coupling. The azo compound formed by coupling at *p*H 8, obtained with saturated bicarbonate, does not yield a color which has the wide absorbance spread between sample and blank that occurs when the coupling is at a higher *p*H. Thus, a low *p*H results in a high blank and low sensitivity. The color obtained with sodium metaborate is theoretically the best because of a low blank. However, it was difficult to obtain uniform lots of sodium metaborate, and different lots of sodium metaborate yielded different *p*Hs in the test upon saturation. The *p*H of 8.6 obtained in the test upon saturation with borax is easy to reproduce, and borax was therefore used. The color is read at 475 mμ, the approximate inflection point of the curve.

The tuna fish samples used in these experiments were frozen portions of authentic canned samples of skipjack, yellowfin, albacore, and bluefin tuna, prepared and packed by Hillig from fish in progressive stages of decomposition. Hillig previously analyzed these samples for histamine by the biological assay method. A description of the fish, the method of packing, and the results of his analyses have already been reported (5).

The biological assay methods used in these experiments were the basic method described by Williams (4) and its modifications, as discussed below.

The chemical method for histamine in canned tuna is as follows:

METHOD FOR HISTAMINE IN FISH

(Use H_2O redistd from glass for prepn of reagents and for detns. Do not clean glassware with soap; use fresh chromic acid cleaning soln, rinsing well with tap H_2O, then 3 times with distd H_2O, and 3 times with redistd H_2O. Alcohol may be used to soak or rinse glassware.)

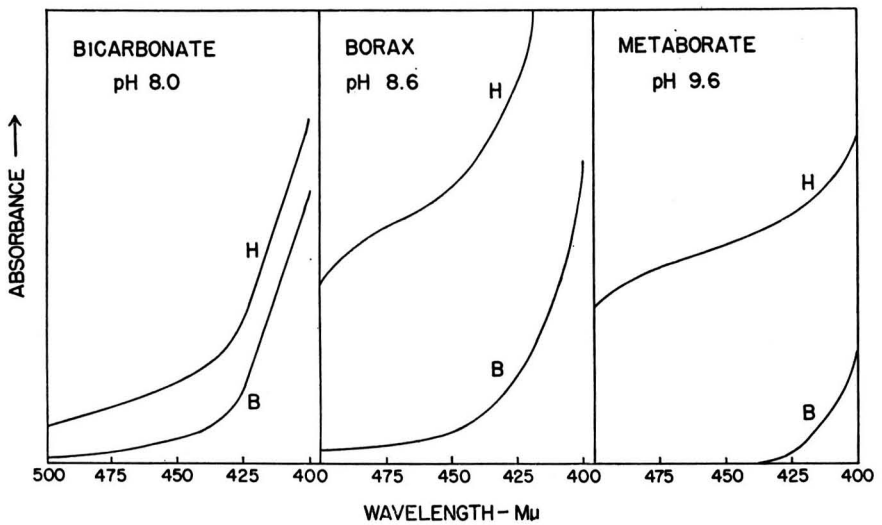

FIG. 2.—Effect of *p*H on the color produced in the histamine-diazonium coupling. **H**: histamine sample; **B**: blank.

REAGENTS

(a) *Benzene-n-butanol mixture.*—(3+2) v/v.

(b) *Cotton acid succinate.*—Dissolve 5 g anhyd. Na acetate, fused just before use, and 40 g succinic anhydride in 300 ml acetic acid in 500 ml erlenmeyer. Immerse 10 g absorbent cotton, cut into strips, in soln; attach drying tube contg drying agent, and heat 48 hr at 100°. (Flask may be immersed to neck in active steam bath.) Filter; wash well with H_2O, HCl (1+9), H_2O, and finally with alcohol. Dry in vacuum oven at 100°.

(c) *Diazonium reagent.*—Dissolve 0.1 g *p*-nitroaniline, recrystd from hot H_2O, and dil. to 100 ml with 0.1*N* HCl. Store in refrigerator. Dissolve 4 g $NaNO_2$ in H_2O and dil. to 100 ml. Store in refrigerator. Just before use place 10 ml of the *p*-nitroaniline soln in ice bath 5 min., add 1 ml of the $NaNO_2$ soln, mix, and let stand in bath at least 5 min. before use.

(d) *Coupling buffer.*—Dissolve 7.15 g Na metaborate ($NaBO_2$) and 5.7 g Na_2CO_3 in H_2O, and dil. to 100 ml. Store in polyethylene bottle.

(e) *Barbital buffer.*—Dissolve 10 g Na barbital in 1 l H_2O and adjust to *p*H 7.7 with acetic acid (1+15) (ca 25–30 ml), using *p*H meter. Store in refrigerator to prevent mold growth. Dissolve any ppt by warming before use. (50–250 ml bottle of the buffer may be kept at room temp. and replenished from main supply when mold growth is apparent.)

(f) *Histamine standard solns.*—Dry histamine dihydrochloride (U.S.P. Reference Standard or material checked against Standard as under DETERMINATION) over H_2SO_4 2 hr. Dissolve 0.1656 g dried histamine dihydrochloride in H_2O and dil. to 100 ml (1 ml = 1 mg histamine). Dil. 10 ml of this stock soln to 100 ml with H_2O (1 ml = 100 mmg histamine). Dil. 5 ml of this dil. std soln and 5 ml MeOH to 100 ml with H_2O (1 ml = 5 mmg histamine). Store in cold. Prep. fresh stds weekly.

(g) *4-Methyl-2-pentanone (methyl isobutyl ketone).*—Commercial purified grade (Eastman No. 416 has been found satisfactory). Used waste ketone may be recovered by washing once with satd $NaHCO_3$ soln and 3 times with H_2O, distg, retaining fraction boiling at 115–118°, and checking absorbance of product at 475 mμ.

(h) *Benzaldehyde.*—Cl-free.

(i) *Dilute sulfuric acid.*—$0.40 \pm 0.02N$, accurately stdzd.

PREPARATION OF SAMPLE

Canned fish.—Comminute sample (include entire contents of canned products) by passing 3 times thru food chopper, mixing after each grinding. Freeze ground sample for storage, if desired.

PREPARATION OF CAS COLUMN

Prep. column by firmly placing small plug of cotton acid succinate (CAS) (ca 50 mg) in column prepared by cutting off or blowing out bottom of 15 ml centrifuge tube. Wash plug with three 15 ml portions H_2O and two 3 ml portions alcohol. Allow solvents to drip thru CAS, blowing out last portion of each solvent, using 10 ml syringe with needle inserted thru rubber stopper. CAS plugs may be re-used for months by washing shortly after use with H_2O and alcohol as above, and protecting from dust with inverted beaker.

DETERMINATION

Transfer 10 g sample to semimicro container of high-speed blender, add ca 50 ml MeOH, and blend ca 2 min. Transfer to 100 ml glass-stoppered vol. flask, rinsing lid and blender jar with MeOH and adding rinsings to flask. Heat in H_2O bath to 60° and allow to stand at this temp. 15 min. Cool to 25°, dil. to vol. with MeOH, and filter thru folded paper. Alcohol filtrate may be stored in refrigerator several weeks (light powdery ppt separating on storage may be ignored). Dil. 5 ml of the filtrate to 100 ml with H_2O (disregard turbidity). Pipet 5 ml aliquot into 150×16 mm glass-stoppered test tube, and add 1 drop benzaldehyde (Cl-free) and 0.2 ml 20% NaOH. (*p*H after adding alkali should be ca 12.4–12.5.) Shake vigorously ca 25 times. Let stand 2 min. and add 5 ml of the benzene-butanol mixt. Shake vigorously ca 25 times and let stand 5 min. to sep. If emulsion forms, centrifuge. Transfer upper layer with fine-tip tube equipped with rubber bulb to previously prepd CAS column, avoiding transfer of any aq. phase. Re-ext. aq. soln with 5 ml of the benzene-butanol mixt. as before, shaking, letting stand 5 min., and transferring upper layer to column. Rinse lip and sides of column with fine stream of alcohol from wash bottle, blowing out excess solvent as before. Wash column with 3 ml alcohol; blow out; wash with two 3 ml portions H_2O, and blow out. Discard solvents and washings. Elute histamine from CAS into 25 ml glass-stoppered erlenmeyer by washing down sides of tube with 2.0 ml $0.40 \pm 0.02N$ H_2SO_4 (vol. and concn of acid are critical) followed by 3 ml H_2O. Blow out with syringe after dripping ceases.

Cool eluate in ice bath, weighting flask with lead ring or clamp to prevent tipping, and let stand 5–10 min. Add 0.5 ml cooled diazonium reagent and let stand 5 min. in ice bath. Add 0.50 ml coupling buffer (vol. is critical; Ostwald pipet is convenient) with continuous shaking or swirling to avoid localized alkalinity (*p*H after addn of coupling buffer, 5–6). Let stand 5 min. in ice bath. Sat. soln with ca 0.25 g

powd. $Na_2B_4O_7.10H_2O$ added in one portion. Shake soln immediately and continuously ca 30 sec. to insure rapid and complete satn (final *p*H ca 8.6). Let stand in ice bath 15 min. Pipet in 5.0 ml methyl isobutyl ketone and shake vigorously 25 times. Immediately transfer both layers to 150×16 mm test tube (do not rinse) and let stand 10 min. at room temp. to sep. and to warm up. Transfer upper layer with fine-tip dropper to second 150×16 mm glass-stoppered test tube contg 5.0 ml of the barbital buffer. Avoid transferring aq. and solid phases (if present) (transfer need not be quant.). Shake vigorously ca 25 times (*p*H of barbital buffer after washing, ca 8.3–8.4). Let stand 10 min. to sep. Transfer upper layer with fine-tip dropper to 1 cm cell and det. absorbance at 475 mμ against methyl isobutyl ketone. Repeat detn on samples yielding absorbance values higher than 25 mmg std by dilg 1 ml MeOH filtrate to 100 ml with H_2O. Alternatively, aq. diln may be dild 1+4 (or more) with H_2O.

Conduct std and blank thru detn as follows: Pipet 5 ml of the 5 mmg/ml histamine std soln into 150×16 mm glass-stoppered test tube and pipet 5 ml 5% MeOH into similar tube for blank. Proceed as in detn, beginning, par. 1, line 8, " . . . add 1 drop benzaldehyde. . . . "

Subtract blank absorbance from absorbance of std and of sample and calc. histamine in sample aliquot as follows:

Mmg histamine = absorbance sample (corr.) ×25/absorbance std (corr.).

RESULTS

Table 1 shows the results obtained with the chemical method for histamine on 54 samples of canned tuna. These values are compared with those previously obtained by Hillig on the same samples with the bioassay.

The values obtained with the bioassay were usually considerably higher than those by the chemical method. It was first thought that the hydrochloric acid extract of the bioassay method was extracting from the fish some stimulating material, other than histamine, which was either not extracted by methanol in the chemical method or which was removed during purification of the filtrate with cotton acid succinate (CAS).

However, when sulfuric acid eluates from the CAS were tested by both the chemical and bioassay methods, similar disparity in results was obtained; thus the discrepancy in the previous results was probably not caused entirely by impurities of this type in the bioassay extracts. Moreover, as described below, the manner of neutralizing the hydrochloric acid extract in the bioassay often caused the results to be nonreproducible and higher than those by the chemical method. Because of the very strong alkalinity of the sodium carbonate neutralizer specified in the bioassay, it was difficult to neutralize the hydrochloric acid extract by "titration" with solid sodium carbonate so as to yield uniformly reproducible physicochemical conditions such as *p*H, sodium ion concentration, carbonate ion concentration, salt concentration, etc. As these conditions varied, response of the ileum segment varied.

In addition, in tests in which too much sodium carbonate was added, the resulting high alkalinity caused "false" stimulation of the ileum segment and results were higher than those actually due to histamine alone. To

TABLE 1.—*Comparison of results on canned tuna fish by the chemical method for histamine with those by the biological method, unmodified (all values are histamine as base, mg/100 g fish)*

FISH NO.	CLASS	BIOLOGICAL, UNMODIFIED	CHEMICAL	FISH NO.	CLASS	BIOLOGICAL, UNMODIFIED	CHEMICAL
55	0	0	2	2	3	70	29
				3	3	45	17
12	1	1	0	4	3	70	21
20	1	2	0	5	3	2	2
26	1	1	1	7	3	54	38
31	1	1	0	8	3	36	10
32	1	18	14	9	3	90	51
32[a]	1	14	22	10	3	45	12
32[a]	1	14	9	11	3	54	22
36	1	0	0	14	3	510	260
41	1	2	2	18	3	90	54
46	1	0	0	19	3	90	25
				25	3	110	111
				28	3	108	111
13	2	1	0	29	3	108	71
21	2	1	3	43	3	72	28
24	2	1	8	49	3	7	12
27	2	7	6	53	3	27	15
33	2	5	4	65	3	54	18
37	2	0	2	66	3	72	19
42	2	0	7	67	3	72	11
48	2	2	2				
				1	4	3	1
				6	4	1080	428
16	2–3[b]	10	3	15	4	580	504
17	2–3	20	32	22	4	72	69
52	2–3	9	2	35	4	108	126
58	2–3	22	22	39	4	144	126
				40	4	1010	448
				44	4	108	66
				56	4	2	2
				59	4	3	0

[a] Repeat determinations on separate cans from the same fish.
[b] Part of the fish was Class 2 and part Class 3.

eliminate this difficulty, the neutralization procedure was modified in the following manner: One ml of the hydrochloric acid extract was partially neutralized with 2 ml of a 1 per cent aqueous sodium bicarbonate solution and the solution was further neutralized by diluting to 10 ml with Ringer-Locke solution (without dextrose). The final uniform *p*H was about 7.5.

In Table 2, results obtained by the chemical method are compared with the results previously obtained by the bioassay method and those by the modified bioassay method on 28 additional samples of canned tuna. The

TABLE 2.—*Comparison of results obtained on canned tuna fish by the chemical method for histamine with results by the biological method and the modified biological method (all values are histamine as base, mg/100 g fish)*

FISH NO.	CLASS	BIOLOGICAL METHODS			CHEMICAL METHOD
		UNMODIFED	METHANOL RESIDUE	RINGER-LOCKE DILUTION	
60	2	0	0	—	0
69	2	18	4	8	2
73	2	2	0	0	0
51	2–3	25	10	—	12
61	2–3	1	0	—	0
23	3	72	10	—	17
34	3	72	40	—	46
38	3	144	100	—	108
50	3	54	20	—	24
54	3	72	40	—	38
57	3	90	60	—	58
62	3	72	60	—	62
68	3	54	10	20	11
71	3	684	210	220	352
72	3	36	2	8	3
74	3	3	2	2	2
75	3	36	8	16	10
76	3	216	120	133	131
77	3	684	300	—	352
78	3	45	16	20	15
79	3	396	260	260	260
30	4	72	50	—	47
63	4	576	600	—	608
64	4	1440	725	—	736
70	4	4	1	6	0
80	4	575	380	380	384
81	4	648	225	200	192
82	4	685	480	480	476

data show that results by the modified bioassay method check well with those by the chemical method.

Table 2 also shows results obtained with the bioassay method modified by preparing methanol extracts of the fish as in the chemical method rather than as hydrochloric acid extracts. The methanol was then evaporated from an aliquot of the extract on the steam bath; the residue was dissolved and brought to volume in Ringer-Locke solution (without dextrose) and used in the bioassay. The data show that results obtained with the bioassay method using methanol extracts also check well with results by the chemical method.

TABLE 3.—*Summary of all results by the chemical method for histamine presented in Tables 1 and 2 (all values are histamine as base, mg/100 g fish)*

	NO. OF SAMPLES TESTED	MIN. VALUE	MAX. VALUE	AV. VALUE
Class 0	1	2	2	2
Class 1	10	0	22	5
Class 2	11	0	8	3
Class 2–3	6	0	32	12
Class 3	37	2	352	66
Class 4	17	0	736	248

Table 3 summarizes all the results on the total of 82 samples of canned tuna presented in Tables 1 and 2. The data show that only small amounts of histamine were found in samples of class 0, class 1, and class 2 fish but that considerable amounts of histamine were found in class 3 and class 4 fish. The wide variations between minimum and maximum values are understandable: not only are bacterial populations different in different fish, but also the conditions of decomposition, and thus the paths of decomposition, are not always the same.

The continuity of the upward trend in the average values is slightly marred by a value of 5 mg for class 1. This high value was caused by one fish, 3 cans of which yielded values from 9 to 22 mg. All other samples of class 1 fish yielded values below 2 mg. Since there was no explanation for this anomaly, the values for this fish were assumed to be correct and were included in the average.

Table 4 presents the results obtained independently on 14 samples

TABLE 4.—*Comparison of results by the chemical method for histamine on 14 samples of canned tuna fish by 2 different analysts (all values are histamine as base, mg/100 g fish)*

FISH NO.	ANALYST 1	ANALYST 2
8	10	11
10	12	13
24	8	7
25	111	114
31	0	0
32	14	13
33	4	2
34	46	47
35	126	126
36	0	0
37	2	1
38	108	99
39	126	136
40	448	468

TABLE 5.—*Comparison of results on canned tuna fish with the chemical and biological methods for histamine by 6 collaborating laboratories (all values are histamine as base, mg/100 g fish)*

LABORATORY	SAMPLE CY2	SAMPLE CY3	50-50 MIXTURE CY2 AND CY3
		Chemical Method	
1	0	131	—
2	1.2, 1.2	150, 152	74, 82
3	1.0, 1.2	124, 121, 133, 137	66, 68, 66, 68
4	1.0, 2.6, 0.2, 0.7	121, 117	54, 59
5	0, 0	135, 136	65, 61
		Biological Method	
3		163, 163	72, 72
6[a]	0.9	120	
	0.5, 0.5	126, 115, 125, 123, 123, 129	
		126, 128, 124, 125	

[a] The results on different lines represent determinations conducted on different days.

of canned tuna fish by two different analysts in the same laboratory. The data indicate the reproducibility of the chemical method.

Table 5 presents a comparison of results obtained with the chemical method for histamine by 5 collaborating laboratories on 2 different samples of canned tuna and a 50-50 mixture of the two samples. Sample CY2 was a class 2 fish and Sample CY3 was class 3. The data show that the values obtained by the different laboratories checked well with each other. The close check between the values obtained on the 50-50 mixture and the values calculated from the results obtained on the 2 individual samples further indicates the quantitative nature of the chemical method.

SUMMARY

In an effort to avoid some of the difficulties of the bioassay method for histamine in which a segment of guinea pig ileum is used, a chemical method for histamine was developed which consists of 5 major steps: (1) histamine is extracted from ground fish in a Waring Blendor with methanol; (2) histamine is purified by the formation of a Schiff base with benzaldehyde and by benzene-butanol extraction of the base with subsequent passage through an ion-exchanger (cotton acid succinate); (3) the eluted, purified histamine is coupled with diazotized *p*-nitroaniline at a carefully controlled *p*H of 8.6 to yield an azo-dye; (4) the azo-dye is purified and stabilized by extraction with methyl isobutyl ketone and washing with a barbital buffer; and (5) the color is read spectrophotometrically.

Results obtained with the new method on 82 samples of canned tuna

fish indicated that fish classified organoleptically in classes 0, 1, and 2 yielded little or no histamine, but that fish in classes 3 and 4 tended to yield considerable, but widely varying, amounts of histamine (0–736 mg/100 g fish).

Discrepancies between the biological and chemical methods for histamine could be eliminated by modifying the bioassay method so that the acid extracts were neutralized with sodium bicarbonate and Ringer-Locke solution rather than with solid sodium carbonate. When the extracts were neutralized in this manner, the *p*H was uniformly about 7.5; thus alkaline stimulation of the guinea pig ileum was avoided, and results checked well with those obtained by the chemical method.

Collaborative results indicated that the method is reproducible.

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MODIFIED SLIDE PREPARATION FOR THE OFFICIAL HOWARD MOLD COUNT METHOD*

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Experience in performing the official Howard mold count method, **35.58** (*Official Methods of Analysis*, 8th Ed., 1955, pp. 781–782), and in instructing analysts in its use has shown that preparing the slide mount still presents some difficulties, such as uneven distribution of the insoluble solids and entrapment of air bubbles between the central disk and cover glass. To overcome these difficulties, the slide preparation has been modified by substituting careful manipulation of the cover glass for the official pre-spreading technique.

The modification has these advantages: (1) distribution of the insoluble material is consistently uniform; (2) air entrapment is virtually eliminated; (3) the mount can be prepared faster and more easily; and (4) scratching of the glass of the central disk is reduced. Those who have used this modified technique agree that it produces no significant difference in the random distribution of mold filaments, other than the normal variation inherent in sampling a product that is a mixture. Table 1 and Figure 1 show a limited comparison of recent data to support this opinion.

Because the Howard mold count method is empirical, it must be used exactly as stated to obtain accurate results. Although the method has been revised several times since its development in 1910, its fundamental principle has remained unchanged.

The present form of the official method contains several improvements not in the original method (1). These revisions (2–6) have helped to standardize the technique of mold counting and have made it more uniform. We feel that the proposed modification serves this same purpose.

This modification of the preparation of the mold count slide was first used in 1945 by Mrs. Gertrude Kissell, of the National Canners Association Research Laboratory. Analysts who have performed it since that time have used two techniques, both of which eliminate spreading the sample portion over the surface of the central disk with the knife blade or scalpel, as in the official method. However, both techniques retain and utilize the spreading, distributing action that results from lowering the cover glass into place. The two techniques are as follows:

A. INCLINED COVER GLASS TECHNIQUE

Using a spatulate instrument, take a portion of a well-mixed sample and transfer to an area on the central disk half-way between the center of the disk and the far

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TABLE 1.—*Comparison of results of Howard mold counts by official and modified methods for preparing slides*

SAMPLE NUMBER	TOTAL FIELDS COUNTED/ METHOD	OFFICIAL METHOD[a]					MODIFIED METHOD[b]				
		POSITIVE FIELDS/SLIDE				% MOLD COUNT	POSITIVE FIELDS/SLIDE				% MOLD COUNT
		NO. 1	NO. 2	NO. 3	NO. 4		NO. 1	NO. 2	NO. 3	NO. 4	
Tomato Juice											
1	50	2	0			4	1	4			10
2	50	0	2			4	2	1			6
3	50	1	3			8	1	2			6
4	50	1	4			10	2	2			8
5	50	6	3			18	6	4			20
6	50	5	7			24	1	7			16
7	50	3	2			10	5	2			14
8	50	2	1			6	3	2			10
9	50	1	1			4	1	0			2
10	50	0	1			2	2	2			8
11	50	4	2			12	4	3			14
12	50	5	3			16	0	2			4
13	50	13	6			38	14	14			56
14	50	4	3			14	6	7			26
15	50	6	3			18	3	2			10
16	50	3	8			22	2	4			12
17	50	2	3			10	2	2			8
18	50	5	6			22	2	3			10
19	50	6	5			22	5	4			18
20	50	3	6			18	3	6			18
21	50	4	6			20	1	7			16
22	50	0	1			2	3	0			6
23	50	4	1			10	3	4			14
24	50	1	1			4	1	0			2
25	50	3	0			6	2	1			6
26	100	10	9	8	11	38	8	10	11	9	38
27	100	3	3	2	3	11	5	1	2	2	10
28	100	4	1	1	3	9	2	2	2	4	10
29	100	1	4	2	2	9	3	4	4	2	13
30	100	0	1	1	0	2	0	1	0	0	1
31	100	0	1	0	0	1	0	0	0	0	0
32	100	1	0	3	2	6	3	0	3	1	7
Catsup											
33	50	8	8			32	7	8			30
34	50	14	14			56	15	15			60
35	100	11	7	9	8	35	8	8	9	9	34
36	100	11	8	9	11	39	12	9	10	11	42
37	100	12	10	9	13	44	12	13	10	12	47
38	100	11	9	10	12	42	13	9	13	11	46

[a] Pre-spreading with knife blade or scalpel.
[b] Spreading by cover glass manipulation.

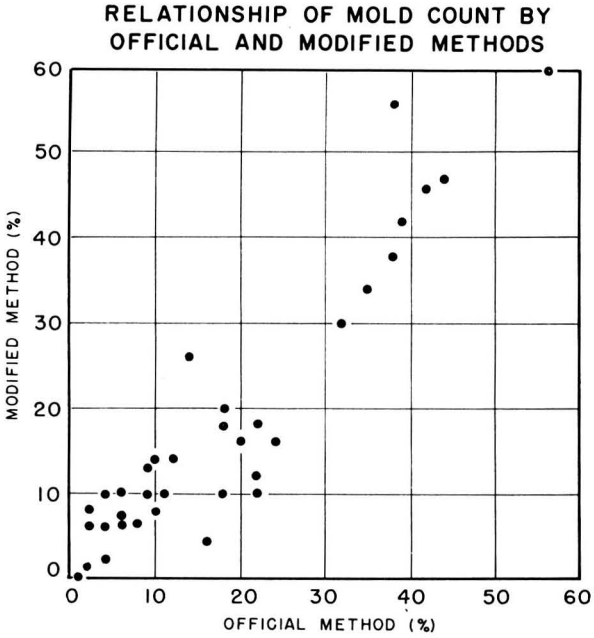

FIG. 1.—Relationship of mold count by official and modified methods.

edge. (A dissecting needle may be used to remove the sample portion from the spatula to the central disk.) Rest one edge of the cover glass in a slanting position on the edges of the slide shoulders nearest the portion of test material. Lower the cover glass slightly until it almost touches the test material on the disk; then lower it into place rapidly, but gently, so that the material spreads evenly over the entire surface of the disk.

B. PARALLEL COVER GLASS TECHNIQUE

Using the transfer method described in technique A, place the sample portion on the approximate center of the central disk. Hold the cover glass parallel to the surface of the central disk and lower it slowly until it just touches the sample portion. While maintaining contact with the test sample, alternately lower and raise the cover glass very slightly two or three times; then without stopping, lower it rapidly but gently until it just touches the shoulders of the slide, so that the test portion spreads evenly over the entire surface of the disk.

DISCUSSION

In both techniques, as in the official method, the cover glass should not be lowered too rapidly or part of the sample may splash over onto one or both shoulders, thus ruining the mount. Neither should it be lowered too slowly, or the insoluble material will not spread uniformly.

With a little practice, this step can soon be controlled so that slides showing evenly distributed insoluble material can be consistently prepared. Although even trained counters have difficulty in preparing slides

by the official (pre-spreading) method, experience shows that the modified techniques are learned quickly and less difficulty is encountered thereafter in slide preparation.

As discussed in the official method, any mount showing uneven distribution of insoluble material, absence of Newton's rings, or liquid that has been drawn or splashed across the moat onto the shoulders should be discarded. No slide should be counted unless it is properly prepared.

The authors have had considerable difficulty with the modified method, as well as with the official method, when 33×28 mm cover glasses[1] are used on the Howard slides constructed with a round central disk. Therefore, square cover glasses (33×33 mm) are recommended for use in the modified method.

If the Association approves, the authors recommend that this modification be incorporated in the official Howard mold count method, **35.58,** as an alternate technique. Only the second paragraph of the present method need be changed. The suggested change in wording is as follows:

Clean the Howard cell, **35.1(j)(1)**, so that Newton's rings are produced between slide and cover glass. With spatulate instrument place portion of well-mixed sample upon central disk; prepare mount, using only sufficient sample to bring material to edge of disk, so that insoluble solids are uniformly distributed in sample on disk. Mount sample by: (a) using spatulate instrument to spread sample evenly over disk and covering with glass so as to give uniform distribution or (*b*) lowering cover glass rapidly but gently over sample on disk to give uniform distribution. (It is of utmost importance that portion be taken from thoroly mixed sample and distributed evenly over slide disk, otherwise insoluble material and consequently molds may be unevenly distributed.) Discard any mount showing uneven distribution or absence of Newton's rings, or liquid that has been drawn across moat and between cover glass and shoulder.

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[1] Designed for use with counting chamber having a rectangular central mounting area.

SEPARATION AND IDENTIFICATION OF FOUR ANTIOXIDANTS, BUTYLATED HYDROXYANISOLE, BUTYLATED HYDROXYTOLUENE, N-PROPYL GALLATE, AND NORDIHYDROGUAIARETIC ACID, BY PAPER CHROMATOGRAPHY*

By LLOYD C. MITCHELL (Division of Food, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D. C.)

BHA[1] constitutes about 55 per cent of the total antioxidants used in foods today, BHT[2] about 25 per cent, and PG[3] about 20 per cent (1). Only a limited amount of NDGA[4] is used because of its poor oil solubility, its low heat stability, and its cost. Generally, two or more of the antioxidants are used together since they are synergistic in their action (2). This paper describes a procedure for the identification of any combination of these four antioxidants.

METHOD

REAGENTS

(a) *Immobile solvent.*—Dilute 10 ml refined soybean oil to 100 ml with ethyl ether, A.C.S.

(b) *Mobile solvents.*—(1) Dilute 20 ml H_2O to 100 ml with methanol, A.C.S. (2) Water.

(c) *Chromogenic agent.*—(1) Dissolve 1 g phosphomolybdic acid in 95% ethanol and dilute to 200 ml with ethanol. (2) Ammonium hydroxide.

(d) *Standards.*—Dissolve 100 mg BHA, 1 g BHT, 100 mg NDGA, and 100 mg PG, as separate solutions, and a mixture of all four, respectively, in ethyl acetate and dilute to 10 ml with ethyl acetate. For sensitivity tests, dilute 1 ml of each standard to 10 ml with ethyl acetate. Store the standards in glass-stoppered containers.

(e) *Paper.*—Whatman No. 1 filter paper, 8×8″ sheets.

PROCEDURE (TWO-DIMENSIONAL)

With a hard pencil, rule a starting line 1″ from the bottom edge of 4 papers and a similar line 1″ from left edge. Dot lower line 1″ from either side edge and the left line 1″ from top edge of paper; designate the lower left hand dot as "A," the lower right hand dot as "B," and the upper dot as "C" (for application of substances to be chromatographed, Fig. 1). Mark the sheet number, date, and other identification data near the top edge and clip an $8\frac{7}{8}$″ glass rod to the top edge of each paper.

With a capillary pipet, apply 0.001 ml portions of the mixed standards on the dots designated A, and apply also 0.001 ml portions of BHA, BHT, NDGA, and PG, respectively, on the dot designated B (only one of the individual standards per sheet). Invert papers and clip bottom edges to a glass rod in the hood. (Handle papers in pairs. Do not remove glass rod from the top edge of the paper.) Impreg-

* Received at the Association of Official Agricultural Chemists on February 1, 1957.

[1] BHA (butylated hydroxyanisole) is 2-*tert.*-butyl-4-hydroxyanisole.

[2] BHT (butylated hydroxytoluene) is 2,6-di-*tert.*-butyl-*p*-cresol.

[3] PG is *n*-propyl gallate.

[4] NDGA(nordihydroguaiaretic acid) is 4,4′(2,3-dimethyltetramethylene) dipyrocatechol.

Purified samples of these four antioxidants were supplied through the courtesy of the Eastman Chemical Products, Inc., subsidiary of Eastman Kodak Company, Kingsport, Tenn.

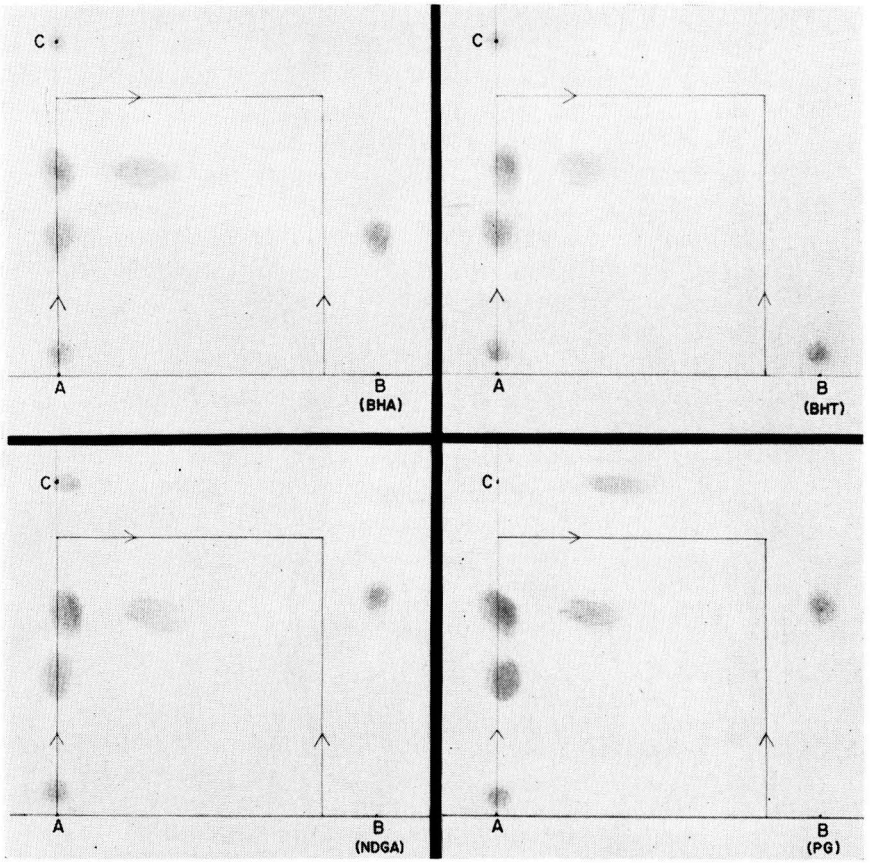

FIG. 1 illustrates the procedure used to separate four antioxidants by two-dimensional chromatography and to identify them by one-dimensional chromatography. For the first run, the unknowns applied at A and the designated known applied at B were developed until the solvent front reached the line above and parallel to the starting line A–B; then the paper was air dried. For the second run, the designated known was then applied at C and the paper was again developed until the solvent front reached the line above and parallel to the second starting line C–A. The identity of the spot (antioxidant) that lies at the intersection of imaginary lines drawn parallel to the respective starting lines from which B and C migrated is the same antioxidant as that applied at B and C.

Solvents.—Immobile: Dilute 10 ml refined soybean oil to 100 ml with ethyl ether, A.C.S. *Mobile, first run:* Dilute 20 ml water to 100 ml with methanol, A.C.S.; *second run:* Water.

Chromogenic agent.—(1) Dissolve 1 g phosphomolybdic acid in 95 per cent ethanol and dilute to 200 ml with ethanol. (2) Ammonium hydroxide.

Temperature: 23°.

nate each paper by spraying uniformly with the immobile solvent, (**a**), with a back-and-forth movement, covering the entire paper.

Add 50 ml mobile solvent, (**b**)(1), to each trough in tank; remove sprayed papers from rod in hood and suspend them in the troughs in tanks. Cover tanks and seal with cellophane tape. Let stand until mobile solvent front approaches to within 2″ (no higher) of top edge of paper. (Fig. 1 shows chromatograms ruled with a 5″ square as a guide for the upper limit of the solvent fronts.)

Break seal, lift cover, and remove one chromatogram. Restore cover, mark solvent front (if necessary) with soft pencil, and transfer paper from glass rod used in tank to glass rod in hood. Repeat the operation for the second paper, and let papers hang until dry. While the papers are drying, remove mobile solvent from troughs; cleanse and dry them.

Attach an $8\frac{7}{8}$″ glass rod to right hand edge of dried papers upon removal from rod in hood. To the dots designated as C, apply, with capillary pipet, 0.001 ml portions of the same standard solutions, respectively, as previously applied to dots marked B. Add 50 ml water (mobile solvent, (**b**)(2)) to each trough in tank, suspend papers therein, cover tank, and proceed as before.

Spray chromatogram with chromogenic agent, (**c**)(1), and immediately, with no drying, hang it in a tank containing about 5 ml NH_4OH in trough, and cover. Repeat process until all chromatograms have been treated with the chromogenic agent and exposed to ammonia fumes.

DISCUSSION

Chromogenic Agent.—The chromogenic agent is phosphomolybdic acid followed by ammonia fumes. The 2 per cent aqueous solution (3) of the acid produced an unsatisfactory background because the excessive quantity of acid caused the paper to darken materially on standing. The aqueous solution also caused some of the spots to run. These defects were largely corrected by employing an ethanolic solution of the acid. The ethanol solution also increased the intensity of the spots. Fuming the paper in a closed container yielded more satisfactory spots than exposure in a hood or blowing the fumes on the paper.

Sensitivity of the four antioxidants to the chromogenic agent was determined by the procedure reported in *This Journal*, **40**, 294 (1957). The results are tabulated in Table 1.

TABLE 1.—*Sensitivity tests, with R_F range and amounts of 4 antioxidants required to yield positive tests or to overload the chromatogram for 2 different solvent systems*

ANTIOXIDANT	AMOUNT IN 1×	SOLVENT SYSTEM[a]			
		AQUEOUS METHANOL		WATER	
		POSITIVE	OVERLOADED	POSITIVE	OVERLOADED
BHA	1 mmg	1×	20×	1×	30×
BHT	10 mmg	2×	50×	2×	40×
NDGA	1 mmg	1×	50×	1×	20×
PG	1 mmg	2×	30×	2×	30×

[a] Temp., 22°. Time: aqueous methanol, $3\frac{3}{4}$ hours; water, $1\frac{3}{4}$ hours.

The sensitivity tests show the approximate amounts of the antioxidants which may be handled. The lower limits reported are probably somewhat less than would normally be observed in individual tests; as conducted, the sensitivity test covers eighteen spots of rapidly increasing amounts of antioxidant across two papers, thus serving as a guide for closer observation and subsequent detection of faint spots in the chromatogram. For this reason the minimum detectable amount of antioxidant given in Table 1 should probably be increased by two to five times. Overloading the paper with the antioxidant causes large spots or streaks and makes separation and identification uncertain or impossible in single tests.

Solvent Systems.—Non-aqueous solvent systems in which a suitable solvent, e.g., dimethylformamide or formamide, is used as the immobile solvent and mixed octanes as the mobile solvent, separate BHA and BHT from each other and from NDGA or PG, but fail to move the latter two substances from the starting line. BHT migrates further up the paper than does BHA. Aqueous solvent systems in which refined soybean oil is used as the immobile solvent and a suitable solvent, e.g., aqueous ethanol or methanol, as the mobile solvent, move all four antioxidants in the reverse order to the non-aqueous systems, but again fail to separate NDGA and PG. However, when water, as the mobile solvent, is paired with oil, the system separates PG from NDGA without moving BHA or BHT. The behavior of the antioxidants thus suggested that they could be separated by two-dimensional chromatography.

The amount of oil in the paper and the quantity of water in the mobile solvent greatly affected the separation of BHA from the NDGA-PG spot for the first (perpendicular) run. Optimum conditions require 10 per cent oil (10 ml diluted to 100 ml with ethyl ether) and 60 per cent ethanol or 80 per cent methanol (40 or 20 ml water diluted to 100 ml with ethanol or methanol, respectively). Ethanol gives a somewhat better separation than methanol, but requires more time than a normal working day to com-

TABLE 2.—*R_F values for 4 antioxidants for both one- and two-dimensional chromatography*[a]

ANTIOXIDANT	TWO-DIMENSIONAL				ONE-DIMENSIONAL			
	PERPENDICULAR (METHANOL)		HORIZONTAL (WATER)		(METHANOL)		(WATER)	
	AVERAGE	RANGE	AVERAGE	RANGE	AVERAGE	RANGE	AVERAGE	RANGE
BHT	0.11	0.07–0.12	0.00	0.00	0.09	0.06–0.11	0.00	0.00
BHA	0.62	0.50–0.69	0.00	0.00	0.56	0.44–0.67	0.00	0.00
NDGA	0.79	0.67–0.87	0.06	0.04–0.11	0.75	0.60–0.86	0.05	0.04–0.06
PG	0.79	0.67–0.87	0.38	0.33–0.44	0.75	0.60–0.86	0.36	0.30–0.41

[a] Temp., 21°. Time: aqueous methanol, 2¾ hours; water, 1¼ hours.

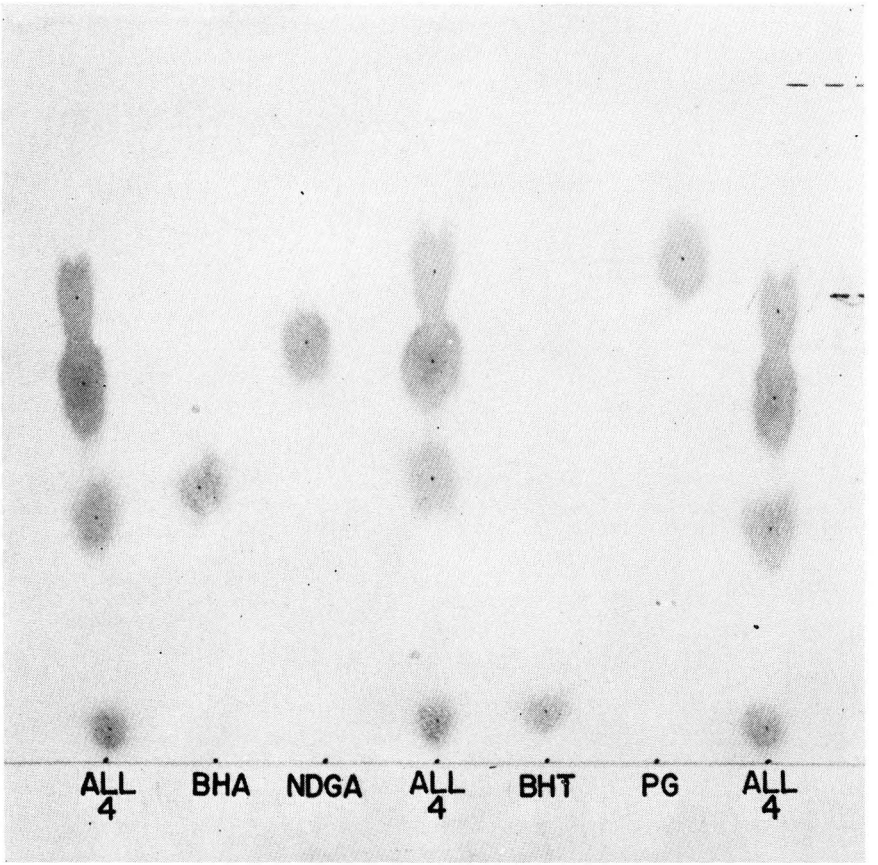

FIG. 2 shows the second procedure employed to separate and identify four antioxidants by means of two successive mobile solvents in the same direction. For the first run, the paper was developed by allowing the aqueous methanol (20 ml water diluted to 100 ml with methanol) to ascend approximately two-thirds of the normal distance for the 8 inch paper; then the paper was air dried. For the second run, the paper was developed by allowing the water to ascend to the normal distance.

Solvents and chromogenic agents: the same as those described for Fig. 1. Temperature, 24°.

plete. Accordingly, methanol was selected since development of the chromatogram required less than half the time required by ethanol.

Table 2 lists the average and range of R_F values obtained from 16 chromatograms developed from a solution of the four antioxidants according to the two-dimensional scheme outlined above. Values are compared to those obtained with the two mobile solvents by the one-dimensional technique.

The aqueous methanol frontal area in the paper retards the upward

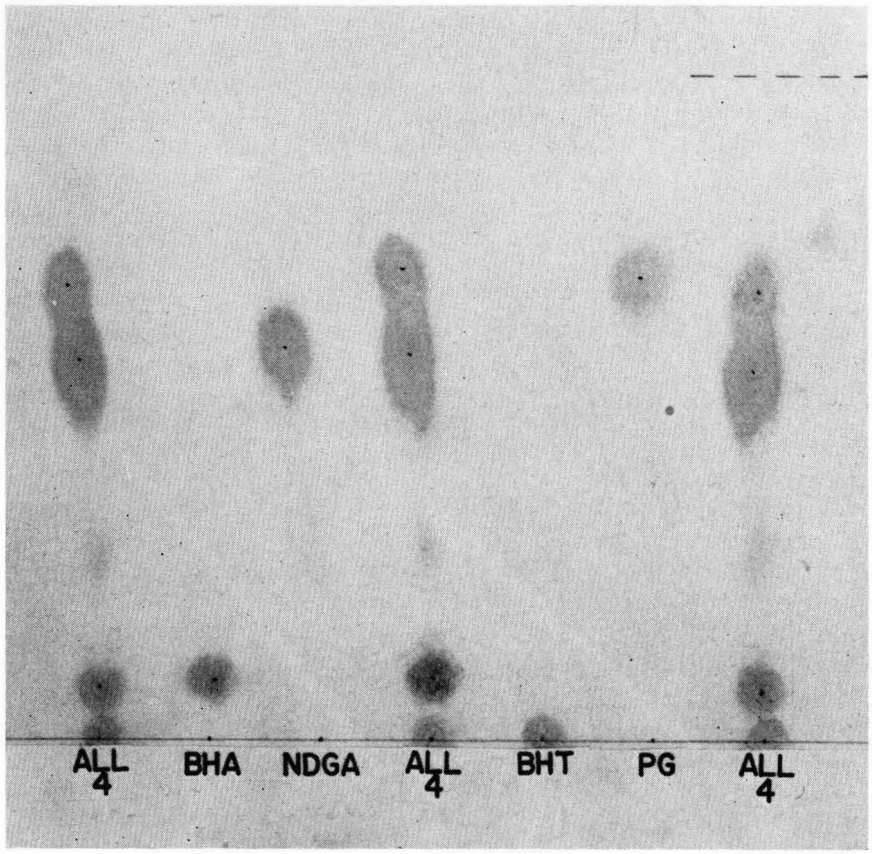

FIG. 3 demonstrates the third procedure used to separate and identify four antioxidants by means of paper chromatography. In this instance, the paper was developed with aqueous methanol whose composition was different from that of the mobile solvent described for Figs. 1 and 2.

Solvent.—Mobile: Dilute 60 ml water to 100 ml with methanol, A.C.S.

Immobile solvent and chromogenic agents: same as those described for Fig. 1. Temperature: 24°.

movement of the water somewhat, thus forming an extended V-shaped front. In practice, the lower part of this V-shaped dip was allowed to approach the 5 inch line.

Figure 2 demonstrates a second procedure, and Figure 3 a third, for the separation and identification of the four antioxidants. The chromatogram in Figure 2 was developed by allowing the aqueous methanol front (20 ml water diluted to 100 ml with methanol) to ascend approximately two-thirds of the normal distance for the 8 inch paper. The paper was then removed from the tank, air-dried, and developed with water to the normal distance. In the third procedure the chromatogram in Figure 3 was de-

veloped with aqueous methanol of a different composition (60 ml water diluted to 100 ml with methanol) to the normal distance.

Incidentally, it is known that BHT does not have as high antioxidant potency at low concentrations as BHA does and that PG at ordinary concentrations has the greatest antioxidant power of any of the food grade products (1). For the quantity of substance involved and from the intensity of the spots obtained, paper chromatography seems to confirm this statement.

CONCLUSIONS

Mixtures of the four common antioxidants, BHA, BHT, NDGA, PG, used as additives to food products, are separated by application of two-dimensional paper chromatography in which (*1*) the paper is pre-impregnated by spraying with an ethyl ether solution of the immobile phase (refined soybean oil), (*2*) aqueous methanol is employed as the first mobile phase (perpendicularly) and water as the second mobile phase (horizontally), and (*3*) the chromatogram is sprayed with the chromogenic agent (0.5 per cent phosphomolybdic acid in ethanol) followed immediately by exposure to ammonia fumes. The four components are also separated with oil as the immobile phase by allowing the aqueous methanol to ascend approximately two-thirds of the normal distance for the 8 inch paper, and, after air-drying followed by water, to ascend the normal distance Further, a third separation is obtained with oil as the immobile phase by allowing dilute methanol to ascend the normal distance for the paper.

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SEPARATION AND IDENTIFICATION OF SOME TERPENES BY GAS PARTITION CHROMATOGRAPHIC ANALYSIS*

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All workers in the field of natural products research will soon feel the dramatic impact of gas partition chromatography (GPC) on analytical chemistry (1, 2). Up to this time, inadequate methods of separating and

* Supported in part by a grant from the Lemon Products Advisory Board, Los Angeles, Calif.
† Employed by Lemon Products Advisory Board under Memorandum of Understanding.

identifying individual constituents has limited the investigation of essential oils and terpene-containing materials. The advent of such a powerful means of separation as GPC will enable the researcher to discriminate between the numerous terpenes. An additional advantage of GPC is that the apparatus involved is reasonably simple in design and relatively inexpensive to construct; thus it is readily available to most laboratories.

In this study a number of authentic terpenes have been conveniently separated by GPC, and the constituents have been qualitatively and quantitatively estimated by this method. This paper will deal mainly with the preliminary work of separating and identifying some of the principal terpenes reported to be present in commercial cold-pressed lemon oil (3).

Several excellent papers deal with the theory (4) and design of gas partition chromatographic apparatus (2, 4, 5). A serious drawback of a number of these designs is that the chromatographic columns are constructed of metal; they may possibly react with or at least contaminate the materials to be analyzed. Equipment developed and designed by Professors H. S. Mosher and R. H. Eastman of Stanford University (6) was found to be the most satisfactory for this work.

THEORY

Any given component requires a characteristic time to reach the exit of a gas partition chromatographic column under a given set of experimental parameters. This time has been designated the retention time. A component can be tentatively identified by comparing its retention time with the abscissa or time axis of recorded retention times of reference materials. Retention times are also a function of (*a*) the nature and concentration of the particular liquid phase employed; (*b*) the temperature of the column; (*c*) the flow rate and column pressure; (*d*) the dead volume between the point of injection and the detector; (*e*) the carrier gas; (*f*) the column dimensions; (*g*) the particle size and surface characteristics of the mechanical support for the liquid phase; and (*h*) the pressure drop through the column. Thus retention times are of only relative value and must be converted to a more useful constant in order to facilitate data transfer.

The retention volume has been used by a large number of workers and is employed in the present study. The use of retention volumes eliminates the effect of flow rate and dead volume in data transfer. Since the instrument parameters (*f*), (*g*), and (*h*) can be conveniently combined into a single constant for each column, a satisfactory degree of standardization can be achieved.

The fundamental equation (4) for the retention volume of a component is:

$$V_R = \frac{2aL}{3R_F}\left[\frac{(p_1/p_0)^3 - 1}{(p_1/p_0)^2 - 1}\right] \qquad \text{(I)}$$

where V_R is the product of the volume of gas emerging from the column outlet in unit time and the time that elapses before the center of the zone emerges from the column; a is the area occupied by the gas phase in any cross-section of the column; L is the length of the column; R_F is the ratio of the movement of the zone of maximum concentration of the component to the movement of the carrier gas (the ratio of linear velocities); p_1 is the pressure of the carrier gas at the column inlet; and p_0 is the pressure of the gas at the column outlet. The retention column V_R is thus dependent upon the ratio:

$$[(p_1/p_0)^3 - 1]/[(p_1/p_0)^2 - 1]$$

for a given column and temperature. James and Martin (4) have shown that as p_1/p_0 approaches unity, a limiting value for V_R can be calculated from:

$$V_R{}^0 = \frac{3}{2} V_R \left[\frac{(p_1/p_0)^2 - 1}{(p_1/p_0)^3 - 1}\right]. \qquad \text{(II)}$$

Since the terms on the right hand side of equation II may be readily evaluated, $V_R{}^0$ can be determined for each component in a mixture.

For routine application of these equations, the relative retention volumes ($V_{R_i}{}^0/V_{R_j}{}^0$) are often calculated; since they may be more easily reproduced by other researchers, they permit easier comparison of data.

EXPERIMENTAL

The gas partition chromatographic column used to separate the terpenes is constructed entirely of glass. The column itself is essentially a Pyrex glass tube 20 feet long and 6 mm i.d. As it is inconvenient to provide effective heating for a tube 20 feet in length, the actual column is constructed of five 4-foot lengths of glass tubing connected to each other by U-shaped sections of glass capillary tubing 1 mm i.d. Joined to the top of the entire tube is a three-way stopcock. An inner ⅋ joint, 7/25, is affixed to the bottom of the tube. This tube is filled with the liquid phase and its mechanical support. Small plugs of glass wool are used to retain the packing in the column. Surrounding the tube is an air bath heated by means of Chromel A resistance tape (1 ohm/ft).

A sensing filament (6) made of tungsten wire is sealed into a small slot cut in the side of a 7 mm Pyrex glass tube 6 inches long attached to an outer ⅋ joint, 7/25. This katharometer assembly may then be conveniently attached to the exit end (bottom) of the chromatographic column and held in place by a small amount of a silicone grease; it is kept at the operating temperature of the column by the electrically controlled air bath which provides a uniform temperature and prevents condensation on the filament and katharometer walls of the materials being analyzed. The sensing device may be readily removed for cleaning and repair. The katharometer is connected to a Wheatstone bridge circuit (6) which

drives a recording potentiometer of either 10 mv or 100 mv full scale deflection.

The mechanical support is a crushed diatomaceous earth firebrick (Johns-Manville C-22) (5). The column is packed with a mixture made up of 100 g of 60–80 mesh C-22 firebrick impregnated with 40 g of the liquid phase.

Helium is the most satisfactory gas phase for this study; in addition to being unreactive, it affords a much higher degree of sensitivity in this system than nitrogen does.

A $\frac{1}{4}$ ml syringe fitted with a 3-inch hollow stainless-steel needle is used to insert samples at the top of the column through the 3-way stopcock.

RESULTS AND DISCUSSION

Silicone fluid (General Electric SF-96-40), Apiezon (type L) high-vacuum stopcock lubricant, di-*n*-octyl phthalate, glycerol, and a polyethylene glycol (Carbowax 600) were examined as liquid phases. Under these conditions Carbowax 600 provided optimum separation of the terpenes.

The importance of selecting the proper liquid phase cannot be overemphasized. This phase is undoubtedly the most powerful experimental parameter in gas partition chromatography. Theoretical considerations (7) help to restrict the list of possible materials, but a suitable liquid phase must often be selected empirically.

Figure 1 provides a graphic example of the powerful influence that the liquid phase exerts upon separation. This is the recording of a chromatogram of a synthetic mixture of five authentic terpene hydrocarbons reported to be present in cold-pressed lemon oil (3): *alpha*-pinene, camphene, *beta*-pinene, D-limonene, and *beta*-phellandrene.

The recordings presented in this paper are plotted as functions of vapor concentration (proportional to the off-balance of the Wheatstone bridge) *versus* time. This sample was run in a column constructed like the one already described, with these specifications: dimensions, 4 feet long and 5 mm i.d.; temperature, 181°; helium flow rate, 30 ml/min.; liquid phase, di-*n*-octyl phthalate.

The recording indicates two broad peaks, 1 and 2, connected to each other by a very shallow trough. (The sharp peak at the extreme left of the recording is the so-called air peak produced by the insertion of the sample.) Careful examination of peak 1 indicates a partial resolution of two or more components in this region. On the basis of retention times for each of the five components under the experimental conditions described, *alpha*-pinene, camphene, and *beta*-pinene are present in peak 1, and D-limonene and *beta*-phellandrene are present in peak 2. Since the five terpenes were only partially resolved, it is apparent that the relative

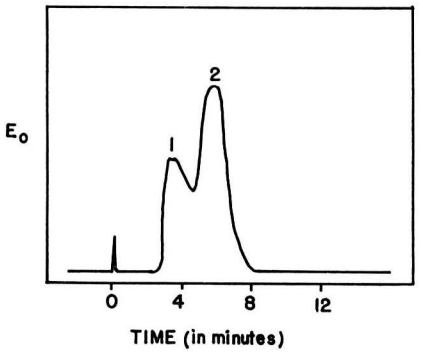

FIG. 1.—Separation of five terpenes: (1) *alpha*-pinene, camphene, *beta*-pinene, (2) D-limonene, *beta*-phellandrene. Temperature: 181°. Helium flow rate: 30 ml/min. Liquid phase: di-*n*-octyl phthalate.

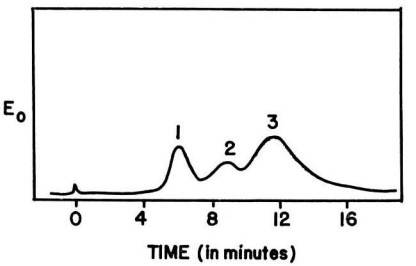

FIG. 2.—Separation of five terpenes: (1) *alpha*-pinene, camphene, (2) *beta*-pinene, (3) D-limonene, *beta*-phellandrene. Temperature: 152°. Helium flow rate: 30 ml/min. Liquid phase: Carbowax 600.

retention volumes of these compounds under these experimental conditions do not differ from each other significantly so as to permit a satisfactory separation with this liquid phase.

The effects of two other operating parameters, temperature and flow rate, were investigated. Decreasing the operating temperature of the column does not measurably increase resolution and only serves to flatten and distend the peaks. A change in flow rate merely compresses or expands the recording and has no effect upon separation.

When the five terpenes were run in a column 4 feet long and 5 mm i.d., at a temperature of 152° and a helium flow rate of 30 ml/min., with Carbowax 600 as the liquid phase, resolution was more complete. Reference to Fig. 2 indicates three broad peaks connected by shallow troughs. On the basis of retention times for each of the five components under the experimental conditions described, *alpha*-pinene and camphene are present in peak 1, *beta*-pinene in peak 2, and D-limonene and *beta*-phellandrene in peak 3.

Changing the temperature within the range 100–200° did not significantly improve resolution.

The resolution was improved still further by using a longer column. The five distinct peaks visible in Fig. 3 show that these terpenes are satisfactorily separated. The peaks are identified as follows: (1) *alpha*-pinene; (2) camphene; (3) *beta*-pinene; (4) D-limonene; and (5) *beta*-phellandrene.

James (8) has reported that relative retention volumes must differ by roughly 20 per cent for complete separation of zones; a difference of 10–15 per cent will show two united peaks; and differences of less than 7 per cent will show a single peak. The relative retention volumes of peaks 4

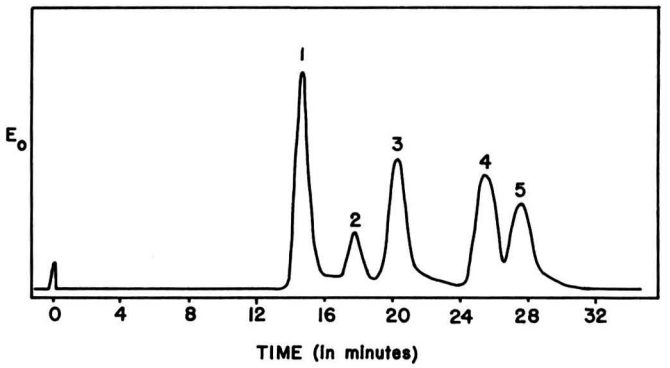

FIG. 3.—Separation of five terpenes: (1) *alpha*-pinene; (2) camphene; (3) *beta*-pinene; (4) D-limonene; (5) *beta*-phellandrene. Temperature: 177°. Helium flow rate: 23 ml/min. Liquid phase: *Carbowax 600.*

and 5 differ by about 7 per cent; thus there is a slight union between the peaks.

This sample was run in the 20 foot column described in detail in the experimental section, at a temperature of 177° and a helium flow rate of 23 ml/min., with Carbowax 600 as the liquid phase. Each peak represents about a 10 microliter sample. Each hydrocarbon is qualitatively identified by its retention volume (4). The concentration of each component may be determined from the area under the peaks, as the response of the thermal conductivity cell for these hydrocarbon isomers is essentially the same.

Table 1 presents the order of elution for the five terpene hydrocarbons, their retention volumes (V_R), their limiting retention volumes (V_R^0), their retention volumes relative to *alpha*-pinene, and the column efficiency for each separation under the experimental conditions described.

The efficiency of separation is expressed in terms of the number of theoretical plates. This can be calculated (9) from the recorded chromato-

TABLE 1.—*Retention volumes for terpene hydrocarbons*[a]

SUBSTANCE	b.p., °C.	V_R[b]	$V_R^{0(b)}$	V_R RELATIVE TO *alpha*-PINENE	THEORETICAL PLATES
alpha-Pinene[c]	156°	625	472	1.00	1170
Camphene	160°	802	606	1.29	1315
beta-Pinene	164°	914	690	1.46	1340
D-Limonene	176°	1270	960	2.03	1810
beta-Phellandrene[c]	179°	1365	1030	2.18	1550
				Av.	1477

[a] Column: Carbowax 600, 20 feet long, temperature 177°, helium flow rate 23 ml/min.
[b] In ml of He at 760 mm of Hg at 177°.
[c] Generously supplied by Dr. N. T. Mirov, Forest Service, U.S.D.A., Berkeley, California.

grams by measuring the height of a peak, h; the area under the peak, A; and its retention distance, d; and using the formula: Number of plates $=2\pi(hd/A)^2$.

It should be noted that for the case at hand, the terpenes are eluted from the column in the order of increasing boiling points.

SUMMARY

An apparatus for gas partition chromatography is described. A commercial polyethyleneglycol is used as the liquid phase with diatomaceous earth as its mechanical support; helium is employed as the gas phase. The separations are carried out at temperatures up to 180°, permitting the use of atmospheric pressure at the outlet. Fractions can be conveniently collected.

Results obtained with this apparatus for some authentic samples of terpenes reported to occur in commercial cold-pressed lemon oil are presented in the form of retention volumes (V_R) and plots of vapor concentration *versus* time.

ACKNOWLEDGMENT

The author wishes to express his gratitude to Professors H. S. Mosher and R. H. Eastman of Stanford University who permitted the author to employ their design for the gas chromatographic equipment prior to publication and to adapt it to his specific problem.

The author also wishes to thank Mr. Lawrence Atkinson of this laboratory for his aid in constructing the equipment.

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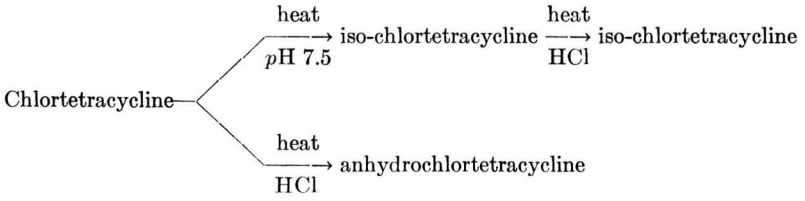
THE COLORIMETRIC DETERMINATION OF CHLORTETRACYCLINE HYDROCHLORIDE IN FEED SUPPLEMENTS, VETERINARY THERAPEUTICS, AND FOOD PRESERVATIVES*

By FORTUNATO S. CHICCARELLI, MASON H. WOOLFORD, JR., and RICHARD W. TROMBITAS (Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.)

The nutritional (1) and therapeutic effects of chlortetracycline have been apparent since 1949. Because chlortetracycline stimulates the growth of swine, chicks, and poults, it cuts down the feed consumption of these animals; they can be sent to market much sooner and in a healthier condition. More recently it has been established that chlortetracycline can be used to preserve poultry (2), meat (3), and fish (4). This laboratory has developed many products containing chlortetracycline for use in the feed industry and also for food preservation.

The quality of these products is controlled by a colorimetric assay. Feed supplement, veterinary therapeutic, and food preservative products are made from mash concentrates, semi-refined, or food grade chlortetracycline that is blended with meals or other diluents, depending upon the purpose for which the particular product is to be used. Some of these blending materials, especially those used in feed supplements, contain components that interfere with the customary colorimetric and fluorimetric methods (5, 6).

The colorimetric assay described in this paper is a modification of the method of Levine, *et al.* (5). It eliminates these interferences without complicated and time-consuming purification or separation techniques. The assay is performed according to the following scheme:



The chlortetracycline in the sample taken for analysis is brought into aqueous solution as described under PROCEDURE. Hydrochloric acid is added to one aliquot of this solution and the mixture is heated, forming colored anhydrochlortetracycline (7), which has an absorption maximum at 445 mμ. This solution is the assay sample.

A second aliquot is heated at *p*H 7.5 (without acid); the chlortetra-

* The trademark of Lederle Laboratories Division, American Cyanamid Co., for chlortetracycline is Aureomycin. Chlortetracycline used as a feed supplement, veterinary therapeutic, and food preservative is distributed by the Farm and Home Division, American Cyanamid Co., under the respective trademarks of "Aurofac," "Aurovim," and "Acronize."

TABLE 1.—*Recommended sample weights*

PRODUCT	LABEL CLAIM[a]	WEIGHT/VOLUME (G/ML)	Ml 5*N* HCl
	g/lb		
Aurofac	1.8	1.0/100	4.0
Aurofac 2A	3.6	1.0/200	8.0
Aurofac D	5.0	1.0/250	10.0
Aurofac 10	10.0	1.0/500	20.0
Aurofac 20	20.0	0.5/500	20.0
A.T.F. No. 5	5.0	1.0/250	10.0
A.T.F. No. 15	15.0	0.75/500	20.0
Auropep Crumbles	2.0	2.0/200	8.0
Aurovim (Poultry)	3.1	0.75/100	4.0
Aurovim (Swine)	2.5	0.75/100	4.0
	per cent		
Acronize B1	16.50	0.3/1000	40.0
Acronize M	2.33	2.5/1000	None
Acronize PD	10.00	0.5/1000	None
Acro-Spray 10	10.00	0.5/1000	None

[a] Chlortetracycline HCl.

cycline is cleaved into iso-chlortetracycline (8). Subsequent acid treatment does not rearrange the iso-chlortetracycline into colored anhydrochlortetracycline; thus the second aliquot can be used as the blank. When both sample and blank are measured at 445 mμ, the iso-chlortetracycline contributes zero absorbance. The difference in absorbance of assay sample and blank can be used to calculate the chlortetracycline present.

METHOD

REAGENTS

(a) *Hydrochloric acid.*—5*N*.

(b) *Sodium hydroxide.*—Dilute 8 ml 5*N* NaOH to 100 ml with H_2O.

(c) *Sodium bisulfite.*—10 g/100 ml H_2O; prepare fresh each time.

(d) *Buffer solution.*—*p*H 7.5. Dissolve 178 g anhydrous K_2HPO_4 and 22 g anhydrous KH_2PO_4 in 1 l H_2O. Filter before using.

PROCEDURE

Take samples for assay of each product as shown in Table 1. Triturate sample with small portion of H_2O, using mortar and pestle. Transfer to volumetric flask and add H_2O equal to about $\frac{2}{3}$ of the specified total volume. Add the recommended amount of acid and shake 3 min. Dilute to volume with H_2O and shake again. If necessary, filter a portion of the sample through Whatman No. 42 paper. Discard the first 10 ml before taking an aliquot for assay. Transfer a 10 ml aliquot to each of two 50 ml volumetric flasks; use one as blank, the other as sample. Upon final dilution, each solution will contain the same salt concentration.

To the sample add in this order: 12 ml 5*N* HCl, 15 ml *p*H 7.5 buffer, 2 ml bisulfite solution,[1] and 3 ml 5*N* NaOH.[2] Suspend in a boiling water bath for exactly 7

[1] The bisulfite solution is not necessary in the assay of Aurovim products.
[2] The hydroxide solution is not necessary in the assay of Acronize M and PD and Acro-Spray 10.

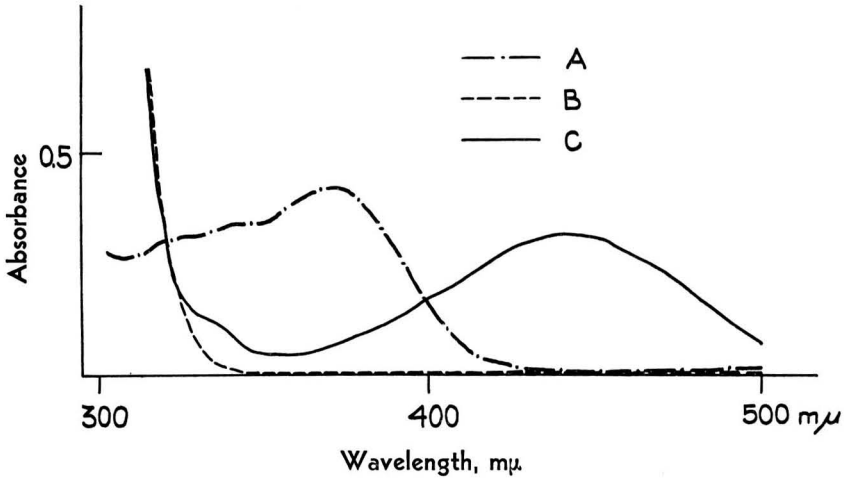


FIG. 1.—Absorption spectrum from 300 mμ to 500 mμ of 20 mmg/ml chlortetracycline: (**A**) in 0.1*N* HCl; (**B**) under conditions of blank preparation; and (**C**) under conditions of sample preparation.

min., swirling occasionally. To the blank add 15 ml *p*H 7.5 buffer, 2 ml bisulfite solution,[1] and 3 ml 5*N* NaOH.[2] Suspend in a boiling water bath for 5 min., swirling occasionally. After exactly 5 min., add 12 ml 5*N* HCl and heat for 2 minutes more.

After completing the heat treatment, immediately cool both the blank and the sample under tap water, dilute to volume with H_2O, and mix well. Determine the absorbance at 445 mμ against H_2O, using a suitable spectrophotometer.

CALCULATION

$$\frac{A_{\text{sample}} - A_{\text{blank}}}{131.0} \times 0.01 \times 50 \times \frac{\text{Initial Volume}}{10} \times \frac{453.6}{\text{Wt in g}}$$

= g chlortetracycline per lb.

DISCUSSION AND RESULTS

Curve A of Fig. 1 is the absorption spectrum of chloretetracycline in 0.1*N* hydrochloric acid; Curve B is the absorption spectrum of iso-chlortetracycline under the conditions of the assay; and Curve C is the absorption spectrum of anhydrochlortetracycline under the conditions of the assay.

In controlling the feed supplements the assay must be performed in the presence of sodium bisulfite, which has a stabilizing effect on the background color during the treatment at *p*H 7.5. Relatively pure products do not require the addition of sodium bisulfite.

Table 2 lists the points of the concentration curve within the limits of the assay. Table 3 lists time as a variable in the preparation of blank and sample solution. At the end of each time interval hydrochloric acid was added and heating was continued in each case for a total of 7 minutes.

TABLE 2.—*Concentration curve*

CONCENTRATION	CORRECTED ABSORBANCE[a]	ABSORPTIVITY AT 445 mμ
mmg/ml		
10.0	0.131	131.0
20.0	0.260	130.0
30.0	0.393	131.0
40.0	0.528	132.0

[a] Absorbance of sample solution minus absorbance of blank solution.

TABLE 3.—*Change in absorbance with time*

BLANK[a]		SAMPLE[a]	
TIME, MIN.[b]	ABSORBANCE AT 445 mμ	TIME, MIN.[c]	ABSORBANCE AT 445 mμ
1	0.070	1	0.017
		2	0.050
2	0.025	3	0.088
		4	0.117
3	0.010	5	0.127
		6	0.134
4	0.006	7	0.134
		8	0.134
5	0.004	9	0.131
		10	0.130
6	0.004	11	0.125
		12	0.122
7	0.004		

[a] Chlortetracycline standard initial concentration: 10 mmg/ml.
[b] This column shows the time during which the solution was heated at *p*H 7.5.
[c] This represents the heat treatment while the solution was acidic.

Thus, on line 2 the solution was heated for 2 minutes at *p*H 7.5; then the acid was added and heating continued for 5 minutes more. It may be seen from Table 3 that 7 minutes is optimum heating time for both the sample and the blank solution.

A well-blended sample of chlortetracycline feed supplement was assayed at intervals over a period of two weeks by the colorimetric and the microbiological assays.[3] The colorimetric assay was performed 27 times and gave a standard deviation of 0.09 gram per pound. The microbiological assay was performed 10 times and gave a standard deviation of 0.28 gram per pound. The average microbiological results were 3.80 grams per pound; the average colorimetric results were 3.77 grams per pound. Label claim is 3.6 grams per pound.

The colorimetric assay is accurate, reproducible, and time-saving; it lends itself to simultaneous determinations. A possible assay for chlortetracycline in finished feeds, based on this assay, is being investigated.

[3] Turbidimetric; *Micrococcus pyogenes* var. *Aureus*.

SUMMARY

A colorimetric assay is presented for chlortetracycline hydrochloride in feed supplement, veterinary therapeutic, and food preservative products. The sample is prepared, diluted, and divided into two equal portions, one the assay sample and the other a blank. The assay sample is treated with acid and heated to convert the chlortetracycline to anhydrochlortetracycline. The blank portion is heated at *p*H 7.5 to cleave the chlortetracycline to iso-chlortetracycline, which cannot be rearranged by acid to develop the color of anhydrochlortetracycline. Sample and blank are measured at 445 mμ. The difference in absorbance is due to chlortetracycline.

ACKNOWLEDGMENT

Many people have helped to complete this work successfully. The authors wish especially to thank R. Adams, M. Avery, S. Farsetta, C. Hotchkiss, N. Knowlden, C. Krieger, E. Matthews, P. Van Gieson, and C. W. Waller.

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DETERMINATION OF THEOPHYLLINE AND AMINOPHYLLINE BY NONAQUEOUS TITRATION

By Mary A. McEniry (Food and Drug Administration, Department of Health, Education, and Welfare, St. Louis 1, Mo.)

Pifer, *et al.* (1) list a number of U.S.P. XIV and N.F. IX compounds that have been successfully titrated in nonaqueous solvents. Among those listed are theobromine and aminophylline. Beckett and Tinley (2) point out that theobromine has been titrated with potassium methoxide azo violet as the indicator and ethylenediamine as the solvent. Theophyl-

line has acidic properties similar to those of theobromine and theoretically it could be titrated in a similar manner.

Theophylline and aminophylline are soluble in dimethylformamide and yield a sharp end point with sodium methoxide and thymol blue. These reagents are used in U.S.P. XV for the assay of at least one preparation. They were chosen for use in this work because of their convenience.

METHOD

REAGENTS

(a) *Dimethylformamide (DMF).*
(b) *Thymol blue indicator.*—1% solution in DMF.
(c) *Sodium methoxide.*—0.1*N*; prepare by U.S.P. XV procedure.

DETERMINATION

Weigh a sample equivalent to about 150 mg theophylline, transfer to a small beaker, add 25 ml DMF, and dissolve. Add 2 drops of thymol blue indicator and cover the beaker with a piece of cardboard containing a small hole (3) through which the buret tip is inserted. Titrate rapidly with $NaOCH_3$, stirring with a magnetic stirrer. Run a blank determination on 25 ml DMF and correct the sample titration accordingly.

EXPERIMENTAL

A commercial sample of aminophylline was analyzed by the proposed method and by the U.S.P. XV method. Results are given in Table 1.

U.S.P. theophylline was recrystallized from 95 per cent alcohol until the melting point was constant. The recrystallized material was assayed by the sodium methoxide method and by the U.S.P. XV method. Results are shown in Table 2.

As indicated by Tables 1 and 2, the results by the U.S.P. assay are consistently lower than those by the proposed method. A solution of aminophylline was prepared and assayed by both methods as a check on the validity of the sodium methoxide titration results. This solution was pre-

TABLE 1.—*Theophylline in aminophylline by $NaOCH_3$ method and by U.S.P. XV method*

$NaOCH_3$ METHOD			U.S.P. XV METHOD		
AMINOPHYLLINE SAMPLE WT.	THEOPHYLLINE FOUND		AMINOPHYLLINE SAMPLE WT.	THEOPHYLLINE FOUND	
mg	*mg*	*per cent*	*mg*	*mg*	*per cent*
210.3	174.6	83.0	256.4	210.8	82.2
217.3	179.9	82.8	273.3	224.3	82.1
206.3	170.9	82.8	264.0	216.6	82.0
206.8	171.6	83.0			
201.9	167.5	82.9			
	Av.	82.9		Av.	82.1

TABLE 2.—*Recoveries on recrystallized theophylline by the $NaOCH_3$ method and by the U.S.P. XV method*

$NaOCH_3$ TITRATION			U.S.P. XV METHOD		
TAKEN	RECOVERED		TAKEN	RECOVERED	
mg	*mg*	*per cent*	*mg*	*mg*	*per cent*
152.8	152.8	100.0	211.5	209.0	98.8
152.8	152.4	99.8	205.7	203.3	98.8
153.5	153.0	99.7	201.7	199.1	98.7
153.4	153.4	100.0			
152.4	151.9	99.7			
	Av.	99.8		Av.	98.8

pared by dissolving 2.6640 grams of recrystallized theophylline in 0.7260 gram of ethylenediamine and water, and diluting with water to a definite volume. The resulting aminophylline contained 21.4 per cent ethylenediamine. This percentage is higher than in U.S.P. aminophylline, but this amount was necessary to take the theophylline into solution conveniently. The nonaqueous titrations were made on the preparation by first evaporating aliquots to dryness. Results of the analysis of this solution are shown in Table 3.

DISCUSSION

In all determinations the sodium methoxide titration gave theophylline values closer to the amount added. The U.S.P. results range from 0.8 to 1.6 per cent lower. The U.S.P. assay involves the precipitation of theophylline with silver nitrate as silver theophyllinate. The lower results obtained could be explained by a slight solubility of the silver precipitate.

Absorption by DMF of any carbon dioxide during the titration with sodium methoxide would yield high results. To investigate the possibility

TABLE 3.—*Determination of theophylline in aminophylline solution by sodium methoxide and U.S.P. XV methods*

THEOPHYLLINE	DETERMINATION							AV.
	1	2	3	4	5	6	7	
$NaOCH_3$ Method: 133.2 mg Theophylline Present								
Found, mg	132.8	132.6	133.2	133.6	133.4	132.8	133.2	
Recovery, %	99.7	99.5	100.0	100.3	100.2	99.7	100.0	99.9
U.S.P. XV Method: 266.4 mg Theophylline Present								
Found, mg	262.0	262.0	262.0					
Recovery, %	98.3	98.3	98.3					98.3

that the higher results by the sodium methoxide method were caused by the absorption of carbon dioxide, four titrations (4, 5, 6, and 7 in Table 3) were made under a nitrogen atmosphere. No difference was found in the results of the titrations.

CONCLUSION

The nonaqueous titration presented gives more accurate results for theophylline and aminophylline than does the U.S.P. XV procedure and requires less time to perform.

ACKNOWLEDGMENT

The author wishes to thank R. L. Herd of the St. Louis District, Food and Drug Administration, for valuable suggestions.

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METHOD FOR DETERMINATION OF MANNITOL HEXANITRATE

By MARY A. MCENIRY (Food and Drug Administration, Department of Health, Education, and Welfare, St. Louis 1, Mo.)

Mannitol hexanitrate in tablets is frequently combined with phenobarbital and has presented an analytical problem. When Cannon and Heuermann presented their rapid assay for nitroglycerine tablets (*This Journal*, **34**, 716 (1951), they noted that the method had been applied to mannitol hexanitrate tablets.

Work was undertaken to determine how reliable the nitroglycerine method is for determining manitol hexanitrate. It was found to be unreliable; recoveries varied and were sometimes low. However, further work showed that continuing the distillation by adding several additional portions of alcohol overcame the difficulty. A side arm was added to the apparatus for this purpose (see Fig. 1).

This assay presents a rapid, accurate method for the determination of mannitol hexanitrate alone in tablets or in combination with phenobarbital.

METHOD

Determine average tablet weight. Grind at least 20 tablets to pass a 60-mesh

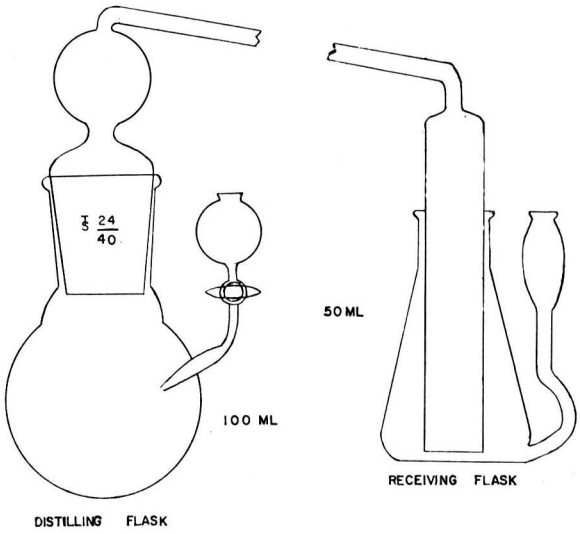

FIG. 1.—Modified distillation apparatus.

sieve. Weigh sample containing 6.0–7.0 mg mannitol hexanitrate, transfer to distillation flask with about 10 ml alcohol, and add 10 ml 0.5*N* alcoholic KOH. To the receiving flask add 5 ml H_2O and a drop of methyl red, place in an ice bath, and connect flasks so that connecting arm dips beneath the H_2O in receiving flask. Heat with a small electric heater controlled by a variable voltage transformer.

Distill the alcohol, neutralizing the distillate by adding 0.01*N* H_2SO_4 through side arm of receiving flask as distillation proceeds. Distill until continued distillation (about 3 min.) fails to produce a yellow color in the receiving flask. (Usually only 0.2–0.3 ml acid is required.)

Discard distillate and add 5 ml H_2O and a drop of methyl red to receiving flask. Cool distillation flask, and add 5–10 ml alcohol and about 50 mg Devarda's metal. Assemble the apparatus, reconnecting the flasks in the same manner as before. Distill as nearly to dryness as possible without charring, titrating as the distillation proceeds. When flask approaches dryness and there appears to be danger of sucking back, begin adding 5–10 ml alcohol dropwise through the side arm opening while still heating, and distill as before.

Continue distilling and adding alcohol until no more than 1 drop of 0.01*N* H_2SO_4 is required to neutralize 5 ml alcoholic distillate. (Usually 1 to 3 additions of alcohol are enough.) Add more indicator if needed. Run a reagent blank on the amount of Devarda's alloy and alcohol added during the second distillation and make correction.

EXPERIMENTAL

Mannitol hexanitrate is not commercially available as a pure compound. It was necessary to use mannitol hexanitrate as it is sold commercially—stabilized with lactose and ammonium phosphate—for most of the experimental work on the method.

The method was applied to a commercial sample (7.00 per cent mannitol hexanitrate declared). Reproducibility is shown by the following re-

sults: 6.97, 6.93, 6.97, 7.00, 6.92, 6.97, 6.93, 6.98, 7.00, and 7.00 per cent; average, 6.97 per cent; standard deviation, ±0.031 or 0.44 per cent.

A simulated tablet mixture was prepared to contain 5.79 per cent mannitol hexanitrate, 3 per cent phenobarbital, starch, and stearic acid. The results of assays of this mixture are shown in Table 1.

TABLE 1.—*Recovery of mannitol hexanitrate from a simulated tablet mixture*

MANNITOL HEXANITRATE IN SAMPLE[a]	RECOVERY	
mg	*mg*	*per cent*
5.92	5.97	100.8
5.90	5.98	101.4
5.79	5.77	99.7
5.95	6.06	101.8
6.64	6.57	98.9
5.88	5.83	99.1
5.95	5.98	100.5
6.08	6.17	101.5
6.14	6.12	99.7
6.17	6.18	100.2
Average		100.4
Standard Deviation		±1.01; 1.01%

[a] Calculated on basis of 6.97% mannitol hexanitrate found in commercial mixture.

To establish the accuracy of the method, mannitol hexanitrate was extracted from the commercial sample with ether and recrystallized from alcohol until a constant melting point was obtained. The pure material was weighed and diluted to volume with alcohol, and aliquots of this solution were analyzed. In this case no preliminary distillation was made. Recoveries of the pure material are given in Table 2.

TABLE 2.—*Recovery of recrystallized mannitol hexanitrate from alcoholic solution*

MANNITOL HEXANITRATE IN ALIQUOT	RECOVERY	
mg	*mg*	*per cent*
6.84	6.82	99.7
6.84	6.79	99.3
6.84	6.86	100.3
6.84	6.72	98.2
6.84	6.79	99.3
6.84	6.77	99.0
6.84	6.72	98.2
Average	6.78	99.1
Standard Deviation	±0.051 mg; 0.75%	

DISCUSSION OF METHOD

The initial alkaline distillation eliminates interference from any ammonium salts present in the tablet mixture and any reagent blank that might otherwise be obtained. In the preliminary work on this method, varying amounts of phenobarbital, up to twice the amount of mannitol hexanitrate present, were added to the mannitol hexanitrate mixture and analyzed. Recoveries were not affected by the phenobarbital (data are not reported). Starch and stearic acid added to the alcoholic extract of the recrystallized material did not affect the recoveries.

No advantage resulted from hydrolyzing the nitrate as a separate step. Distillation of successive small portions of alcohol was necessary to recover the ammonia completely.

The original apparatus, described in the nitroglycerine method, can be used. However, the side-arm titrating flask and side-arm distilling flask make the determination easier.

The experimental portion of this work was completed before Sarnoff's Associate Referee report was published (*This Journal*, **39**, 630 (1956)). Sarnoff's method has not been compared with the one presented in this paper.

ACKNOWLEDGMENT

The receiving flask with the arm for titrating was designed by J. H. Cannon (St. Louis District Laboratory); he has used it for many years.

DETERMINATION OF ADDED DISTINCTIVE CATIONS IN WHISKY.* IX. ULTRAVIOLET SPECTROPHOTOMETRIC DETERMINATION OF CERIUM

By MAYNARD J. PRO and RAYMOND A. NELSON (Alcohol and Tobacco Tax Division Laboratory, Internal Revenue Service, Washington 25, D.C.)

This is the ninth in a series of papers that describe the determination of markers added to whisky to identify different brands. The following investigation describes the determination of cerium by ultraviolet spectrophotometry.

Existing colorimetric methods (1–6) are not sensitive or specific enough to determine cerium in this work. Moreover, some of these reagents form intensely colored oxidation products with cerium which change in hue or fade on standing (1, 2, 6).

Since Freed and Mesirow (7) noted diffuse cerium bands in the ultra-

* Series 1, 5, 8 represent inorganic and series 6 and 7 organic markers.

violet region of the spectrum, several procedures based on the ultraviolet absorption characteristics of cerium have been reported. Medalia and Byrne (8) and Freedman and Hume (9) determined the ceric ion in a sulfuric acid solution and found 320 and 315 mμ, respectively, to be the absorption maxima. Telep and Boltz (10) determined the ceric ion in a carbonate-peroxide solution; Conca and Merritt (11), repeating the work, found that quadrivalent cerium gave approximately the same absorption values. However, the procedure reported by Conca and Merritt is more desirable because it permits a broader range of *p*H and eliminates the use of hydrogen peroxide. Also, it was shown that the absorbance was only slightly affected by 2.0–5.0*M* potassium carbonate solutions.

In the proposed spectrophotometric method, the organic material is destroyed by burning, the cerium is dissolved in hot concentrated sulfuric acid, and a cerium carbonate complex is formed with concentrated potassium carbonate. Absorbance is measured at 340 mμ.

ABSORBANCE SPECTRA AND CALIBRATION CURVE

Although in the following experimental work 305 mμ was found to be the absorbance maximum for the ceric carbonate complex, the wavelength at 340 mμ was selected because it offered a means of avoiding absorbance contributed by the presence of diverse ions. Also, at this wavelength no marked decrease in sensitivity was noted.

Aqueous solutions containing 1.0–5.0 ppm cerium were prepared and determined by the procedure subsequently described. The calibration curve, illustrated in Fig. 1, shows that the absorbance is a direct function of concentration. Also it was found that the cerium carbonate complex was stable for several days.

METHOD

INSTRUMENTS AND APPARATUS

(a) *Spectrophotometer.*—Beckman Model DU with ultraviolet attachment.

(b) *Platinum dishes.*—75–100 ml capacity.

REAGENTS AND STANDARDS

(a) *Cerium standard solution.*—Dissolve 2.6590 g reagent grade cerous chloride ($CeCl_3.7H_2O$) in H_2O and dilute to 1 l. This solution contains approximately 1000 ppm cerium. Standardize the cerium solution by the gravimetric procedure reported by Brinton and James (12).

(b) *Potassium carbonate solution.*—Dissolve 500 g reagent grade K_2CO_3 in 500 ml H_2O.

(c) *Reference solution.*—Add 5.0 ml H_2O to 5.0 ml K_2CO_3 solution, (b).

DETERMINATION

Pipet 50 ml whisky into a platinum dish (thoroughly cleaned with boiling concentrated HCl followed by hot concentrated H_2SO_4), evaporate to dryness on a steam bath, and then completely destroy the organic material with a Meker flame. Add 1 ml concentrated H_2SO_4 to the residue in the dish and heat to fumes on an asbestos-covered hot plate (a low Meker flame under an asbestos-covered wire

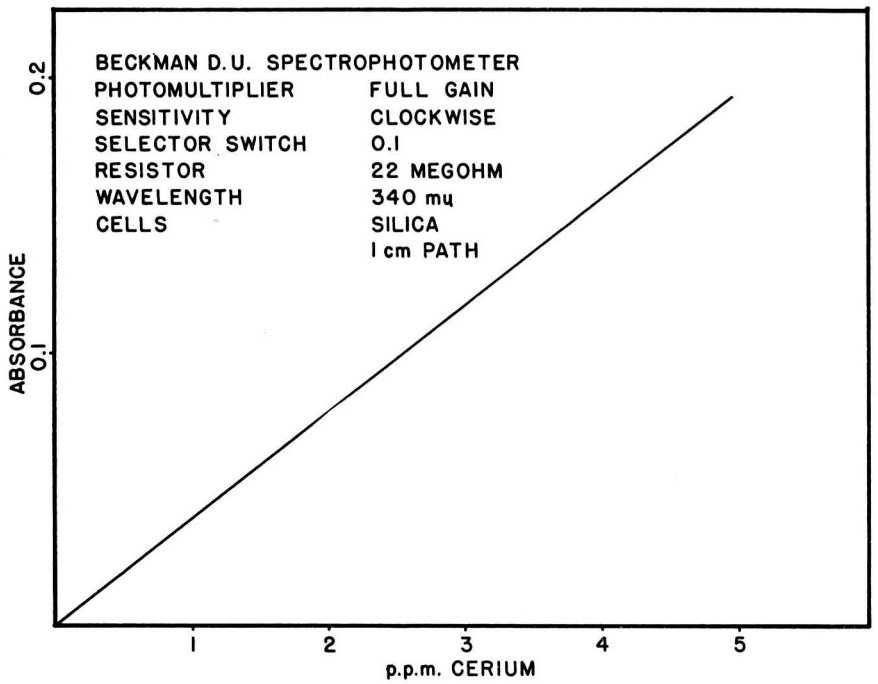

FIG. 1.—Calibration curve for cerium.

gauze may be used). Heat the dish until the dense fumes almost cease, or until only a trace of H_2SO_4 remains in the dish. Add 5.0 ml H_2O to the dish (solution should be acid at this point), followed by 5.0 ml K_2CO_3 solution, and mix well. Read the absorbance of the sample against the reference solution in clean silica cells at 340 mμ.

Calculations.—Ppm cerium $= C_{std} \times (10/50) \times (A_{sample} - 0.020)/A_{std}$, where C_{std} = concentration of standard and 0.020 = average blank obtained for 30 whiskies.

RESULTS AND DISCUSSION

The accuracy and reproducibility of this procedure was studied by adding increments of cerium (0.1–1.0 ppm) to bourbon, corn, rye, and unaged whiskies and determining the cerium concentration by the procedure previously described. The data presented in Table 1 show that these quantities of cerium can be determined with an accuracy of at least 0.1 ppm.

INTERFERENCES

To study the effect of many ions, some of which are normally found in whisky and others are introduced as markers, 1 ppm cerium and 1 ppm each of aluminum, barium, boron, cadmium, calcium, cesium, chromium, cobalt, copper, gold, indium, iron, lithium, lanthanum, magnesium, manganese, molybdenum, neodymium, nickel, platinum, praesodymium,

TABLE 1.—*Cerium determinations in whisky*

CERIUM ADDED, PPM	CERIUM FOUND, PPM			
	BOURBON	RYE	CORN	UNAGED SPIRITS
0.10	0.10	0.07	0.05	0.10
0.20	0.28	0.23	0.23	0.21
0.30	0.37	0.31	0.30	0.26
0.40	0.45	0.48	0.37	0.44
0.50	0.54	0.47	0.56	0.51
0.60	0.66	0.63	0.60	0.65
0.70	0.76	0.75	0.76	0.67
0.80	0.80	0.79	0.76	0.74
0.90	0.82	0.82	0.83	0.84
1.00	0.97	1.03	0.93	0.96

rubidium, samarium, selenium, strontium, thallium, thorium, tungsten, vanadium, zinc, and zirconium were added to whisky and the cerium was determined by the foregoing procedure. Iron was the only cation found to affect the cerium recovery by as much as 0.1 ppm.

Since some iron is generally found in whisky, the absorbance it contributed at 340 mμ was studied in detail. Varying concentrations of iron were added to iron-free whisky containing 1 ppm cerium and the cerium was determined by the procedure previously described. The following tabulation shows that iron concentrations up to 0.5 ppm do not affect the accuracy claimed for the cerium determination:

Iron added, ppm	0.10	0.20	0.30	0.40	0.50	1.00
Cerium found, ppm	1.01	1.04	0.97	1.03	1.05	1.30

No normal whisky contains this much iron because it gives an off-color to the spirits, and thus manufacturers try to prevent iron contamination. However, if the whisky does contain more than 0.5 ppm, the iron can be determined by the *o*-phenanthroline procedure (13), and a blank containing the predetermined amount of iron is then carried through the procedure.

SUMMARY

A rapid and simple ultraviolet spectrophotometric method has been presented for the determination of cerium in the presence of many diverse ions. The results indicate that this method is accurate enough to determine cerium within 0.1 ppm.

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SIEVE ANALYSIS OF SOME FERTILIZER MIXTURES*

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Sampling of mixed fertilizer containing pelleted materials, particularly those mixes without phosphate, has been recognized as a serious problem with some of the high analysis grades used in Florida. Failure to meet the tolerance for nitrogen was found more frequently in these mixes than in lower analysis mixes that derive most of their nitrogen from ammoniated superphosphate.

In order to study this problem a Florida group, representing the State Department of Agriculture, the fertilizer industry, and the Agricultural Experiment Station, met and outlined a Fertilizer Research Control Project. Interested consumer groups were also invited as observers.

Seven fertilizer mixtures were made in bag and bulk at two mixing plants; the mixing and the sampling, both of the materials for the fertilizers and of the mixed fertilizer, were observed closely. Bag and bulk goods were loaded on trucks and driven about 45 miles, after which samples were drawn again by the official samplers.

Samples taken for sieve analysis were drawn from 10 bags by the official sample tube and composited from the cores. In the case of samples of materials and of the bulk goods, the portion for sieve analysis was obtained by passing the official sample twice through a riffle-splitter. The purpose of the sieve analysis was to compare the distribution of particle sizes of the mix with that of the materials entering the mix to determine the efficiency of the sampling.

This paper presents the theoretical considerations, the procedure used in the sieve analysis, and the interpretation of the data.

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STATISTICAL CONSIDERATIONS AND PROCEDURE

True samples of mixed fertilizer contain each ingredient in the same proportionate weight as that entering the mix. This can happen only if the fertilizer is homogeneous after proper mixing and if the sampling tool withdraws the particles of each material in the correct amount. This sampling is subject to a standard error which must be much less than the legal tolerance, since sampling itself represents only a part of the deviation from the true values sought in the chemical analysis of the sample.

If a material such as a pelleted nitrogen source is not taken into the sample in the ideal proportion, then the sample is biased negatively or positively, depending on whether too few or too many pellets were retained in the sample drawn from the mix. The materials remaining in the mix must show a corresponding bias inverse to that of the pelleted material.

This physical relationship of the particles is such that the sum of the distributions of particle sizes of the materials proportionately entering the mix is the distribution of particle sizes of the fertilizer. Sieve analysis of fertilizer materials and of fertilizer mixtures can determine these distributions if the method reduces both materials and mix to particle sizes that are consistently characteristic of each material. This applies to all types of material entering the mix, whether pelleted, caked, or granular, and to the fertilizer mixture in which particles adhering to one another are all reduced to the originally stable particle sizes. Sieve analysis is subject to the usual statistical errors and to special errors due to the properties of the materials.

Sieving errors have been studied by Herdan (1). He concluded that reducing the sample size was far more effective than prolonging the sieving, and that the smaller the sieve mesh the more serious the error introduced by overloading the sieves.

Another source of error was found to be random orientation of particles: angular particles were subject to oriented passage through a sieve, whereas spherical particles were not affected. Errors arising from slight variations in the apertures in the sieves and errors of observation he classified as having a Normal or Gaussian distribution.

The error due to random sampling of material to be sieved applies both to drawing the official sample from the fertilizer mixture and to subsampling for the sieve or chemical analysis. Particles in mixed fertilizer are so varied in shape and size that a perfectly homogeneous mix cannot be attained. A composited sample obtained by withdrawing many cores of the fertilizer is more likely to be representative of the fertilizer mix than a composite sample from single or few cores. Splitting the sample by a riffle likewise is more accurate than withdrawing a sample by a scoop or other single act of sampling.

According to Herdan (1) if the percentage of particles that pass a specified sieve is p and the percentage retained on the sieve is q, and if the

number of particles is N, then the standard error of the percentage of the particles retained on the sieve is given by $\sqrt{pq/N}$.

This relationship shows that the sampling error can be reduced by increasing the size of the sample. Assuming that the Binomial or Bernoullian Law is valid for the sample, then in repeated trials the true mean sample percentage lies within twice the standard error of the percentage with a 95 per cent probability.

The sieve analyses of the fertilizers used in the present study were examined statistically in developing the procedure and in evaluating the ensuing data.

PROCEDURE

Preparation of the Sample and Sieve Analysis.—Samples drawn at the mixing plant were placed in metal cans and sealed with masking tape. Setting up of the sample was slight and varied slightly between mixtures. Materials such as calcium nitrate, ammonium nitrate, and nitrate of soda which are deliquescent showed little or no caking; apparently little moisture had entered the cans.

All samples and materials were exposed to 250 watt reflector-type infrared lamps placed 12 inches above the sample which was spread out on aluminum pans $6\frac{1}{2} \times 10\frac{1}{2}$ inches at the base. Drying time was usually 10 minutes, although samples covering the pan to a depth of more than $\frac{1}{4}$ inch had to be stirred and heated longer to insure dryness. (It was not desirable to dry samples at 100°C. in an oven because of chemical reaction.) Sieves were also dried by the infrared lamps.

Samples dried in this fashion were put through a riffle-splitter. A Ro-tap Testing Sieve Shaker was connected to an automatic timing device and fitted with the No. 10, 20, 40, 60, 80, 100, and 140 U.S. Standard sieves. Sieve fractions were emptied onto dry aluminum foil, and any fertilizer was carefully removed from the screen by a wide camel's hair brush.

Visual inspection and low-power lens showed that 3 minutes' shaking was sufficient to break down the sample to the primary particle sizes found in the materials. Longer shaking, up to 12 minutes, altered by less than 2 per cent the sieve fractions found for ammonium nitrate, calcium nitrate, ammonium sulfate, dolomite, vermiculite, sulfate of potash-magnesia, superphosphate, peanut hulls, and castor pomace. Caked materials such as muriate of potash and N.S. base showed a 2 per cent increase in the material passing the No. 140 sieve when they were shaken another 3 minutes, with a corresponding 0.5 per cent loss on the No. 10 sieve and the remainder from the finer sieves. Fertilizer mixtures showed less than 1 per cent change in weight on any sieve when they were shaken for 6 minutes instead of 3 minutes.

Sieve Fractions, Formulations, and Reproducibility of Materials and Fertilizers.—Each sample was put through the sieving process in triplicate

by using the riffle-splitter to obtain one ½ and two ¼ portions of the sample weighing about 500 grams. These subsamples were weighed before sieving, and the combined sieve weights agreed to within 0.1 per cent with the weight of the sample.

The sieve analyses of materials used in the 7 fertilizer grades are shown in Table 1. Sieve fraction is the weight of the material remaining on the sieve as percentage of the total after the shaking period, and material passing the No. 140 sieve is designated as fines.

Agreement between triplicate determinations was within 7 per cent for

TABLE 1.—*Sieve analysis of fertilizer materials*

FERTILIZER MATERIALS	PER CENT IN SIEVE FRACTION:						
	#10	#20	#40	#60	#80	#140	FINES
Materials Used for Fertilizers without Phosphate							
Calcium nitrate	65.77	29.72	1.27	0.70	0.77	1.11	0.66
Ammonium nitrate	7.55	88.22	3.25	0.55	0.17	0.16	0.10
Nitrate of soda	16.60	61.65	15.65	2.69	0.98	1.06	1.37
Sulfate of potash Magnesia (Sul-Po-Mag)	16.00	39.21	22.16	10.14	3.84	4.17	4.48
Sulfate of ammonia	0.86	29.38	44.28	18.57	3.86	2.12	0.93
Muriate of potash	0.00	2.51	19.92	28.07	14.94	20.27	14.29
Vermiculate fines	0.00	4.63	30.27	25.35	12.66	18.24	8.85
Dolomite	5.06	9.82	9.56	7.79	5.08	9.77	52.92
Materials Used for Fertilizers with Phosphate							
N. S. base	16.07	18.68	20.21	15.20	8.70	5.32	15.82
Superphosphate	9.96	12.80	14.72	14.92	9.41	6.54	31.65
Muriate of potash	2.46	2.05	20.24	28.51	17.57	9.94	19.23
Castor pomace	0.94	41.83	35.68	12.30	3.84	1.84	3.57
Peanut hulls	3.17	31.18	35.04	12.40	5.12	2.88	10.21
Dolomite	3.79	12.03	12.10	8.49	4.83	4.41	54.35

the largest sieve fraction and, almost without exception, to within 2 per cent for the other sieve fractions. Larger deviation resulted if the sample of material was not dried sufficiently, in which cases the sieving was repeated after further exposure of the sample to infrared heat. The range of deviation for the mixed fertilizer samples was similar.

The theoretical sieve analyses for the 7 fertilizer grades, given in Tables 2 and 3, were calculated from the data in Table 1 and from the formulation sheets. The column for total material entering the mixture shows the formulation of each fertilizer.

Sampling and Sieving of Mixed Fertilizer.—Seven fertilizer grades in largest demand in Florida were chosen for the sampling study. Ten tons of each fertilizer was bagged and stacked on a truck. Composite samples

TABLE 2.—*Calculated sieve analysis of the mixes containing phosphate*

MATERIAL	PER CENT IN SIEVE FRACTION:							
	#10	#20	#40	#60	#80	#100	FINES	TOTAL
4–7–5 Mixture (25% Organic)								
N.S. base	7.18	8.34	9.02	6.79	3.88	2.38	7.06	44.65
Muriate of potash	0.19	0.16	1.59	2.24	1.38	0.78	1.51	7.85
Castor pomace	0.16	7.35	6.26	2.16	0.67	0.32	0.63	17.55
Dolomite	1.13	3.60	3.62	2.54	1.44	1.32	16.30	29.95
Total	8.66	19.45	20.49	13.73	7.37	4.80	25.50	100.00
4–8–8 Mixture (20% Organic)								
N. S. base	7.53	8.75	9.47	7.12	4.08	2.49	7.41	46.85
Muriate of potash	0.32	0.27	2.61	3.69	2.28	1.29	2.49	12.95
Superphosphate	0.34	0.44	0.50	0.50	0.32	0.22	1.08	3.40
Castor pomace	0.13	5.88	5.01	1.73	0.54	0.26	0.50	14.05
Dolomite	0.86	2.74	2.75	1.93	1.10	1.00	12.37	22.75
Total	9.18	18.08	20.34	14.97	8.32	5.26	23.85	100.00
4–10–7 Mixture								
N. S. base	9.36	10.89	11.78	8.86	5.07	3.10	9.24	58.30
Muriate of potash	0.28	0.24	2.35	3.31	2.04	1.15	2.23	11.60
Superphosphate	0.44	0.57	0.66	0.66	0.42	0.29	1.41	4.45
Peanut hulls	0.16	1.56	1.75	0.62	0.26	0.14	0.51	5.00
Dolomite	0.78	2.48	2.50	1.75	1.00	0.91	11.23	20.65
Total	11.02	15.74	19.04	15.20	8.79	5.59	24.62	100.00

MATERIAL	PER CENT IN SIEVE FRACTION:							
	#10	#20	#40	#60	#80	#140	FINES	TOTAL
8–0–12 Mixture								
Ammonium nitrate	0.94	11.03	0.41	0.07	0.02	0.02	0.01	12.50
Calcium nitrate	17.82	8.05	0.35	0.19	0.21	0.30	0.18	27.10
Sulfate of potash magnesia	5.28	12.94	7.31	3.34	1.27	1.38	1.48	33.00
Muriate of potash	0.00	0.21	1.64	2.31	1.23	1.66	1.15	8.20
Vermiculite fines	0.00	0.11	0.76	0.64	0.31	0.46	0.22	2.50
Dolomite	0.85	1.64	1.60	1.30	0.85	1.63	8.83	16.70
Total	24.89	33.98	12.07	7.85	3.89	5.45	11.87	100.00
10–0–10 Mixture								
Ammonium nitrate	1.42	16.59	0.61	0.10	0.03	0.03	0.02	18.80
Ammonium sulfate	0.17	5.73	8.64	3.62	0.75	0.41	0.18	19.50
Sulfate of potash magnesia	5.28	12.94	7.31	3.34	1.27	1.38	1.48	33.00
Muriate of potash	0.00	0.12	0.98	1.38	0.73	0.99	0.70	4.90
Vermiculite fines	0.00	0.11	0.76	0.64	0.31	0.46	0.22	2.50
Dolomite	1.08	2.09	2.04	1.66	1.08	2.08	11.27	21.30
Total	7.95	37.58	20.34	10.74	4.17	5.35	13.87	100.00
12–0–10 Mixture								
Ammonium nitrate	1.89	22.14	0.82	0.14	0.04	0.04	0.03	25.10
Ammonium sulfate	0.16	5.58	8.43	3.52	0.73	0.40	0.18	19.00
Sulfate of potash magnesia	5.28	12.94	7.31	3.34	1.27	1.38	1.48	33.00
Muriate of potash	0.00	0.13	1.00	1.40	0.75	1.01	0.71	5.00
Vermiculite fines	0.00	0.11	0.76	0.64	0.31	0.46	0.22	2.50
Dolomite	0.78	1.51	1.47	1.20	0.78	1.50	8.16	15.40
Total	8.11	42.41	19.79	10.24	3.88	4.79	10.78	100.00
8–0–8 Mixture								
Ammonium nitrate	0.94	11.03	0.41	0.07	0.02	0.02	0.01	12.50
Nitrate of soda	4.36	16.21	4.12	0.71	0.26	0.28	0.36	26.30
Sulfate of potash magnesia	5.28	12.94	7.31	3.34	1.27	1.38	1.48	33.00
Muriate of potash	0.00	0.04	0.34	0.48	0.25	0.35	0.24	1.70
Vermiculite fines	0.00	0.11	0.76	0.64	0.31	0.46	0.22	2.50
Dolomite	1.21	2.36	2.29	1.87	1.22	2.34	12.71	24.00
Total	11.79	42.69	15.23	7.11	3.33	4.83	15.02	100.00

for 10 bags at the bottom, for 10 bags in the center, and for 10 bags at the top of the pile were sampled with the official sampling tool; this provided three samples termed "bagged goods plant" for sieve analysis. After shipment by truck, 3 such samples, termed "bagged goods destination," were drawn.

Bulk goods were made for the 4 grades without phosphate, and 40 cores were withdrawn for each composite sample. This was riffled so that the State chemist and a commercial laboratory received part of the same sample used for sieve analysis. Triplicate samples were drawn. Bulk goods at destination were sampled and split similarly. Samples were taken within the plant by using a cup-like container and cutting the fertilizer stream from the mixer near the beginning, middle, and end of the batch. Triplicate composited samples were split for sieve and chemical analysis. Samples of mixed fertilizer were dried by infrared and split by the riffle, and the sieve analysis was done in the same manner as for the fertilizer materials.

Statistical Treatment of Sieve Analyses.—The difference between the theoretical sieve analysis and the observed percentage was defined as the bias. The bias for each sieve fraction of each sample was calculated and the resulting data were tested by analysis of variance with 83 degrees of freedom for samples taken within the plant, 167 for bagged goods, and 167 for bulk goods. In each case the biases for the different sieve fractions were found to be statistically different from each other; these differences depended on the particular fertilizer grade.

From chemical information on the materials entering the fertilizer, the sieve analysis, and its bias, the calculated chemical value in the fertilizer sample can be obtained. Thus for the 8–0–8 mixture the No. 10, 20, and 40 sieves should hold 1.01, 6.30, and 0.80 per cent nitrogen, respectively, and 11.79, 42.69, and 15.23 per cent of the total material. Biases found on these sieves were: −.043, −0.90, and +0.27. Bias for total nitrogen then is

$$\frac{1.01}{11.79}(-0.43) + \frac{6.30}{42.69}(-0.90) + \frac{0.80}{15.23} \times 0.27 = -0.152.$$

This compares with a bias of −0.280 reported from the chemical analysis of the same group of samples (2). Where statistically significant differences can be separated from the main body of the data, the interpretation of the data, such as the biases in sieve analysis, is made an easier task and is on a sound basis.

RESULTS AND DISCUSSION

The data for one of the fertilizer samples, the 12–0–16 mix, are shown in Table 4 to illustrate the type of information found by the sieve analysis and the variability found between samples.

Bias of observed values from the theoretical values for the mixtures without phosphate are given in Table 5. Differences for sampling bags

TABLE 4.—*Sieve analysis of samples of bagged and bulk goods of 12–0–10 fertilizers*

SAMPLE	PER CENT IN SIEVE FRACTIONS:						
	#10	#20	#40	#60	#80	#140	FINES
Within plant							
#1	7.27	40.04	20.73	11.41	4.69	5.59	10.27
#2	7.10	38.64	20.15	11.75	5.09	6.17	11.10
#3	6.47	38.37	20.64	12.22	5.17	6.17	10.96
Mean	6.95	39.02	20.51	11.79	4.98	5.98	10.78
Bagged goods, plant							
Bottom	6.44	39.52	20.32	11.95	4.95	5.88	10.94
Center	6.19	38.50	21.63	12.34	4.91	5.70	10.73
Top	6.44	39.33	21.38	12.02	4.81	5.62	10.40
Mean	6.36	39.12	21.11	12.10	4.89	5.73	10.69
Bagged goods, destination							
Bottom	6.36	40.28	20.60	11.91	4.74	5.57	10.54
Center	6.01	39.13	22.16	11.74	5.18	5.62	10.16
Top	6.36	41.30	21.09	11.45	4.56	5.39	9.85
Mean	6.24	40.24	21.28	11.70	4.83	5.53	10.18
Bulk goods, plant							
#1	5.64	36.93	21.94	13.32	5.57	6.29	10.31
#2	6.27	37.21	21.85	12.88	5.46	6.12	10.21
#3	5.55	37.11	22.08	12.94	5.47	6.21	10.64
Mean	5.82	37.08	21.96	13.05	5.50	6.21	10.39
Bulk goods, destination							
#1	5.88	36.79	21.97	13.43	5.16	6.45	10.32
#2	5.79	38.09	21.56	12.39	5.24	6.14	10.79
#3	5.97	36.47	21.81	13.04	5.55	6.28	10.88
Mean	5.88	37.12	21.78	12.95	5.32	6.29	10.66
Theoretical value	8.11	42.41	19.79	10.24	3.88	4.79	10.78

TABLE 5.—*Average biases in sieve analysis of fertilizer mixtures without phosphate*

FERTILIZER GRADE	SIEVE FRACTIONS							
	#10	#20	#40	#60	#80	#140	FINES	STANDARD ERROR
Within Plant								
8–0–8	−0.73	−0.67	0.81	1.16	0.68	0.31	−1.56	
8–0–12	+1.46	−4.73	3.46	3.11	2.12	1.56	−6.94	
10–0–10	−1.88	−4.54	2.14	2.36	1.25	1.18	−0.52	±0.394
12–0–10	−1.16	−3.39	0.72	1.55	1.10	1.19	−0.00	
Means	−0.58	−3.33	1.78	2.05	1.29	1.06	−2.26	±0.197
In Bags								
8–0–8	−0.43	−0.90	0.27	0.87	0.66	0.40	−1.05	
8–0–12	+1.20	−3.15	2.15	2.55	1.76	1.88	−6.39	
10–0–10	−0.67	−1.30	0.79	1.37	0.88	0.39	−1.45	±0.280
12–0–10	−1.81	−2.73	1.41	1.66	0.98	0.84	−0.34	
Means	−0.43	−2.02	1.15	1.61	1.07	0.88	−2.31	±0.140
In Bulk								
8–0–8	−1.63	−1.80	1.62	1.07	1.07	0.57	−0.94	
8–0–8	−0.33	−4.89	3.82	3.89	2.38	1.70	−6.61	
10–0–10	−2.45	−4.69	2.75	2.70	1.58	1.29	−1.19	±0.280
12–0–10	−2.26	−5.31	2.08	2.76	1.53	1.46	−0.26	
Means	−1.67	−4.17	2.56	2.61	1.64	1.25	−2.25	±0.140

located in the bottom, middle, or top of the pile on the truck and differences between samples drawn at the plant and at destination were not statistically significant, and are averaged in the table. These data show that particle sizes larger than those that can pass the No. 20 sieve were insufficiently sampled and the particles ranging in size between passing the No. 20 sieve and refusing the No. 140 sieve were sampled excessively. Negative bias of the fines is attributed to adhesion to larger particles. The standard error applies to each average value.

In Table 6 the average biases are shown for the fertilizer grades containing phosphate. These data again indicate some larger particles were being missed in drawing the sample, and particles passing the No. 40 sieve were less than expected.

In Table 7 the over-all sieve biases for total nitrogen and potash are indicated. The average biases found from the chemical analysis of the two

TABLE 6.—*Average biases in fertilizer mixtures containing phosphate*

FERTILIZER	SIEVE FRACTIONS							
	#10	#20	#40	#60	#80	#100	FINES	STANDARD ERROR
Within Plant								
4–7–5	+0.07	2.18	1.63	−0.83	−1.34	−0.67	−1.03	
4–8–8	−1.56	2.63	4.00	+0.63	−1.39	−1.01	−3.30	
4–10–7	−1.69	2.45	3.36	−0.50	−1.40	−0.57	−1.64	±0.357
Average	−1.06	2.42	3.00	−0.23	−1.38	−0.75	−1.99	±0.206
In Bags								
4–7–5	−1.88	2.38	1.62	−0.90	−1.03	−0.49	+0.29	
4–8–8	−2.59	2.91	3.87	+0.32	−1.24	−0.90	−2.36	
4–10–7	−3.12	2.17	3.76	−0.04	−0.95	−0.68	−1.13	±0.252
Average	−2.53	2.48	3.08	−0.21	−1.07	−0.69	−1.07	±0.146

laboratories are included for purposes of comparison. Agreement between the methods of sieve analysis and of chemical analysis is apparent; each is subject to rather large standard errors, especially for the mixtures without phosphate. Particularly important are the large negative biases for total nitrogen in the fertilizers containing a large amount of pelleted nitrogen and the large positive bias for potash found in such cases. Samples of bagged and bulk goods show similar bias to samples drawn at the mixer discharge, indicating the difficulty of sampling these mixtures.

The data on nitrate nitrogen, calculated in like fashion, gave similar negative biases that are not reported in this paper. Consistently negative bias is more likely to result from inability to draw the true distribution of particle sizes from the fertilizer mixture than from insufficient mixing, which would give high standard errors. The biases from the chemical analysis support calculated chemical biases from the sieve analysis sufficiently closely for the fertilizers containing phosphate.

The pattern of over-all sieve biases of bagged goods for fertilizer grades without phosphate is shown in Figure 1 and those for the three grades containing phosphate are presented in Figure 2. Since most of the pelleted nitrogen particles are expected to be found on the No. 10 or No. 20 sieves, the negative sieve biases can mean with reasonable certainty that not as much nitrogen is contained in the sample as in the fertilizer bag. In the case of the fertilizers containing superphosphate which was ammoniated or treated with nitrogen solution, failure to sample the larger particle sizes sufficiently is not likely to result in nitrogen deficiency, since the N.S. base

TABLE 7.—*Over-all biases for total nitrogen and potash by sieve, and chemical analysis of fertilizers without phosphate and with phosphate*

FERTILIZER GRADE	SIEVE ANALYSIS METHOD				CHEMICAL METHOD AVERAGE
	WITHIN PLANT	BAGGED GOODS	BULK GOODS	AVERAGE	
			Total Nitrogen		
8–0–8	−0.090	−0.129	−0.281	−0.167	−0.292
8–0–12	−0.409	−0.236	−0.635	−0.427	−0.860
10–0–10	−0.487	−0.064	−0.454	−0.335	−1.083
12–0–10	−0.518	−0.373	−0.742	−0.528	−1.183
Average	−0.376	−0.200	−0.528	−0.368	−0.856
4–7–5	+0.054	−0.010		+0.022	+0.175
4–8–8	+0.331	+0.062		+0.196	+0.192
4–10–7	+0.023	−0.014		+0.004	+0.308
Average	+0.136	+0.013		+0.074	+0.225
			Potash		
8–0–8	0.219	0.150	0.236	0.202	0.346
8–0–12	1.602	1.319	1.928	1.616	1.033
10–0–10	0.344	0.230	0.405	0.326	0.587
12–0–10	0.360	0.293	0.527	0.393	0.671
Average	+0.631	+0.498	+0.774	0.634	+0.660
4–7–5	−0.224	−0.159		−0.192	−0.095
4–8–8	−0.162	−0.137		−0.150	−0.294
4–10–7	+0.207	−0.030		+0.088	+0.066
Average	−0.060	−0.109		−0.084	−0.108

has a wide distribution of particle sizes; it is the dominant material on each sieve fraction except the fines.

Sub-sampling and preparation of fertilizer samples has been studied by Randle (3) and Allen (4, 5). The sub-sample deviations for a large number of fertilizer mixtures were reported by Miles and Quackenbush (6). Haven and Jacob (7) have reported a study of wet and dry sieving in the analysis of ground Florida pebble phosphate. These papers dealt with refinements in methodology and in reproducibility of results. Studies on sampling bias as such have not been reported previously, to the knowledge of the authors. Work on other fertilizer mixtures and methods of sampling has been continued under the Florida Fertilizer Control Research Project.[1]

[1] Work now in progress by G. M. Volk indicates that at least part of the bias may be eliminated by improved sampling tubes.

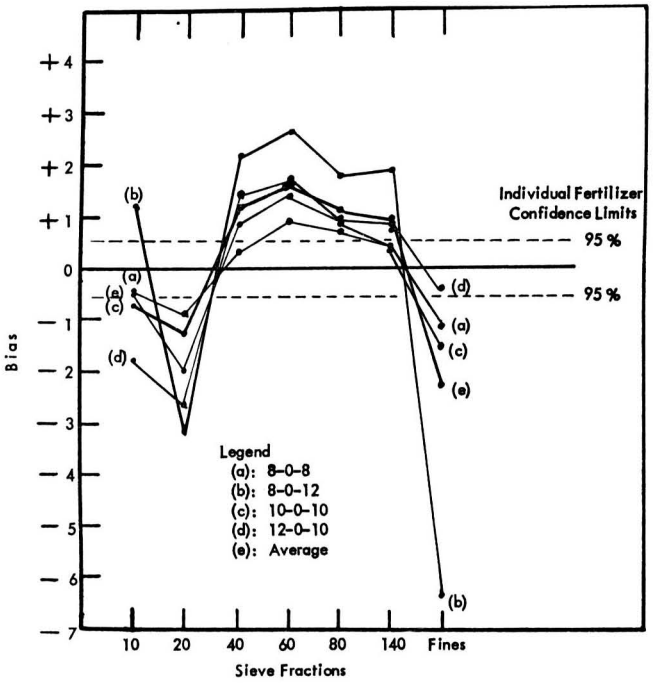

FIG. 1.—Graphs of biases against sieve numbers for grades of fertilizer that contain no phosphate.

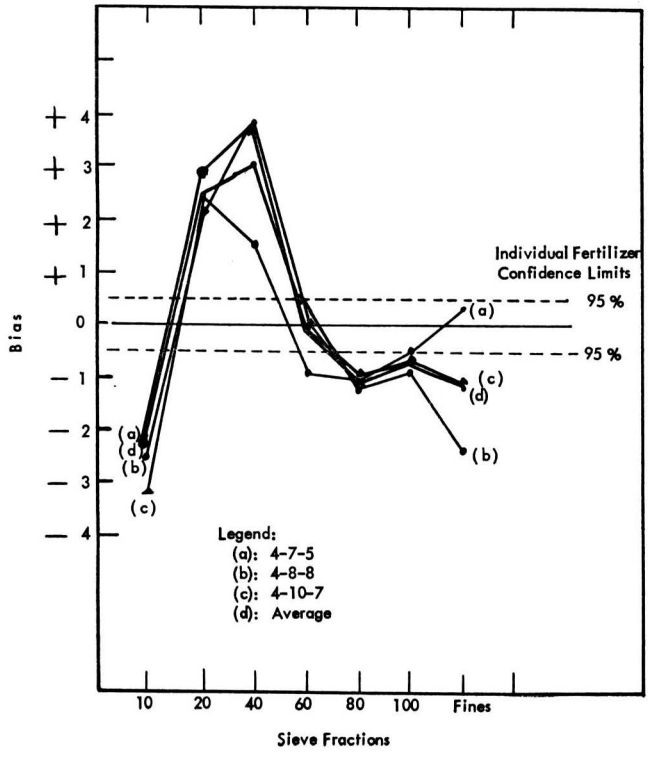

FIG. 2.—Graphs of biases against sieve numbers for grades of fertilizer that contain phosphate.

Results to date have led to legislative action on fertilizer tolerances with adoption of new tolerances based on the Model State Fertilizer Bill (8).

SUMMARY

Certain methodology and statistical considerations are required for accurate sieve analysis of fertilizer materials and mixed fertilizer. Samples were kept dry and subjected to infrared heat prior to sieving. Sieve analysis gave a reproducible particle size distribution for each material entering the fertilizer. Particle size distributions of seven fertilizers were calculated from the sieve analysis of materials and the formulation. Sieve analysis of fertilizer samples was made of bagged and bulk goods, and samples taken within the plant showed good agreement.

Conclusions for samples from within plant, from bagged goods, and from bulk goods were statistically identical. Biases of the values found from the theoretical values were significantly different between sieve fractions, and differed with fertilizer mixtures. The sieve biases were calculated in terms of total nitrogen and potash and these biases were like those found on the samples drawn for chemical analysis. Sieve analysis of mixed fertilizer was found to be a rapid method of getting information on sampling of particle size, particularly when the data were statistically analyzed.

ACKNOWLEDGMENT

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COMPARISON OF SPECTROPHOTOMETRIC AND VISUAL TITRATION FOR THE DETERMINATION OF FLUORINE*

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In a report on the determination of fluorine in soils (1), it was concluded that the lack of agreement among analysts was due to the titration step. The desirability of an instrumental method was pointed out.

It has long been recognized that the ability to judge the end point of the visual titration of fluoride with thorium nitrate requires considerable training, experience, and patience. Results obtained by different individuals vary widely. The present investigation was undertaken to ascertain the general utility and reproducibility of the photometric titration method when applied to a variety of fluoride-bearing samples. This procedure has been investigated by Chepelevetskii (2) and by Nichols and Kindt (3). As these workers confined their studies to milligram levels of fluorine, it seemed desirable to try to extend the method to microgram concentrations. Mavrodineanu and Gwirtsman (4) worked in the microgram range with solutions of pure fluorides, using two techniques: (*a*) they titrated directly; and (*b*) they added an excess of thorium nitrate and back-titrated with standard fluoride in a comparison solution.

Photometric titration has distinct advantages over direct colorimetric determination. The presence of other substances that absorb at the same wavelength does not necessarily cause interference, because only the change in absorbance is significant. Locating the titration line by pooling the information derived from several points is more precise than using any single point.

The fundamental law of monochromatic-light absorption underlying photometric titrations is Beer's law: $A_s = -\log T = abc$, in which A_s signifies absorbance; T, transmittance; a, absorbance index; b, length of light path through the absorbing medium; and c, concentration of light-absorbing constituent. Absorbance, the quantity directly measured on a spectrophotometer, is linearly proportional to the concentration of the absorbing ions. This means that in a titration the plot of absorbance *versus* titrant added will consist of two straight lines that intersect at the end point. Because of this linear relation of absorbance to concentration, the plot of a photometric titration will often show an appreciable break, even though the changes in concentration are too small to show a clearly defined color change in visual titration.

The photometric titration method should be particularly applicable to

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the titration of the fluoride with thorium nitrate in the presence of alizarin red S. The fluoride is present in solutions of low concentration and the reaction is appreciably incomplete at the equivalence point. Thus the plot of the titration tends to be rounded, rather than linear, near the end point. However, no particular importance need be attached to points taken in the vicinity of the end point. The end point can be located accurately by extrapolating the straight-line portions of the curve until they intersect.

The absorbance of the solution will depend on the absorbant characteristics of the thorium-alizarin red S lake. A relatively large amount of indicator should be added to obtain a good straight line beyond the equivalence point. However, the actual amount of indicator added must be carefully controlled, as will be shown later.

EXPERIMENTAL

Photometric Titrimeter.—A satisfactory arrangement was obtained by slightly modifying the Beckman Model B spectrophotometer, as illustrated by Goddu and Hume (5). The cell carriage, the cover, and the floor to the cell compartment were removed. The 150 ml beakers (selected for uniformity), used as titration vessels, were held by a wooden support that rested on the cell carriage rods. The spectrophotometer was raised by setting it on a wooden base in order to accommodate a conventional magnetic stirrer with its housing removed. The cover of the cell compartment was replaced with one made of masonite, fastened to the hinge with two short bolts. This cover *must* be light-tight. The tip of a microburet was inserted through a $\frac{3}{8}$ inch hole drilled in the cover so that it dipped into the sample solution near the side of the beaker without obstructing the light path.

Equally suitable are any narrow band-pass filter photometers, such as the Klett-Summerson colorimeter, or abridged spectrophotometers such as the Bausch & Lomb Spectronic 20. The test tube holder of the Spectronic 20 is replaced by a tube 1 inch in diameter. An overhead stirrer is employed with these two instruments.

PROCEDURE

Set the spectrophotometer to a wavelength of 540 mμ and let it warm up; then adjust the dark current. Add the proper amount of indicator and buffer, place the beaker in the light path, and close the cover. Lower the buret so that the tip extends through the hole and into the solution; then set the instrument to read zero absorbance. Start the stirrer and adjust the speed so that the stirring cone does not obstruct the light path. Begin the titration by slowly adding the titrant from the buret until the needle indicates that 0.20 absorbance has been reached; then record this volume. Repeat the addition for 0.30 and 0.40 absorbance, recording these volumes likewise. Plot the three readings against the three absorbance points and take the intersection of the extrapolated line with the volume axis as the end point. Use this procedure to prepare a standard curve by titrating aliquots of sodium fluoride, ranging from 0 to 200 mmg fluorine in 10 mmg increments, and plotting the

end point values *vs.* mmg fluorine. Read the amount of fluorine in unknown samples from this standard calibration curve.

Selection of Wavelength.—The operating wavelength is selected to satisfy two considerations: to avoid interference by other absorbing substances, and to obtain an absorbance index such that the change in absorbance during the titration falls within a convenient range. The second consideration is important because serious photometric error is possible in high-absorbance regions.

The yellow acid-form of alizarin red S shows no absorption at wavelengths longer than 530 mμ. The lake exhibits a rather broad absorbance maximum centered around 515 mμ (3). However, in working with pure standards, a wavelength between 515 and 580 mμ is suitable for spectrophotometric titrations. For a spectrophotometer and with distillates that sometimes contain gray color bodies, the wavelength chosen was 540 mμ, whereas for photoelectric colorimeters a narrow band-pass filter centering at that wavelength should be selected.

Optimum Amount of Indicator.—In the titration of fluoride with thorium nitrate the color reaction involves formation of a thorium-lake, beginning in the vicinity of the equivalence point. For a photometric titration a relatively large quantity of indicator must be added, so that the whole amount is not converted until well past the equivalence point.

Since the thorium-fluoride reaction is not quantitative at the equivalence point, nor does the stability constant of the thorium-alizarin lake differ appreciably from the constant for thorium fluoride formation, the amount of indicator to be used must be decided carefully. If too little indicator is used, the curvature around the equivalence point will overlap the region where the excess of indicator is exhausted and no straight portion will be obtained in the rising branch of the titration curve. On the other hand, as more indicator is added the apparent end point occurs at smaller volumes of titrant; this is probably due to common ion effect. Therefore, the amount of indicator added must be measured out within 5 per cent.

For the preliminary work with the Klett-Summerson colorimeter and 40 mm cells containing 40 ml of solution, 0.5 ml of 0.1 per cent alizarin red S was used; for the Beckman Model B spectrophotometer and 100 ml solution in 150 ml beakers, 1.5 ml of 0.1 per cent indicator was used. These quantities provided the steepest slope in the rising branch of the titration curve without unduly compressing the ratio of the volume of thorium nitrate to fluoride present.

In any case, enough indicator should be present to provide a linear portion on the rising branch of the titration curve that extends from 0.20 to 0.40 absorbance units.

Nature of the Titration Curve.—The titration curve consists of two branches; the portion preceding the equivalence point shows a gradually

increasing absorbance that becomes more pronounced in the vicinity of the equivalence point and the rising portion after the equivalence point. The latter part of the curve exhibits a linear region between 0.20 and 0.40 absorbance for amounts of fluorine less than 200 mmg per 100 ml when the recommended quantity of indicator is used.

The extrapolated part of the rising branch always passes through zero for blank titrations. For simplicity, the end point was always taken as the point of intersection of the rising branch when extrapolated to the volume axis. This seemed justified because the earlier portion of the curve preceding the end point actually showed 0 absorbance when reasonably large quantities of fluoride were titrated. The calibration curve, prepared as described in the procedure, was linear for amounts of fluorine less than 120 mmg but showed curvature towards the concentration axis for larger amounts.

With increasing amounts of fluoride, the slope of the rising branch becomes progressively smaller and the curvature in the vicinity of the end point larger. These two factors limit the fluorine concentrations to 150 mmg or less for optimum precision and reproducibility.

Figure 1 shows a typical set of photometric titration curves for stand-

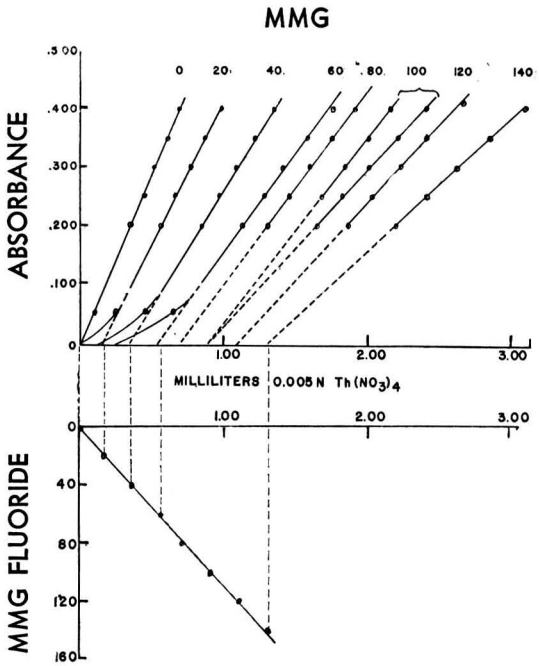

FIG. 1.—Typical series of photometric titration curves and corresponding calibration curve.

ards at different levels of fluorine concentration. The slope of the curve may change somewhat without altering the intercept on the volume axis. (Note the 100 mmg curves.)

DISCUSSION OF RESULTS

As a basis upon which to judge the results obtained by the photometric titration method in relation to the visual method, it seemed desirable to estimate the standard deviation for the visual thorium nitrate titration. The results reported in 1953 by nine collaborators (1) are given in Table 1. These are the average values of triplicate titrations on 50 and 100 ml

TABLE 1.—*Collaborative results for fluorine on a composite of soil distillates*

	AVERAGE FLUORINE CONTENT,[a] PPM								
100 ml aliquot	290	293	312	259	296	277	302	285	281
50 ml aliquot	290	320	300	265	285	272	330	287	281

	100 ml aliquot	*50 ml aliquot*
Number of observations, n:	9	9
Mean, $\bar{x}$:	288	292
Estimate of variance, s^2 $=S(x-\bar{x})^2/(n-1)$:	236	453
Estimate of standard deviation, s:	15	21

[a] Average of triplicate titrations from each collaborating laboratory.

aliquots of the composite of soil distillates. In aliquots that contained 58 mmg of fluorine, the *lsd* at the 5 per cent level was 6 mmg. Similarly, for 29 mmg of fluorine the *lsd* was 4 mmg. Deviations in the same order of magnitude have been observed repeatedly in comparing replicated routine samples at these levels of fluorine concentration.

The first series of comparisons between the photometric titration method and the usual visual method were made on 250 ml composites of replicated distillations from perchloric acid at 137°C.

The results obtained on the replications are shown in Table 2. Although the replicate values are given in parts per million, the statistical treatment, shown in Table 3, involved the actual micrograms of fluorine obtained in the titration of each aliquot before this value was adjusted by any factor to reflect the sample weight. Except for the urine results by photometric titration, the standard deviation is equal to, or less than, the value obtained in the earlier collaborative study of the visual titration used on the soil distillate. Thus, there is no evidence that either method is more reproducible than the other.

No bias was found between the two titration methods for the alfalfa

TABLE 2.—*Comparison of replicate photometric and visual titrations on distillates from various types of samples*

REPLICATIONS	FLUORINE CONTENT, PPM							
	URINE		GRAIN		BONE		ALFALFA	
	INSTRUMENTAL	VISUAL	INSTRUMENTAL	VISUAL	INSTRUMENTAL	VISUAL	INSTRUMENTAL	VISUAL
1	30	37	405	410	8200	9200	58	64
2	31	39	390	410	8300	9200	57	62
3	31	37	385	395	8600	9500	57	55
4	36	38	375	435	9500	9500	62	54
5	35	37	375	420	8300	9200	59	58
6	33	38	395	355	8100	8600	59	58
7	33	37	385	390	8300	9200	59	58
8	34	40	375	410	8800	8600	61	59
9	43	37	385	420	8900	9200	57	61
10	37	38	375	405	9100	9400	59	—
11	—	—	390	—	—	—	—	—
12	—	—	390	—	—	—	—	—
Mean, $\bar{x}$	34	38	385	410	8600	9160	59	59

samples. However, in the case of the urine, grain, and bone samples, the fact that zero is outside the limits of the bias indicates that the photometric titration method gives results with a negative bias in comparison with the visual method. It should be noted, however, that zero is close to the lower limit of the bias. Furthermore, the actual standard deviations are smaller than would be expected in routine work. Were the deviations "normal," the bias for the grain and the bone series would be on the borderline of significance at the 5 per cent level. The higher apparent reproducibility is undoubtedly due to the greater care exercised in these analyses than is usual under routine conditions.

During the preparation of the calibration curve for the photometric titration method, numerous sets of standards were run. From the different levels of concentrations of these standards, and also from the results in Table 2, the random errors are approximately proportional to the mean (at least where the titration aliquot contained more than 60 mmg fluorine) and are 4 per cent of the mean. Below 60 mmg of fluorine, the random error appears to be essentially constant and equal to ± 3 mmg, or 6 mmg at the 5 per cent level of significance.

As a final comparison of the two methods, a number of results from routine titrations are assembled in Tables 4 and 5. From Table 4 the relative bias for the series of vegetation samples is $+1$ with the uncertainty at the 5 per cent level of ± 4 mmg. When the soil results shown in Table 5 are grouped at two levels of fluoride concentration, a negative bias

TABLE 3.—*Statistical treatment of data from Table 2*

	URINE[a]		GRAIN[b]		BONE[c]		ALFALFA	
	INSTRUMENTAL	VISUAL	INSTRUMENTAL	VISUAL	INSTRUMENTAL	VISUAL	INSTRUMENTAL[d]	VISUAL[e]
Mean, $\bar{x}$, and Std Dev., ppm	34 ± 4	38 ± 1	385 ± 9	410 ± 14	8600 ± 460	9150 ± 340	59 ± 2	59 ± 3
Mean, $\bar{x}$, mmg per aliquot titrated	86 ± 10	95 ± 3	77 ± 2	81 ± 3	86 ± 5	92 ± 3	59 ± 2	118 ± 6
Bias, instrumental to visual method, mmg	−9		−4		−6		None	
Limits of bias at 95% probability level	−2 to −16		−1 to −7		−2 to −9		None exists	

[a] 25 g sample digested and distilled; 25 ml portion used for titration from 250 ml distillate.
[b] 5 g sample taken and 10 ml aliquot titrated.
[c] 0.25 g sample taken and 10 ml aliquot titrated.
[d] 5 g sample taken and 50 ml aliquot titrated.
[e] 5 g sample taken and 100 ml aliquot titrated.

TABLE 4.—*Comparison of photometric and visual titration on distillates from vegetation samples*

SAMPLE NO.	FLUORINE CONTENT, MMG		x^a	x^2
	INSTRUMENTAL	VISUAL		
1	114	121	− 7	49
2	130	136	− 6	36
3	132	118	+14	196
4	122	113	+ 9	81
5	145	127	+18	324
6	128	127	+ 1	1
7	103	103	0	0
8	132	131	+ 1	1
9	137	138	− 1	1
10	106	94	+12	144
11	153	163	−10	100
12	154	163	− 9	81
13	167	182	−15	225
14	186	183	+ 3	9
15	148	147	+ 1	1
16	149	147	+ 2	4
		Totals	+13	1253

Observations, $n = 16$
Mean bias, $\bar{x} = +1$
Estimates of variance and standard deviation: $s^2 = 83$
$s = 9$
$s_m^2 = 5$
$s_m = 2.2$

[a] x = photometric minus visual.

appears to exist at the lower level (78 mmg per aliquot), whereas no bias exists at the higher level (approximately 130 mmg per aliquot):

Samples 1 thru 10	Samples 11 thru 18	All the Samples
Relative bias = −5	Relative bias = −2	Relative bias = −4
Limits of relative bias:	Limits of relative bias:	Limits of relative bias:
−2.5 to −7.5	−16 to +12	−10 to +2

Taken together, there appears to be no bias between the methods. More work is needed to clarify this point.

SUMMARY AND CONCLUSIONS

The comparisons of results of fluorine determinations made by the visual titration and by the spectrophotometric titration showed significant agreement on a variety of samples. The results are considered comparable, although there was a slight negative bias with the spectrophotometer.

TALE 5.—*Comparison of photometric and visual titrations for the determination of fluorine in soils*

SAMPLE NO.	FLUORINE CONTENT, MMG		x^a	x^2
	INTSRUMENTAL	VISUAL		
1	73	73	0	
2	82	86	− 4	16
3	75	78	− 3	9
4	88	100	−12	144
5	73	84	−11	121
6	87	94	− 7	49
7	65	66	− 1	1
8	73	76	− 3	9
9	67	71	− 4	16
10	68	73	− 5	25
		Subtotals	−50	390
11	130	118	+12	144
12	104	104	0	
13	120	156	−36	1296
14	112	133	−21	441
15	137	136	+ 1	1
16	100	97	+ 3	9
17	156	150	+ 6	36
18	165	146	+19	361
		Subtotals	−16	2288
		Totals	−66	2678

[a] x = photometric minus visual.

It is estimated that the visual titration by an experienced operator is about twice as rapid as the spectrophotometric titration. The use of the instrument, however, should be a valuable adjunct in laboratories where fluorine titrations are intermittent and where the analyst may not be experienced in the visual titration. Adaptation of an automatic buret and recording titrimeter might increase the speed of the titration.

ACKNOWLEDGMENT

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FATE OF AIR-BORNE FLUORIDES AND ATTENDANT EFFECTS UPON SOIL REACTION AND FERTILITY*

By WALTER H. MACINTIRE†

INTRODUCTION

The seriousness of fluoric contamination of the atmosphere received recognition through the requested papers that were presented at the conference called by President Truman at Washington in 1952 (6a, 9a, 27, 70).

Chemical, biochemical, and mechanical techniques have been used to determine air-borne fluorides at certain locales where aluminum is manufactured, and where rock phosphate is processed into phosphorus, phosphoric acid, and fertilizers (1, 2, 6, 6a, 7, 9, 9a, 15, 18, 21, 24, 27, 33, 38, 39, 49, 50, 51, 70). Abnormal amounts of fluorine in forage vegetation near such operations have been attributed to air-derived fluorides, ingestion of which is purported to injure livestock (9, 19, 24, 51a, 53a). In the United States, however, 27 other industrial operations cause emissions of fluorine (27).

Before the remarkable expansion of the aluminum and phosphate industries in the past decade, the fluoric effluents were scrubbed and either neutralized to waste calcium compounds or converted into other fluoric materials. More recently, however, the necessity to eliminate the growing nuisance of fluoric contamination has led to the conversion of the emitted fluorine into industrial commodities, including synthetic cryolite and potassium silicofluoride. Hignett and Siegal (18) demonstrated that oolitic limestone in packed towers effectively absorbed effluent fluorides, and olivine is rated as an efficacious fixative through the formation of magnesium silicofluoride; from this highly soluble material, hydrofluoric acid, magnesium hydroxide, ammonium fluoride, and other products can be obtained (67, 68).

Although atmospheric fluorides may cause foliar effects upon certain types of vegetation (70) such as gladiolas, pines (51a), and tomatoes (6a),

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field-grown forage may retain an abnormal content of fluorine from air-borne fluorides without visible foliar damage. However, determinations of total fluorine in unwashed samples of field vegetation and experimentally exposed solid fixatives do not establish the proportions of fluoric gases and nonacidic particulates that are respectively responsible for air-derived pollution. Approximate proportions of the two forms can be determined through parallel analyses of the washed and unwashed portions of particular samples of vegetation. This is an important consideration in evaluating analyses of fluorine in vegetation sampled in those localities where fluoride contaminations may result from dusts of rock phosphate or phosphatic soils (9). In one locality, however, washdown of fluorine from soot-contaminated urban atmosphere was equal to the corresponding washdown within a few miles of a location where industrial emissions of fluorides are known to occur (28, 33, 38).

Until recent years nothing was known of the fate of fluorides that reach the soil through incorporations of superphosphate, or from the atmosphere, either directly or through rainfall. In particular there was no evidence as to whether such acquired fluorides affect a soil's reaction and fertility. Although accumulations of apatite dusts on vegetation will raise its fluorine content, the relatively small per acre additions of the material of such low degree of solubility could exert no measurable effect upon plant response, nor upon either soil reactions or fertility.

Therefore, the presently used term "air-borne fluorides" connotes acidic forms.

Obviously, acquired acidic fluorides do not function identically on soils of different chemical composition, nor under different meteorological conditions. They do not function alike in the rock-derived soils of the humid regions and the alkaline soils of arid and semi-arid regions. However, virtually nothing is known about the fate of fluorides that reach the Coastal Plains soils, which are characterized by a high content of quartz, with and without shell residues. It is stressed that the findings reported here are effects induced by additive fluorides upon rock-derived soils in the greenhouse and upon field vegetation, and upon such soils under humid conditions.

In the Tennessee county where fluoride emissions were due to the manufacture of aluminum, the nearby soils contained only 100 to 200 ppm of fluorine in mineral forms. Consequently, dusts from those soils caused no determinable increases in the fluorine content of collected samples of field vegetation. However, in the other locality, where phosphate mining and processing operations are extensive, many of the soils were highly phosphatic and therefore relatively rich in fluorine. Consequently, the dusts from such soils cause increases in the fluorine registered through analyses of unwashed leaves. The insoluble dusts can be removed through washing and also by means of air suction, as in the use of the "Electrolux" that was reported by Gaud, Charnot, and Langlais (9).

As indicated by the title, the two objectives of the present paper were to determinine what happens to the air-borne fluorides that reach the soil and the resultant effects upon uptake of fluorine by vegetation and upon soil reaction and fertility. A particular object was to ascertain the amounts and any effect of the acidic fluorides washed down to the soil.

HISTORICAL

For many years, little was known about the natural occurrence of fluorine in soils, and no function was attributed to the element. In 1919, Steinkoenig (62) reported the fluorine contents of several soils, and in 1921, Hilgard (17) estimated a normal incidence of 0.02 per cent. In 1946 Robinson and Edington (58) reported the fluorine content of a substantial number of soils of various types. In the past decade fluorine and associated elements were determined in many hundred soil samples at the Tennessee Station and the findings are embodied in an extensive manuscript, to be published.

In 1906, Aso (5) concluded that the fluoride content of incorporated superphosphate was beneficial to a specific crop, but in 1952 Morse arrived at an opposite conclusion (52). In 1933, Hart, *et al.* (16) raised the question as to whether incorporations of phosphatic fertilizers and raw rock phosphate would impart to forage crops fluorine levels that would be toxic to livestock, or detrimental to human health (54). However, their analyses of samples of forage grown on phosphated soils at several experiment stations were inconclusive. The Wisconsin workers then obtained rainwater leachings from added phosphates in lysimeter experiments that were under way at the Tennessee Station, and determined the fourth year outgo of fluorides. Integrating the leachings of fluorine from the lysimeters with the failure of incorporated superphosphate to cause increase in the fluorine contents of the sampled forage crops, Hart, *et al.*, suggested that the component fluoride had passed out in the rainwater leachings (16).

The first report upon the stability of an added fluoride in the soil was the 1929–1933 findings (40) on the leaching of fluorine from 1500 and 6000 pound incorporations of barium silicofluoride. This material had been utilized in Pennsylvania and in New Jersey for the eradication of the larvae of the Japanese beetle on golf greens (10, 22). Thus, as late as 1933 there was no certainty that added fertilizer fluorides affect plant growth and composition. In 1942, however, pioneer findings demonstrated that the fluoride content of a heavy rate incorporation of superphosphate did not affect crop response adversely and caused no increase in fluorine uptake (45, 46). Even then, other fluoride-induced soil reactions had not been suggested, but in 1943, Sherman, McHargue, and Hageman (61) reported that the fluorine of added fluorides had induced the oxidation of manganous compounds in the soil, whereas the other halogens had not.

(The citations given in this paper are selected for their bearing on the question of what happens to the air-borne fluorides that may reach vegetation and soil).

EXPERIMENTAL

The initial approach was to determine incidence of fluorides in the atmosphere and their periodic concentrations at locales where emissions were known to come from manufacturing operations. The fluorides that reach the soil through infusion or as droplets through the rainwater washdown and washings from vegetation are the acidic forms or their salts. Obviously, the acidic forms react upon a soil's supply of either limestone, dolomite, or silicate, as well as upon such coatings on vegetation, so that the soil acquires a content of either calcium fluoride, magnesium fluoride, or calcium silicofluoride. It will be shown that in soils poor in calcium content the dominant reaction is between the increments of the fluoride ion and the alumino-complex of the soil.

Also to be considered is the specific effect of potassium fluoride that may result from the action of air-borne fluorides upon leaves of vegetation rich in potassium content. Therefore, the behavior and effects of additive potassium fluoride will be considered, along with fluorides of calcium, magnesium, and hydrogen, through plant response, fluorine uptake, induced soil reaction, and outgo of potassium, calcium, and magnesium fluorides in rainwater leachings.

DETERMINATIONS OF AIR-BORNE FLUORIDES

Analyses of Filtered Field Atmosphere.—In exploratory investigations in Tennessee, large volumes of field atmosphere were pumped through dry paper filters and on through water scrubbers in 12 hour periods on a farm five miles distant from an aluminum plant. Negligible amounts of the farm soil—one that had a low content of fluorine—were stopped on the paper filters. The acidic fluorides caught in the water traps were attributed to gaseous phases. But because of the low concentration in terms of parts per billion, the results did not justify the long continuous operations and the requirements for personnel and equipment, so the direct determinations of the fluorine content of the pumped and filtered atmosphere were discontinued.

The next approach was through the analysis of quarterly composites of rainfall and computation of fluorine washdown (33). Later, however, the washdown of fluorine was determined on every rainfall. The collections were obtained at six points in East and in Middle Tennessee during the years 1948–1954 (38). The rainwater analyses were made by the Sanchis procedure (60) or by the American Public Health method (3) as modified by Shaw (60a).

The washdown was in the range of 1.2–1.5 pounds of fluorine per acre.

Surprisingly, the washdown at Knoxville was as much as at each of six locales that included aluminum and rock phosphate operations. In contrast was the small washdown at Crossville on the Cumberland Plateau —between the East and Middle Tennessee collection points and 100 miles equidistant to them.

Occurrences of acidic fluorides in the atmosphere were also determined in an inverse manner. The fluorine acquired by plants grown outdoors was compared with the uptake of those growing in an adjacent chamber, the atmosphere of which was scrubbed and freed of fluorine. The acidity of the scrub water and its fluorine content were determined to show the amount removed from the known volume of the washed air. The plants, a legume and a non-legume, had been transferred from origin to a location near fluoric emissions and were then allowed to grow three weeks more. During that interval the outdoor plants derived their fluorine content from the atmosphere and soil, whereas the plants in the chamber obtained their supply of fluorine from the soil alone. After the further growth, fluorine contents of the outdoor plants had become significantly greater than the fluorine contents of the companion plants that were grown in the fluorine-free atmospheres (39).

Although the fluorine totals did not prove that the acidic forms were solely responsible for the gains by the plant-tops during their further growth outdoors, the comparisons demonstrated that the washing of the atmosphere entering the chamber prevented an increase in the intake of fluorine by the above-ground growth of the vegetation.

Acquirement of acidic fluorides from the atmosphere was also verified through analyses of alfalfa grown in the field on soils that were blanketed with a layer of quartz to prevent contamination by upspatter of a soil rich in fluorine. Protection against upsplash contamination resulted also when dense vegetation was allowed to grow on plots without the quartz cover.

Occurrence of air-borne fluorides was also demonstrated through the uptake of fluorine by Spanish moss in month-long successive multiple exposures at three wide-apart locations (38, 39). The exposures of the epiphyte registered the variations in the 30-day occurrences of atmospheric fluorides at the several locations and in the different seasons. After being dried at 50°C the moss suffered a substantial decrease in its capacity to take up fluoride. Upon subsequent exposure to moist air, the moss did not regain its physical structure and hence there was no certainty as to whether stomatal function was a factor in the larger uptakes of fluorine by the unheated moss.

When the exposed moss was washed, fractions of the fluoride uptakes were recovered, but no recovery was obtained in simultaneous ethanol extractions. Therefore, it appeared probable that a fraction of the absorbed fluorides persisted in unchanged form or as soluble derivatives,

whereas the retained fraction had been converted into insoluble forms.

The occurrence of air-borne fluorides was determined also through the uptake by quadruplicated exposures of limed 12.5 cm filter papers that were sheltered in inverted buckets at 12 locations. The variations in the fluorine contents at the several points, and at different seasons, were substantial (39a).

Analytical Determinations.—Considerable experience in the analyses of pure fluorides, organics, greenhouse cultures, waters, and soils had been gained prior to extensive investigation of the various aspects of fluorine contamination of the atmosphere and field vegetation (26). An early observation was that the fluorine content of the relatively clean greenhouse vegetation could be determined accurately through distillations from the calcines of limed charges, and that the calcines of vegetation grown outdoors on nearby soils, poor in natural fluorides, always registered a higher content of fluorine. Although the fluorine content of the lime-ash of uncontaminated greenhouse vegetation was distilled readily and completely without prior fusion with hydroxide of either sodium or potassium, such prior fusion proved imperative for perchloric distillations from ash of vegetation that carried coatings of argillaceous dusts (15, 38, 57, 59). However, *the NaOH fusion of soils,* prefatory to perchloric distillation, is *inadmissible.*

In 1927 Shuey compared four methods for the determination of fluorine through distillations from pure fluorides, and in 1928 (63) he demonstrated the feasibility of sulfuric acid distillation of SiF_4 from pure fluoride and quartz at 165°C. In 1931 he developed the ideal reaction flask that has proved so well adapted for analytical distillations of SiF_4 (64). In 1933 (65, 66) he obtained a preview of the manuscript of the Willard and Winter procedure (69), whose novel feature was the use of thorium nitrate in the titrations of the distillates. In using that procedure he found that the perchloric distillations and thorium nitrate titrations gave virtually complete recoveries of fluorine from pure fluorides. However, he found that recovery from pure fluorides plus ash from organic material was not complete. Similar observations were made by Willard and Winter in the subsequent publication of their procedure (69).

MacIntire and Hammond obtained complete distillations of fluorine from fluoride-impregnated charges of vegetation calcined with peroxide of either calcium or magnesium, and from calcium peroxide calcinations of fluoride-fortified soils (29, 30). However, the use of the two peroxides was handicapped by the difficulty of obtaining them as reagents of high purity. In 1951 the double distillation—sulfuric from raw soil and perchloric acid distillations of the concentrated distillates—was demonstrated to be effective (37) and now is the A.O.A.C. official procedure (4). Its adoption was based upon studies conducted by the Referees of that Association (12, 13, 14, 30, 32, 36, 37).

In the Tennessee studies, all distillations from calcines were conducted with either sulfuric acid or perchloric acid, using the modifications of Hammond and MacIntire (11) and the distillates were titrated against thorium nitrate according to the Willard and Winter procedure (69). The initial multiple unit apparatus for fluorine distillations was developed by Reynolds, Kershaw, and Jacob in 1949 (56). The 12-unit apparatus in use at Wilson Dam, and in the U.T.-T.V.A. cooperative research laboratory at Knoxville, was devised by Drisdell (8). Determinations of fluoride solutes in rain and pond waters and in the lysimeter leachings were made by either the Sanchis procedure (60) or the U. S. Public Health Association procedure (3), as modified by Shaw (60a).

FATE OF FLUORINE INCREMENTS

The meager solubility of apatite and its stability as a soil component was definitely proved by the minimal outgo of phosphates in lysimeter leachings (47). Hence the behavior of the fluorine component of rock phosphate increments is disregarded as not being germane to the present objectives.

Although the calcium fluoride added to or engendered in limestoned soil is not highly soluble, it is far more soluble than that of rock phosphate, whereas the three effluent fluorides are highly soluble and their component fluorine has been found to be retained to a large extent against rain-wash by the leaves of vegetation. It will be shown that certain soils have capacities to tie up the most potent fluoride that may be brought to their surfaces, through direct absorption, through the settlings of mists and droplets, or in rain washdowns.

In zones near the seacoast or in arid and semi-arid regions, the occurrence of sodium fluorides in effluents would not be extensive enough to affect adversely either vegetation uptake or soil reactions. Nevertheless, incorporations of sodium fluoride and sodium silicofluoride were compared with equivalent incorporations of magnesium fluoride, cryolite, and rock phosphate in lysimeter experiments (47), to determine uptake of fluorine by vegetation of different types, and fluorine "leachability." Incorporated at the rate of 300 pounds of fluorine per acre on limestoned soils, neither incorporation caused an increase in the fluorine contents of three successive crops.

Although plant response was not affected adversely by incorporations of calcium fluoride in pot cultures (46) or in lysimeters (40), inputs of potassium fluoride underwent disintegration and release of potassium that proved toxic to vegetation and resulted in decided elevations in the *p*H of the experimental soils.

In soils well supplied with calcium, additive hydrofluoric acid reacts to develop calcium fluoride, and additions of hydrofluosilicic acid develop calcium silicofluoride. But neither of those fluorides has proved toxic to

plants. This fact was demonstrated when the calcium fluoride content of large inputs of superphosphate did not adversely affect the growth of nine successive crops and did not cause an aggregate increase of their fluorine uptake. The single incorporation of superphosphate (45) carried the quantity of fluorine that would have been supplied by 448 annual quarter-ton incorporations of the same superphosphate. However, the plant responses to and the uptakes of fluorine from the normal superphosphate were virtually identical to the responses and uptakes from the fluoride-free superphosphate. The two superphosphates were modified in reaction through soil inputs of calcium silicate as Wollastonite (45). Furthermore, calcium fluoride caused no toxicity when incorporations of it were compared with equivalent incorporations of calcium carbonate to ascertain whether the fluoride would provide an adequate supply of nutrient calcium (46). The fact that incorporations of fluorides of calcium do not repress plant growth, and do not cause increases in the uptake of fluorine, has also been demonstrated conclusively through the beneficial effect of calcium silicate slags of 4 per cent fluorine content upon vegetation that registered no increase in fluorine uptake (37a, 46). Moreover, meager leachings of fluorine resulted from additions of calcium fluoride and accompanying inputs of either mono-, di-, or tertiary phosphate, and calcium as either carbonate or silicate (43).

Against the single observation by Aso in 1906 that additions of calcium fluoride exerted beneficial effects upon plant growth (5), was the conclusion of Morse in 1935 that the fluoride content of superphosphate had proved toxic to the germination of maize (52).

It has been established that HF predominates in the emissions from the electric cells in aluminum manufacture (49) and also in the defluorination of washed rock phosphate in closed systems (18). Both of the starting materials are almost devoid of free silica. But the acidulations and nodulizations of unwashed rock phosphate are influenced by presence of silicates and therefore the resultant effluents occur chiefly as hydrofluosilicic acid, as evidenced by the precipitation of calcium silicofluoride when the complex acid is neutralized by liming. Consequently, there is a distinct difference between the relative stability of incorporated calcium fluoride and the behavior of equivalent incorporations of the more soluble calcium silicofluoride. The decided difference between the properties of the two fluorides was shown through their respective releases of fluorine solutes to rainwater leachings.

The apparent stability of additive calcium fluoride in the soil and the appreciable mobility of additive silicofluoride were registered emphatically through disparity in their respective releases of fluorides to rainwater drainage in a 1 year lysimeter study. The outgo of fluorine from per acre inputs of 2400 pounds of fluorine as 4932 pounds of calcium fluoride was only 7 per cent as much as the 6 year outgo from 1280 pound inputs

of fluorine as calcium silicofluoride, which was liberated from the slag upon its disintegration in the soil (43). The attendant recoveries of calcium from the fluoride and silicofluoride inputs were in like order. Because of the relatively small leachings of fluorine and calcium from the incorporated calcium fluoride, in contrast to the substantially larger leachings of fluorine and calcium, along with solvated silica, from equivalent incorporations of the silicofluoride of slag, it appears that a good supply of calcium will exert "common-ion" effect upon the solubility of the soil's content of calcium fluoride, but not upon the solubility of an equivalent content of calcium silicofluoride that is engendered when the slag undergoes decomposition.

The ready outgo of calcium silicofluoride was established also through the differential leachings from the parallel incorporations of electric furnace slag and limestone (35). The 10 year increases in fluorine leachings from two limestoned soils were only four pounds per acre from each of two soils, in contrast to four cases of fluorine leachings that exceeded 500 pounds per acre, with maximum of 800 pounds, from one soil; and four cases of fluorine outgo beyond 400 pounds per acre, and a maximal increase of 600 pounds, from the other soil.

Of especial interest has been the finding that incidence of solvated silica in the soil leachings is conducive to greater outgo of air-derived fluorine, and that the effect is most pronounced when the soil is well supplied with calcium (34). This observed role of solvated silica is registered also through enhancements in concomitant leachings of calcium and phosphorus. The phenomenon may mean that soil retentions of fluorine emitted as hydrofluoric acid in the manufacture of aluminum, and from the defluorination of rock phosphate "sands" (18), are not identical to the corresponding retentions of fluorine from hydrofluosilicic acid that is emitted in superphosphate manufacture and in thermal nodulizations of unwashed rock. To reason otherwise would mean that the emitted SiF_4 and H_2SiF_6 undergo hydrolysis to HF in the soil with resultant uniformity in the behavior and fate of the fluorine from the three forms—HF, SiF_4, H_2SiF_6, and to the exclusion of engendered calcium silicofluoride. But the postulation is negated by the slight "leachability" of the calcium fluoride added as such or engendered from additions of HF in calcium-rich soils, in contrast to the greater leaching of the silicofluoride of calcium following the dissolution of incorporated furnace slag.

Although the silicofluoride complex occurred at relatively high concentration in the drainage from slagged soils, there was no increase in uptake of fluorine by the vegetation of pot culture parallels. The larger outgo of fluorine from incorporations of the phosphate-reduction slag carried with it a calcium outgo much larger than the one from limestone (35). Moreover, the solvation of silica from the slag into the complex fluoride resulted in larger leaching of phosphates and calcium as well as fluorine (34).

However, it will be shown that unless speedy neutralization of the air-derived acidic fluorides is effected by the calcium in the soil, their fluoride ion will be precipitated by the alumino-silica complexes and retained tenaciously in combinations that virtually preclude uptake of fluorine by vegetation plant tops and greatly diminish the outgo of fluorides in rain-water drainage (41, 42).

BEHAVIOR OF EXPERIMENTAL APPLICATIONS AND INCORPORATIONS OF HF

In Pot Cultures: At a certain location fluorine emissions were known to occur as HF because of the reaction between nascent fluorine released from the cryolite in the electric cells in the manufacture of aluminum (49). In relatively minor operations HF is also emitted when rock phosphate is defluorinated in closed systems (18). Moreover, HF may result from the hydrolysis of the silicon tetrafluoride and hydrofluosilicic acid that are emitted in the manufacture of superphosphate. Consequently HF was deemed the air-borne fluoride most likely to reach the soil and was used to provide additions of fluorine in exploratory pot cultures of four soils, to ascertain effects upon plant growth and fluorine uptake (48). Each potted soil received highly diluted acid to supply 100 pounds, 400 pounds, and 800 pounds of fluorine per acre. The 100 pound input was equivalent to more than 50 years of rainwater increments but it registered virtually no effect upon vegetation nor upon soil reaction; however, the 400 and 800 pound inputs registered decreases in plant growth and decided depressions in *p*H values (48).

Using additions of "greatly diluted" hydrofluoric acid to soil in bench experiments, Hurd-Karrer found that liming prevented any detrimental effect upon plant response (20). Corrective effects of liming upon potted soils that received additions of hydrofluoric acid were reported also by Leone, *et al.* (23), by Prince, *et al.* (55), and by Daines, *et al.* (6a), and by workers at the Tennessee Experiment Station (48).

In Lysimeters: Although the pot cultures registered immediate effects of HF upon plant response and fluorine uptake, they did not reveal the full story of the persistence of soil reactions and resistance of the additive fluorine against rain-water leaching. Therefore, a 4 year lysimeter study was made to determine the "leachability" of 200 and 800 pound inputs of fluorine as applications and as incorporations of diluted solutions of HF to four soils in lysimeters that were subjected to a rainfall of 50 inches per annum. The ideal for additions of HF would have been the approximately two pounds of fluorine per acre in conformity to annual increments through rainwaters. But that ideal could not be implemented experimentally because the soils retained those increments completely against 50 inch annual rainfalls. Moreover, it was necessary that the HF additions be sufficient to effect measurable changes in *p*H values and yield determinable quantities of exchange bases and fluorine in the rainwater

leachings throughout the four years of the experiment. Therefore, greatly diluted solutions of HF were added to the upper zone and to full 12 inch depth of soil at the above-mentioned rates of 200 pounds and 800 pounds of *fluorine* per acre.

After four years the fluorine retentions by the upper zone and by the 12 inch depth of four soils were as much as 99 per cent of the 200 and 800 pound inputs. Upon examination of the compositions of the several soils and the leachings of fluorides from them it was obvious that the fixations of the added fluoride ion were in direct proportions to soil contents of alumina, as totals and as silico complexes (42). Maximal fixation of the fluoride ion occurred in the soil that had the highest content of Al_2O_3 and lowest content of calcium.

The near-complete fixation of the fluorine of HF additions was opposite to the ready release of the chloride ion from additions of hydrochloric acid to the same soils. Moreover, the 4 year retentions of fluorine from the additions of HF were as much as 720 pounds, without increases in the leachings of calcium, magnesium, or potassium (42). Therefore, it appears certain that the increments of air-borne acidic fluorides that reach the soil become fixed so readily and are held so tenaciously by the fixatives, as to be virtually absent from the soil solution and hence without effect upon plant response and soil fertility.

OBSERVATIONS

Related laboratory studies had indicated the possibility that the fixation of the fluoride ion in the soil might diminish the proportion of the more reactive soil content of Al_2O_3 and thereby lessen soil capacity to effect phosphate fixation. But although decrease in phosphate fixation might ensue from the heavy experimental inputs of hydrofluoric acid, the increments of air-borne fluorides would be so minute that any possible decrease they might cause in phosphate-fixation capacity would be negligible under field conditions.

An earlier postulation (25) was that preliming caused additive water-soluble phosphates to become dibasic quickly, to assume the tertiary form more slowly, and then to undergo conversion slowly to apatite. Although only slightly soluble, the precipitated apatite would be more soluble than the tertiary phosphates that result from reaction between water-soluble phosphates and the amphoteric elements in the soil. The postulation was based upon evidence from extensive small scale trials (31) and integrated with demonstrations of the development of apatite in mixtures of superphosphate and slag (44). The suggested function of additive fluorides upon the development of apatite was thought by Nagelsmidt and Nixon (53) to have occurred in the heavily phosphated calcareous soils at Rothamsted. However, subsequent pot culture (42, 47, 48) and lysimeter findings served to point the strong tendency of the alumino complexes to effect

the formation of the alumino-silicofluoride in soils not well supplied with calcium. Unless forestalled by the soils' content of calcium, the alumina fixation of fluorine from incorporations of *potassium fluoride* is also rapid and extensive.

But whether the fluoride ion reaches unlimed soil in combination with hydrogen, or as solutes of calcium, magnesium, or potassium washed from vegetation, the fixation of the fluorine component by the soil is governed by the solubility of the fluoric solid and the degree of the ionization of its solute. When judged by the slight effects that incorporations of calcium fluoride exert upon the rainwater leachings of fluorine and calcium, however, and without indication of base interchange, the added fluoride appears relatively stable in the rock-derived soil systems, either limed or unlimed. Although fixation of the fluoride ion of additive CaF_2 may appear to be slow and greatly delimited by its low degree of dissolution, the leachings by rainwaters indicate that, regardless of the speed with which the solid CaF_2 may dissolve, the fixation of the resultant fluoride ion by alumina becomes virtually absolute.

In the fixation of the fluoride ion of additive HF the *p*H lowering (42) was not so extensive as the reverse elevation effect that ensued from the liberation of the K ion of added potassium fluoride. Substantial inputs of that fluoride caused soil alkalinity, induced toxicity upon plant growth, even to lethal extent (41), and did not induce exchanges with the soil's content of either calcium or magnesium. Although the fluorides of hydrogen and potassium induce opposite effects upon soil reaction they possess a common potency in that both additions caused decisive repressions in the leachings of calcium and magnesium from the four soils of the 4 year (42) and 6 year lysimeter experiments.

SUMMARY

Approach to fate of air-borne fluorides and attendant effects upon soil reaction and fertility was through adaptation of specific analytical techniques for determination of fluorine present in atmosphere; rain, river, and pond waters; vegetation grown in greenhouse, in field, and in fluorine-free outdoor air; as minerals in soils; in rainwater leachings; and in multiple monthly exposures of Spanish moss and limed paper at widely separated locales, where fluoride emissions could be attributed to either the manufacture of aluminum or phosphate operations.

The air samples, filtered free of particulates, were meagerly acidic from fluorides in low concentration.

Fluorine washdowns were up to two pounds per acre per annum and were retained completely by each of the four unlimed soils against six years' rainfall.

Those fractions of the abnormal occurrences of air-borne fluorides that were caught by forage gave no visual indication of foliar damage or con-

tamination. Those fractions that reached limestoned soil directly through infusion, or as droplets, in rainwaters, and as washdown from foliage, did not affect forage growth, fluorine uptake, or soil reaction and fertility. Such increments to rock-derived acidic soil became fixed and were held tenaciously in alumino-complexes, without measurable effect upon soil reaction.

The fluorides that reached the soils, limestoned or slagged, were converted to either the simple fluoride of calcium or the corresponding silicofluoride; but neither fluoride caused increases of fluorine in forage crops, nor discernible injury to them.

The relative inertness of calcium fluoride in the soil was evidenced when a heavy incorporation of concentrated superphosphate caused no increase in fluorine uptake by nine successive crops on calcium silicated soil, although the superphosphate introduced calcium fluoride equivalent to that of 448 quarter-ton annual incorporations of like input.

As possible atmospheric contaminants, either directly or through foliar washings, fluorides of calcium, magnesium, sodium, potassium, and hydrogen were compared in greenhouse cultures and through leachings in outdoor lysimeters. In combination with either of the first three of these bases, fluorine incorporations of 300 pounds per acre induced no effect upon plant response and no increase in fluorine uptake by three successive crops, caused no determinable base exchange, and had little effect upon the reaction of limestoned soils.

Equivalent incorporations of the fluorides of hydrogen and potassium resulted in opposite effects in the soil. Reasonable incorporations of the former reacted quickly in alkaline and in acidic soils, without measurable base exchange or alteration of *p*H values. But incorporations of the latter caused alkalinity, toxicity to vegetation, partial leaching of the potassium of engendered hydroxide and carbonate, and complete fixation of the additive fluorine.

In simulation of rainwater washdown, 100 pound applications and incorporations of fluorine as a highly diluted solution of HF were of little effect upon plant response and fluorine uptake from four unlimed soils in pot cultures.

After four years' leaching from 50 inch annual rainfall the fluorine retentions from 200–800 pound per acre additions as HF were as much as 99.5 per cent of the inputs.

Retentions of additive fluorine by acidic soils in the lysimeters were proportionate to soil contents of Al_2O_3, which tied up the fluoride ion through development of aluminum silicofluoride, and without attendant increase in the leaching of bases.

Stability of calcium fluoride, added to or engendered in the soil, was indicated by the meager release of its fluorine and calcium components to rainwater leachings. In contrast was the mobility of the silicofluoride

engendered in slagged soils. However, the greater solubility of the silicofluoride did not induce a greater uptake of fluorine.

Fate of air-borne fluorides is accounted for partly by foliar lodgment that may be either minor or substantial on particular leafage and retained to various degree under different meteorological conditions. Such stoppage may enhance the fluorine content of forage to a level toxic to livestock (19).

Air-borne fluorides will not induce measurable effects upon either the reaction or fertility of a rock-derived soil, nor upon uptake of fluorine from it.

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IDENTIFICATION OF STORED PRODUCTS INSECTS BY THE MICROMORPHOLOGY OF THE EXOSKELETON

IV. ADULT LEGS

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Background information on micromorphological studies of storage insects that contribute identifiable fragments to food products has been discussed in earlier papers (1–4). As a result of food processing and breakage through the normal activity of insect life in food materials, the adult legs are seldom recovered from comminuted foods as intact appendages; they are more frequently isolated as single component segments, two or more joined component segments, or fragments of a single segment. In the latter category, they can be identified only if the isolated fragments have characteristics that are diagnostic of the intact segment.

Many of the leg segments of species within the same family show a relationship. In this paper[1] leg segments of related species are discussed and illustrated under their respective family classification.

GENERAL LEG MORPHOLOGY—PLATE I

To assist in locating and orienting leg areas and characteristic features, an artificial terminology, illustrated in Plate I, has been used in some of the description. Except for some spines, spurs, or setae of the tibiae and certain tarsal segments, the characteristics illustrated can be detected by the widefield microscope (30–75×).

Coxa: Fig. 1. This is the first or proximal leg segment; it is much used in the classification of whole insects because its size and shape varies among the three of pairs of legs of a single species. This segment is readily shattered during comminution; usually neither the resulting fragments nor the intact segment bear any distinctive landmarks that permit the structural origin to be determined,

[1] This paper is one of a series in which insect fragments found or likely to occur in cereal grain, cereal products, and stored products in general are discussed and illustrated (see References 1, 2, and 3).

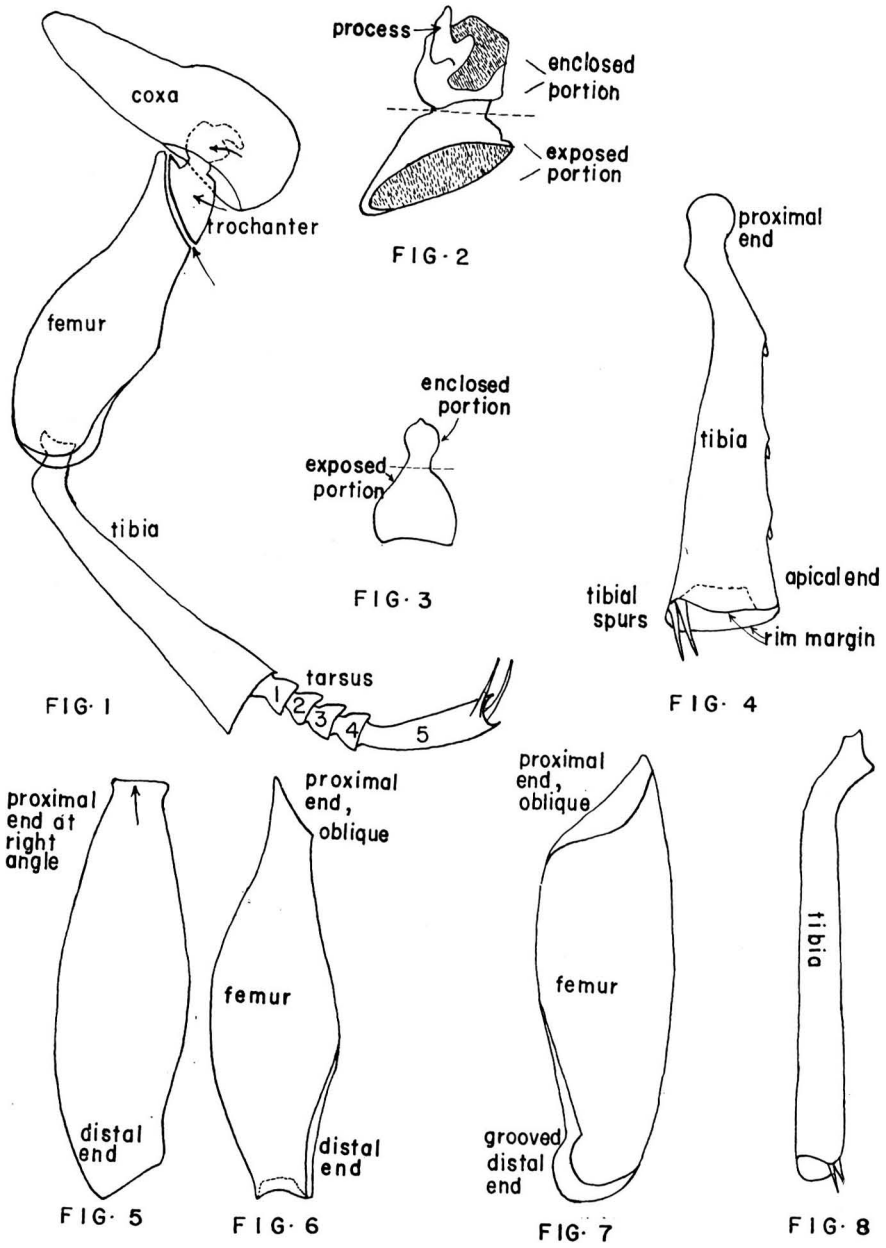

PLATE I.—General leg morphology.

except the pro- or meso-coxae of the rice and granary weevils (Plate II, Fig. 4).

Trochanter: Figs. 2 and 3. In most species this segment is a relatively minute and variously shaped structure. It is usually joined obliquely to the proximal end of the femur and in its axis (Fig. 1; *see* arrow). However, the trochanter and

femur of several species treated in this paper are usually joined more or less along a straight line (Fig. 5; *see* arrow) at a right angle to the longitudinal axis of the femur. Here the trochanter appears more as a "cap" at the proximal end of the femur (Fig. 3).

Only a portion of the trochanter is visible in the intact leg (Fig. 1; *see* arrow). This portion (Figs. 2 and 3; *see* legend), generally the color of the adjoining leg segments, is hollow; it may be roughly triangular, square, oval, spherical, or hemispherical in shape; this portion has been termed the "exposed portion." A smaller portion of it (Fig. 1; *see* arrow) is enclosed by the coxal shell of the intact leg and is exposed only when the coxa is shattered or when the trochanter is withdrawn. This portion (Figs. 2 and 3; *see* legend) is light-colored (it may be membranous in some species), bulky, rounded and grooved, or flattened; it has been termed the "enclosed portion" of the trochanter. One or two minute but distinct "processes" (Fig. 2) may project from this portion. In judging the trochanter as an isolated fragment, the fragment must frequently be manipulated to expose the shapes and landmarks illustrated for the particular species.

Femur: Figs. 5, 6, and 7. This is usually the most robust of the leg segments and can often be recognized by its thigh-like character (usually at least one longitudinal margin is moderately to strongly convex). The opposite margin may be straight and sometimes grooved along part or all of the length. The distal (apical) end is usually narrower; it sometimes is slightly to strongly expanded to one side (Figs. 6 and 7) and slightly to strongly grooved along this expansion. The proximal end may be gradually narrowed (Fig. 5) or abruptly constricted (Fig. 6) and is obliquely margined (Fig. 6), except where the trochanter joins as a "cap" (Fig. 5). With few exceptions, an essentially intact segment must be isolated for positive identification, and even then identification is usually limited to broad groups (families).

Tibia: In contrast to the femur, the tibia is characteristically a slender segment, enlarging slightly to strongly in the distal (apical) direction (Fig. 4) or remaining relatively uniform in width throughout most of the length (Fig. 8). The tibia is also recognized by the character of the proximal end (Figs. 4 and 8): it is distinctly bent to one side, at a right angle in some species, so that it may be flexed close against the ventral surface of the femur. The tibia usually compares roughly to a hollow cylinder and, by raising and lowering the microscope focus, the apical end appears to have an "upper and lower rim" (Fig. 4; *see* arrow). This effect is not seen in tibiae that are appreciably flattened (Plate III, Figs. 2, 5, and 8; Plate X, Fig. 1*d*). A pair of minute to large tibial spurs (Fig. 4) is usually present at one lateral angle of the apical end. They generally can be readily differentiated from any other large spurs or setae along the apical margins. The over-all shape, position of the apical articulating area (tibia-tarsus), presence or absence of setae, spines, and spurs, and relative length are used to differentiate and identify the species.

Tarsus: Fig. 1. This constitutes the foot portion of the insect leg; the number of segments varies. Isolated tarsal segments are unique in some species; in others, the essentially intact tarsus must be present to permit differentiation. Like other leg segments, identification is often limited to broad groups.

RICE OR GRANARY WEEVIL (SITOPHILUS ORYZA OR GRANARIUS)—PLATE II

Coxa: Fig. 4. A grooved band (*see* arrow) encircles the proximal end and is characteristic of the pro- and mesothoracic legs. Fragments that contain sections of the grooved band (Figs. 1 and 2) are commonly found in comminuted products. The spherical portion of the coxa also contains a deep pit (Fig. 4; *see* arrow) that seems to be associated with the enclosed portion of the trochanter articulation. A fragment containing this pit is frequently recovered (Fig. 3). Note the

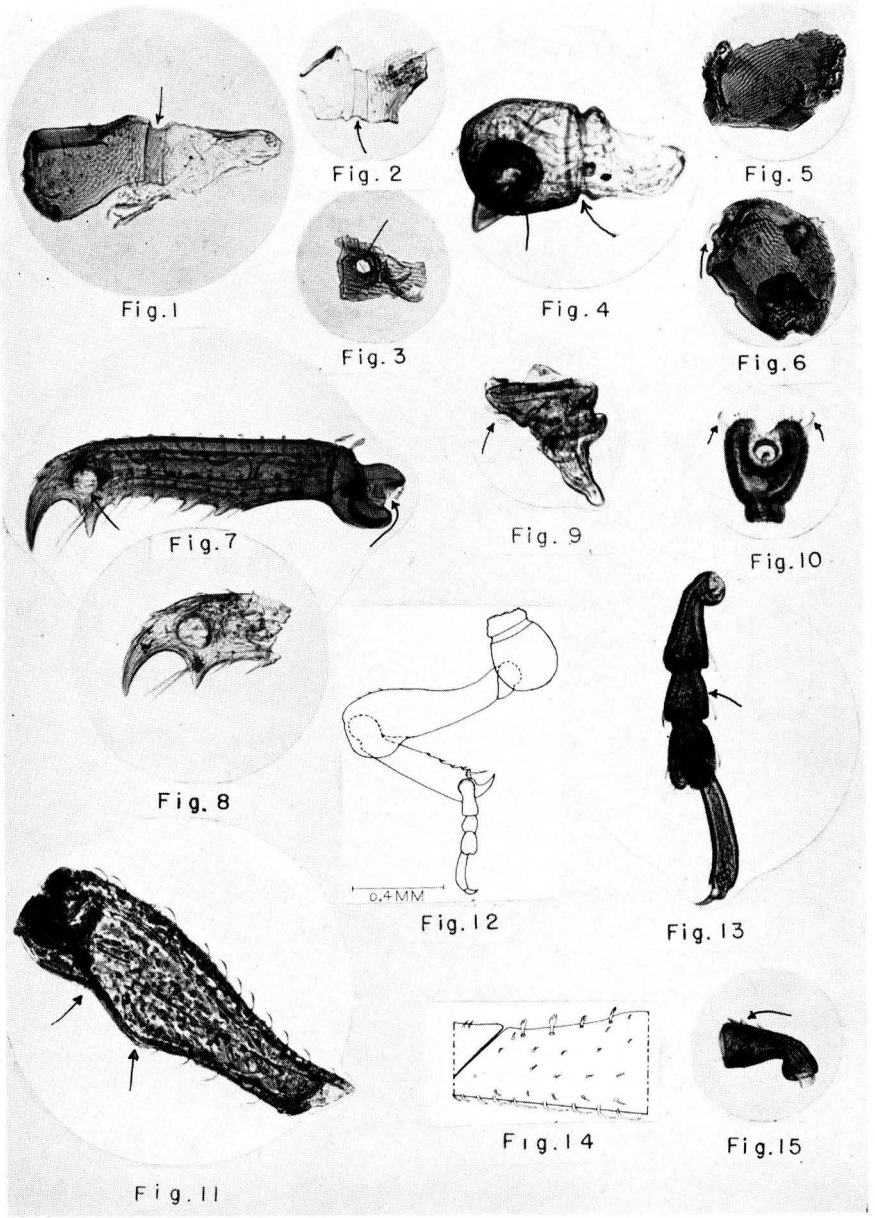

PLATE II.—Figs. 1, 2, 3, 5, 6: Pro- and mesothoracic coxal fragments. Fig. 4: Pro- or mesothoracic coxa. Fig. 7: Pro- or mesothoracic tibia. Fig. 8: Tibial fragment. Fig. 9: Trochanter. Fig. 10: Bilobed, third tarsal segment. Fig. 11: Femur. Fig. 12: Intact leg (drawing). Fig. 13: Intact tarsus. Fig. 14: Section of femur with types of setae (drawing). Fig. 15: First tarsal segment.

distally enlarged or brush-like setae (Figs. 4 and 6; *see* arrows) and the cuticular pattern of the coxal fragments (Figs. 5 and 6).

Trochanter: Fig. 9. The shape of this segment is readily distinguished from all other trochanters illustrated. The proximal half (enclosed portion) appears to be strongly grooved and to spiral somewhat to the pointed end. The distal half (exposed portion) contains 5–6 brush-like setae (100–200×, transmitted illumination).

Femur: Fig. 11. Slight differences occur among the three pairs of a single species and between the femora of the rice and granary weevils, but these differences do not alter the general form. The distal end is enlarged, expanded to one side, and strongly grooved to receive the tibial articulation. One longitudinal margin has a distinct bulge about midway (*see* arrow), followed distally by a broad but shallow concavity or V-shaped indention (*see* arrow) depending upon the microscope focus. Two types of setae occur on the femur (Fig. 14): brush-like (seen on the coxa and trochanter), and a somewhat recumbent type, fringed along one side. The over-all cuticle of the femur is dark brown to nearly black with oval pits that bear the setae. *Length in millimeters.*—Rice Weevil: pro-, 0.66; meso-, 0.53; meta-, 0.60. Granary Weevil: pro-, 0.90–0.97; meso-, 0.70–0.74; meta-, 0.89–0.92.

Tibia: Fig. 7. The most distinctive feature of this segment is the distal end; it terminates in a strongly curved hook projecting from one side and a smaller, conical projection on the opposite side that together form a U-shaped margin. On the upper surface, in this immediate area, is a circular area (*see* arrow) for the articulation of the first tarsal segment. The entire apical region (Fig. 8) is commonly found as a leg fragment. All but the metathoracic tibiae bear 4–6 stout spines (continuous with the general cuticle) along one longitudinal margin. A long seta rises from near the base of each spine; the opposite longitudinal margin is straight. Note the large, strongly-grooved proximal end (*see* arrow). *Length in millimeters.*—Rice Weevil: pro-, 0.58; meso-, 0.45; meta-, 0.53. Granary Weevil: pro-, 0.77–0.84; meso-, 0.61–0.68; meta-, 0.74–0.79.

Tarsus: Fig. 13. The first three segments are either characteristically shaped or have diagnostic landmarks. The first tarsal segment (Fig. 15) is readily recognized by its almost right-angle bend near the point where it articulates with the circular area of the tibia. Note the coarse, minute spines (*see* arrow) near the distal end of this segment. The second segment (Fig. 13; *see* arrow) is symmetrical and carries several flattened setae (*see* arrow). The third tarsal segment (Fig. 10) is bi-lobed and contains a depressed circular articular area surrounded by a dark crescent-like formation. Each lobe carries numerous fine setae and a tuft of setae beneath it (*see* arrow).

DRIED FRUIT BEETLE (CARPOPHILUS HEMIPTERUS)—PLATE III

Trochanter: Fig. 7. Resembles the trochanters of the Tenebrionid group (Plate IV), but differs in its smaller size, yellow color, the acute angles of the exposed portion, the deep notch on one side between the exposed and enclosed portion (*see* arrow), and the terminal, thumb-like process (*see* arrow) projecting straight forward from the side of the enclosed portion.

Femur: Figs. 3–6. This segment also resembles the femora of the Tenebrionids somewhat, but its oblique, proximal margin extends fully to each longitudinal margin (*see* arrow). In general the proximal end looks sharply pointed. The distal end is slightly to appreciably narrowed; it does not expand to one side and does not appear to be grooved to receive the tibia. *Length in millimeters:* pro-, 0.59–0.62; meso-, 0.65–0.67; meta-, 0.71–0.73.

Tibia: The three thoracic tibiae are much alike in shape, color, and size; each

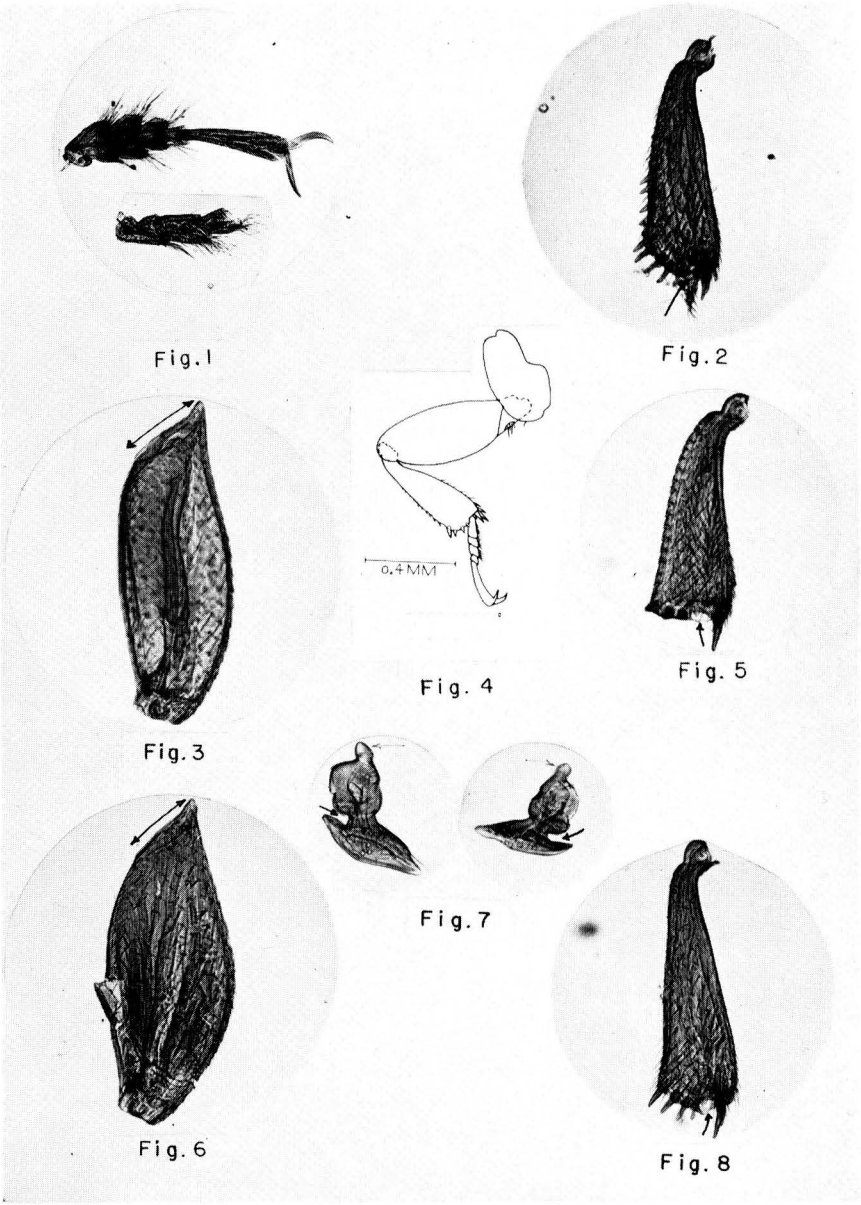

PLATE III.—Fig. 1: Intact tarsus and tarsal segments. Fig. 2: Mesothoracic tibia. Figs. 3 and 6: Femora. Fig. 4: Intact leg (drawing). Fig. 5: Prothoracic tibia. Fig. 7: Trochanters. Fig. 8: metathoracic tibia.

enlarges distally to produce a broad, club-like structure. The tibia-tarsus articulation (*see* arrow) is located distinctly to one side of the apical margin and separates the pair of long tibial spurs from other coarse spurs that occupy the greater length of the apical margin.

Protibia: Fig. 5. Apical margin is truncate and has three coarse, stubby spurs; one longitudinal margin is minutely saw-like.

Mesotibia: Fig. 2. Apical end is obliquely margined and has 4–5 long spurs; one longitudinal margin has coarse spurs.

Metatibia: Fig. 8. Apical end is obliquely margined and has 4 long spurs; only inconspicuous hairs are on the longitudinal margins. The tibiae of the three legs are appreciably flattened, in contrast to the more cylindrical form commonly seen. *Length in millimeters:* pro-, 0.50–0.52; meso-, 0.52–0.55; meta-, 0.61–0.65.

Tarsus: Fig. 1. The underside of each segment is densely clothed with long fine hairs. The size of segments decreases distally (*see* upper photograph).

CONFUSED AND RUST-RED FLOUR BEETLES (TRIBOLIUM CONFUSUM AND CASTANEUM); BROAD-HORNED FLOUR BEETLE (GNATHOCERUS CORNUTUS); TRIBOLIUM DESTRUCTOR; MEAL WORM ADULT (TENEBRIO OBSCURUS OR MOLITOR)—PLATE IV

These species are discussed as a group because their family (Tenebrionidae) relationship is similar in general appearance and details of the leg segments. The confused and rust-red flour beetles are the two most commonly found to infest food plants and to contaminate finished products.

Trochanter: The general shape of this segment among these species is similar. The trochanter of the Confused Flour Beetle (Fig. 5) is illustrated in four views to show the curved process projecting from the end of the enclosed portion, the prominent grooves and cavities, and the sharp angles of the exposed portion. The process of the Broad-Horned trochanter (Fig. 2) does not project from the extreme tip but springs from the surface of the enclosed portion in the form of a saddle horn. In *Tribolium destructor* (Fig. 3) the process is somewhat intermediate. The exposed portion resembles the Confused Flour Beetle trochanter and the enclosed portion resembles the Broad-Horned trochanter.

Femur: The over-all femoral shape of the four species is similar (Figs. 11 and 12). The most distinctive feature common to all is the oblique proximal margin which does not extend the full width of the femur; a small portion of the proximal end is not occupied by the trochanter (*see* arrow). This is readily seen in the upper photo of Fig. 12 (*see* arrow) where the trochanter is attached. The longitudinal margins are slightly sinuate (Fig. 11) to convex (Fig. 12, lower photo); the distal end expands somewhat to one side and is moderately grooved to receive the tibial articulation. *Length in millimeters.*—Confused or Rust-Red Flour Beetle: pro-, 0.45–0.53; meso-, 0.52–0.58; meta-, 0.61–0.67. Broad-Horned Flour Beetle: pro-, 0.62–0.67; meso-, 0.65–0.70; meta-, 0.72–0.80. Meal Worm: pro-, 2.75; meso-, 2.80; meta-, 3.23. Tribolium destructor: pro-, 0.60–0.63; meso-, 0.61–0.65; meta-, 0.72–0.80.

Tibia: Although the general shape of the tibia of these species is similar, individual differences can be detected.

Confused or Rust-Red Flour Beetle: Fig. 6. Longitudinal margins are straight and the segment enlarges distally, especially the prothoracic tibiae (Fig. 6*b*). The apical end of the meso- or metathoracic tibia is truncate; one margin has a fringe of coarse setae along one apical rim (Fig. 6*a*). The apical end of the protibia is somewhat concave; it does not have the fringe of coarse setae (Fig. 6*b*).

Broad-Horned Flour Beetle: Fig. 10. The most distinctive feature is the minute

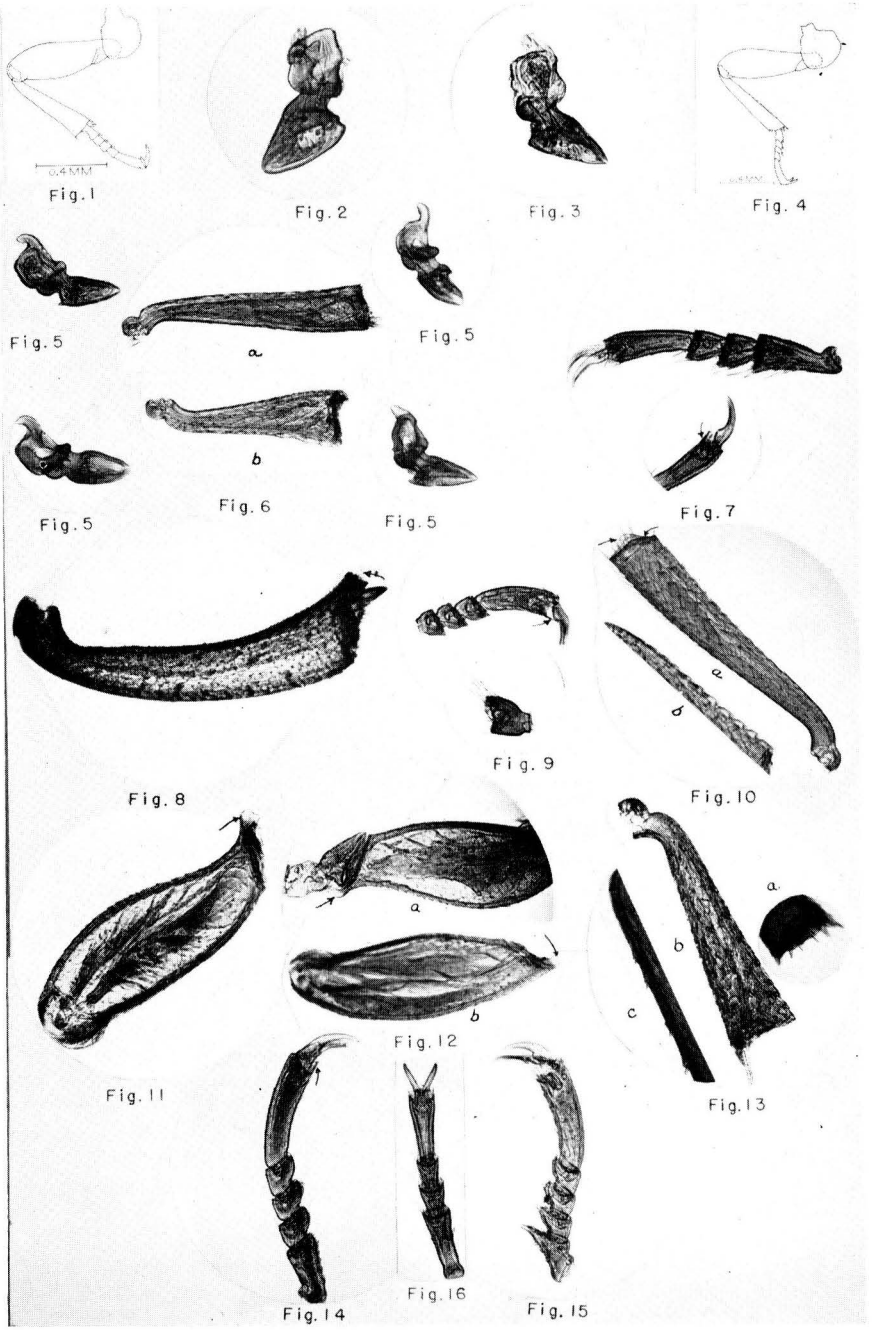

PLATE IV.—Fig. 1: Confused or Rust-Red Flour Beetle, intact leg (drawing). Fig. 2: Broad-Horned Flour Beetle, trochanter. Fig. 3: *Tribolium destructor*, trochanter. Fig. 4. Broad-Horned Flour Beetle, intact leg (drawing). Fig. 5: Confused or Rust-Red Flour Beetle, trochanters. Fig. 6: Confused or Rust-Red Flour Beetle;

but distinct angular cut of the apical end (*see* arrow) accentuated by a membranous, dome-like extension of one apical rim (*see* arrow). One longitudinal margin of the protibia, especially, is minutely notched or serrated; each notch carries a minute seta (Fig. 10*b*). *Length in millimeters:* pro-, 0.62–0.67; meso-, 0.65–0.70; meta-, 0.72–0.80.

Tribolium destructor: Fig. 13. Longitudinal margins are straight and the segment enlarges distally; apical margin is truncate (slightly concave along one rim) and bears a pair of long tibial spurs at one lateral angle associated with a group of long golden hairs that do not occupy the full width of the apical rim (Fig. 13*b*); the opposite apical rim has a fringe of coarse setae (Fig. 13*a*). One longitudinal margin has widely-spaced, short, stubby setae rising from the base of minute projections (Fig. 13*c*). The tibiae of this species are essentially like those of the Confused or Rust-Red Flour Beetle. *Length in millimeters:* pro-, 0.65–0.70; meso-, 0.60–0.65; meta-, 0.60–0.65.

Meal Worm Adult: Except for the protibia and the large size of all its leg segments, the tibiae are similar to the foregoing species. The protibia (Fig. 8) is strongly concave on one longitudinal margin; it extends beyond the tibia-tarsus articulation (*see* arrow) and has a dense fringe of hairs. The apical end (Fig. 8) is obliquely margined and clothed with long needle-like setae. *Length in millimeters:* pro-, 2.84; meso-, 2.73; meta-, 3.34.

Tarsus: Terminal segments of all four species end in a prominent spine beneath the paired claws (Figs. 7, 9, and 14; *see* arrows). The intermediate tarsal segments, except for the first long segment, are roughly bell-shaped; when viewed from the side position, however, one margin diverges slighly to strongly. When viewed from the top (Fig. 16) the segments are roughly tubular. Segments of the Broad-Horned protarsus are distorted by the slight to pronounced projections from the divergent margin (Fig. 15). These segments are illustrated, except Fig. 16, as they normally fall on the filter paper for examination. The stiff hairs spring from the ventral surface.

CADELLE BEETLE (TENEBROIDES MAURITANICUS)—PLATE V

Trochanter: Fig. 7. Superficially resembles the trochanters of the Tenebrionid species (Plate IV). The enclosed portion is "spherical" and large; it contains a distinct cavity and does not terminate in a process.

Femur (not illustrated): Proximal end resembles the Tenebrionid group (Plate IV); apical area is expanded rather strongly to one side and is deeply grooved; longitudinal margins are prominently convex. *Length in millimeters:* pro- 0.65; meso-, 0.65; meta-, 0.67–0.80.

Tibia: Fig. 2. Over-all shape resembles the prothoracic tibiae of the Tenebrionid group (Plate IV). One rim of the apical margin extends beyond the opposite rim in a flap-like extension (not seen in photo); paired tibial spurs of the apical lateral angle end are large and heavy (Fig. 4; large tibial spur detached). Several coarse, stout spurs are also present along one apical rim. Note, along one longitudinal margin, the flattened, light-yellow setae, minutely serrated along one setae margin (Fig. 6, 100–200× with transmitted illumination); the opposite longitudinal margin has 3–6 minute, stubby spurs in depressions (Fig. 3; 100–

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a—meso- or metatibia; *b*—protibia. Fig. 7: Confused or Rust-Red Flour Beetle, metathoracic tarsus. Fig. 8: Mealworms, prothoracic tibia. Fig. 9: *Tribolium destructor*, tarsus. Fig. 10: Broad-Horned Flour Beetle, tibia. Fig. 11: Prothoracic tibia, typical of all species of Plate IV. Fig. 12: *a*—profemur with trochanter attached; *b*—meso- or metafemur (typical). Fig. 13: *Tribolium destructor*, tibia. Fig. 14: Broad-Horned Flour Beetle, mesothoracic tarsus. Fig. 15: Broad-Horned Flour Beetle, prothoracic tarsus. Fig. 16: Typical shape of Tenebrionid tarsal segment from top view.

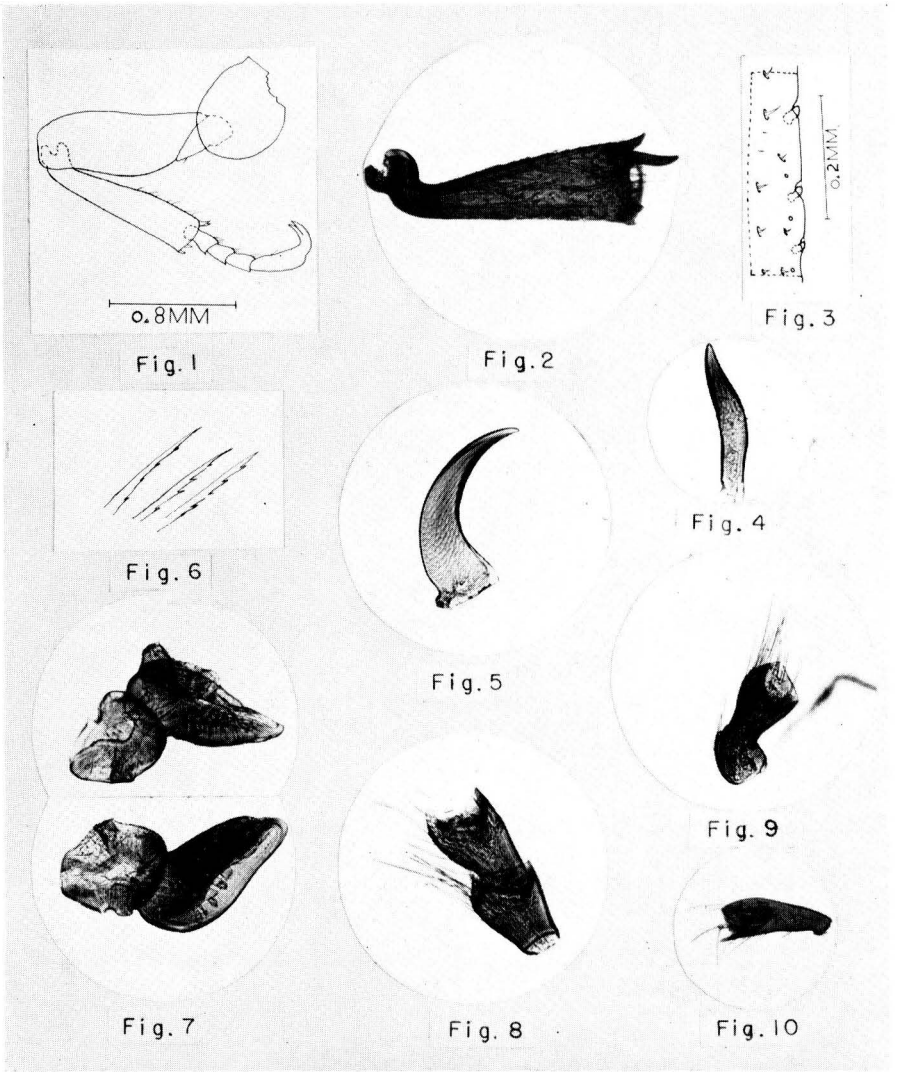

PLATE V.—Fig. 1: Intact leg (drawing). Fig. 2: Tibia. Fig. 3: Section of tibia showing stubby spur in depression. Fig. 4: Tibial spur. Fig. 5: Terminal tarsal claw. Fig. 6: Serrated setae of tibiae. Fig. 7: Trochanter. Fig. 8: Second and third tarsal segments. Fig. 9: First tarsal segment. Fig. 10: Terminal tarsal segment.

200× with transmitted illumination). *Length in millimeters:* pro-, 0.70–0.80; meso-, 0.65–0.72; meta-, 0.70–0.80.

Tarsus: The first segment (Fig. 9) is constricted near the middle and is angular at the apex. The intermediate segments (Fig. 8) are concave at the apex and the terminal segment ends in a stalked pair of setae and a stout spine (Fig. 10). Note the large terminal claw (Fig. 5).

SAWTOOTHED GRAIN BEETLE (ORYZAEPHILUS SURINAMENSUS), FLAT GRAIN BEETLE (LAEMOPHLOEUS MINUTUS), FOREIGN GRAIN BEETLE (CARTHARTUS ADVENA), AND SQUARE-NECKED GRAIN BEETLE (CARTHARTUS QUADRICOLLIS)—PLATES VI AND VII

The family relationship is apparent among these four species in the over-all shape and in certain details of the leg segments. The Sawtoothed Grain Beetle and the Flat Grain Beetle are by far the most often encountered in food plants.

Trochanter: Plate VI, Figs. 5, 9, and 10, and Plate VII, Figs. 6–8. The general shape of all four species is much the same. The portion of the trochanter that is enclosed by the coxal shell is small, somewhat flattened, slightly grooved, and lightly pigmented or membranous; it tapers to a somewhat pointed, finger-like process. The metathoracic trochanters of the Square-Necked Grain Beetle (Plate VII, Fig. 6) and the Sawtoothed Grain Beetle male (Plate VI, Fig. 5) are larger than the other thoracic trochanters and contain an acute tooth-like projection (*see* arrows). Pro- and mesothoracic trochanters of the Foreign Grain and Square-Necked Grain Beetles are essentially alike and are grouped together (Plate VII, Fig. 8). The trochanters of the Flat Grain Beetle (Plate VI, Fig. 10, 200×) are so minute and lightly pigmented that it is unlikely they will be readily detected unless they are associated with one or both of the adjoining leg segments.

Femur: The proximal region of all four species is strongly constricted and obliquely margined. The over-all form is shaped like a bowling pin.

Sawtoothed Grain Beetle: Plate VI, Fig. 1. Color is brick red; pro- and mesothoracic femora are strongly convex along one longitudinal margin and straight to slightly convex along the opposite margin; metathoracic femur of the male has an acute tooth (Plate VI, Fig. 1*a* and *b; see* arrow). Note the enlarged metathoracic trochanter which expands beyond the femoral margin (Fig. 1*a*). *Length in millimeters:* pro-, 0.43–0.49; meso-, 0.40–0.47; meta-, 0.44–0.53.

Square-Necked Grain Beetle: Essentially like the Sawtoothed Grain Beetle, but colored light golden (Plate VII, Fig. 9). *Length in millimeters:* pro-, 0.49; meso-, 0.41; meta-, 0.52.

Foreign Grain Beetle: Plate VII, Fig. 5. Similar to the foregoing two species; colored like the Square-Necked Grain Beetle. *Length in millimeters:* pro-, 0.40; meso-, 0.42; meta-, 0.45.

Flat Grain Beetle: Although it resembles the foregoing species, it is readily distinguished because it is smaller and shaped more like a bowling pin as a result of the more equal convexity of both longitudinal margins (Plate VI, Fig. 4). *Length in millimeters:* pro-, 0.33; meso-, 0.29; meta-, 0.35.

Tibiae: With the exception of the Flat Grain Beetle tibiae (Plate VI, Fig. 6), the tibiae are characterized by an appreciable enlargement in the distal direction and an abrupt narrowing at one side near the apical end. A similar angular cut is present on the prothoracic tibiae of the Cigarette Beetle (Plate X) and the tibiae of the Broad-Horned Flour Beetle (Plate IV) but are readily differentiated by other tibial characters.

Sawtoothed Grain Beetle: Plate VI, Fig. 7. The apical constriction is a shallow concavity (*see* arrow) which is moderately grooved. Note the distinct bulge at the point of constriction (*see* arrow). A small, stout conical spur occurs on or near one rim of the apical end (*see* arrow) in addition to the lateral paired tibial spurs on all species of this group. Color is brick red. *Length in millimeters:* pro-, 0.31–0.37; meso-, 0.28–0.37; meta-, 0.31–0.40.

Square-Necked Grain Beetle: Plate VII, Fig. 1. General shape is similar to the Sawtoothed Grain Beetle; color is golden yellow. The angular cut of the apical end is straight and is clothed with a fringe of slender golden hairs;

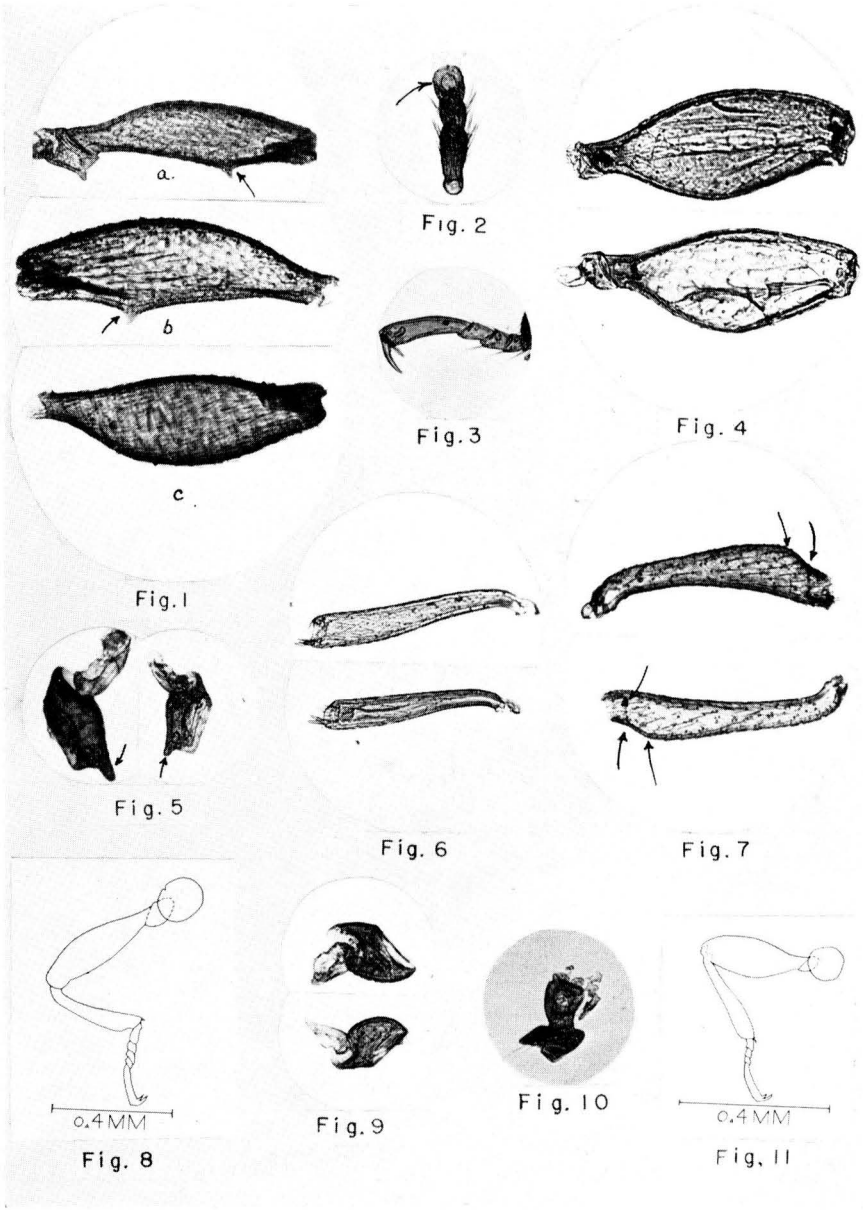

PLATE VI.—Fig. 1: Sawtoothed Grain Beetle; *a* and *b*—metathoracic male femora; *c*—pro- or mesothoracic femur. Fig. 2: Sawtoothed Grain Beetle tarsus, terminal segment missing. Fig. 3: Flat Grain Beetle tarsus. Fig. 4: Flat Grain Beetle femora. Fig. 5: Sawtoothed Grain Beetle metathoracic trochanters. Fig. 6: Flat Grain Beetle tibiae. Fig. 7: Sawtoothed Grain Beetle tibiae. Fig. 8: Sawtoothed Grain Beetle intact leg (drawing). Fig. 9: Sawtoothed Grain Beetle pro- or mesothoracic trochanters. Fig. 10: Flat Grain Beetle trochanter. Fig. 11: Flat Grain Beetle intact leg (drawing).

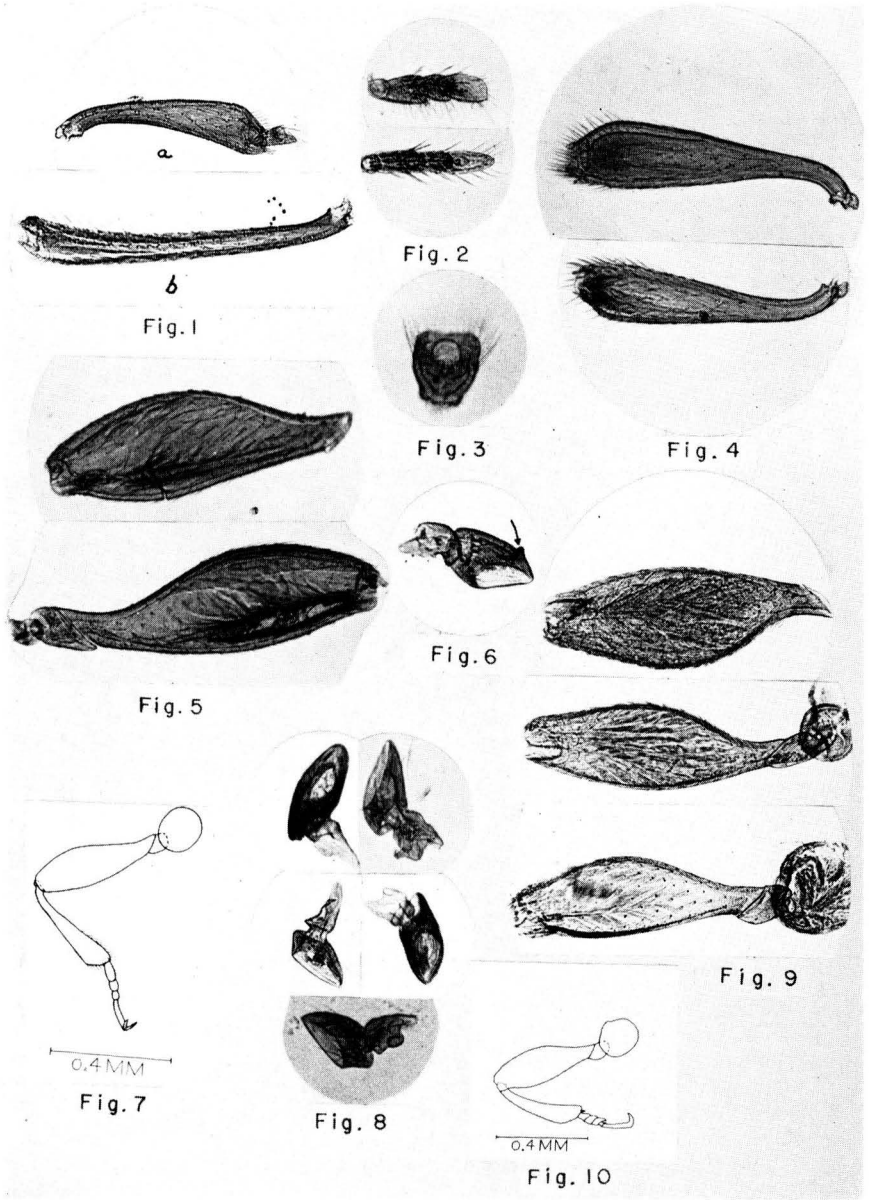

PLATE VII.—Fig. 1: Square-Necked Grain Beetle; *a*—pro- or mesothoracic tibia; *b*—metathoracic tibia. Fig. 2: Foreign Grain Beetle tarsi, terminal segment missing. Fig. 3: Square-Necked Grain Beetle, subterminal tarsal segment. Fig. 4: Foreign Grain Beetle tibiae. Fig. 5: Foreign Grain Beetle femora. Fig. 6: Square-Necked Grain Beetle, metathoracic trochanter. Fig. 7: Foreign Grain Beetle intact leg (drawing). Fig. 8: Foreign and Square-Necked Grain Beetles, trochanters. Fig. 9: Square-Necked Grain Beetle, femora. Fig. 10: Square-Necked Grain Beetle intact leg (drawing).

the metathoracic tibia is long (Fig. 1*b*) and the entire apical region bends somewhat. *Length in millimeters:* pro-, 0.37; meso-, 0.34; meta-, 0.43.

Foreign Grain Beetle: Plate VII, Fig. 4. The angular cut of the apical end is straight, but unlike the foregoing two species, both longitudinal margins are relatively straight; apical margin bears long needle-like setae. *Length in millimeters:* pro-, 0.30–0.34; meso-, 0.28–0.35; meta-, 0.32–0.38.

Flat Grain Beetle: Plate VI, Fig. 6. Entire length is relatively uniform in width; no angular cut at the apical end. *Length in millimeters:* pro-, 0.30; meso-, 0.28; meta-, 0.31.

Tarsus: The individual tarsal segments are so minute and light-colored that they are seldom detected in isolation. The simple sub-terminal segments are characteristically shaped in all but the Flat Grain Beetle, but are generally seen only when joined to the other tarsal segments. (*See* Plate VI, Figs. 2 and 3; Plate VII, Figs. 2 and 3.) In all but the Flat Grain tarsus (Plate VI, Fig. 3) the terminal, claw-bearing segment is missing.

LESSER GRAIN BORER (RHYZOPERTHA DOMINICA)—PLATE VIII

Trochanter: Fig. 3. The exposed portion is dentate around the margin which joins the femur, appearing somewhat like the crown of a tooth. Note its union with the proximal end of the femur (Fig. 8); it does not join along an oblique margin.

Femur: Figs. 5 and 8. Rather uniform in width; distal end slightly expanded to one side. *Length in millimeters:* pro-, 0.35; meso-, 0.37; meta-, 0.38.

Tibia: Figs. 1 and 2. This segment is distinctive. One longitudinal margin bears prominent spines that enlarge progressively in the distal direction. The flat, enlarged spines of the apical end look like a cock's comb (Figs. 1 and 2); this end is frequently isolated as a fragment (Fig. 4). The prothoracic tibiae terminate apically from the spine-free longitudinal margin in a large tibial spur (Fig. 2; *see* arrow). *Length in millimeters:* pro-, 0.41; meso-, 0.43; meta-, 0.42.

Tarsus: Fig. 6. Individual segments are not significant in isolation; intact tarsal segments look somewhat twisted. Each segment fits like a cap over the proximal end of the succeeding segment.

BLACK CARPET BEETLE (ATTEGENUS PICEUS), LARDER BEETLE (DERMESTES LARDARIUS), AND BUFFALO CARPET BEETLE (ANTHRENUS SCROPHULLARIAE)—PLATE IX

These species are discussed as a group because of their family (Dermestidae) relationships which can be observed in the general appearance and in certain details of the leg segments.

Trochanters:

Black Carpet Beetle: The distinctive character is the paired "processes" of the enclosed portion (Fig. 4; *see* arrow), especially those of the pro- and mesothorax. Note the somewhat hemispherical exposed portion of the metathoracic trochanter (Fig. 2) and the distinct constriction between the exposed and enclosed portions.

Buffalo Carpet Beetle: Fig. 3. The large exposed portion is roughly square; the connection between the exposed and enclosed portions is strongly constricted and terminates in a bulb grooved on one side. This segment is profusely clothed with characteristically shaped setae (Fig. 14).

Larder Beetle: (not illustrated). It is similar to the trochanter of the Black Carpet Beetle but is considerably larger; the exposed portion is roughly spherical.

Femur: A common feature of the Dermestid femora is the grooved longitudinal margin extending the entire length to receive the tibia and the thin sharp margin along this same side.

Black Carpet Beetle: Fig. 1. The proximal end is broad (not constricted) and

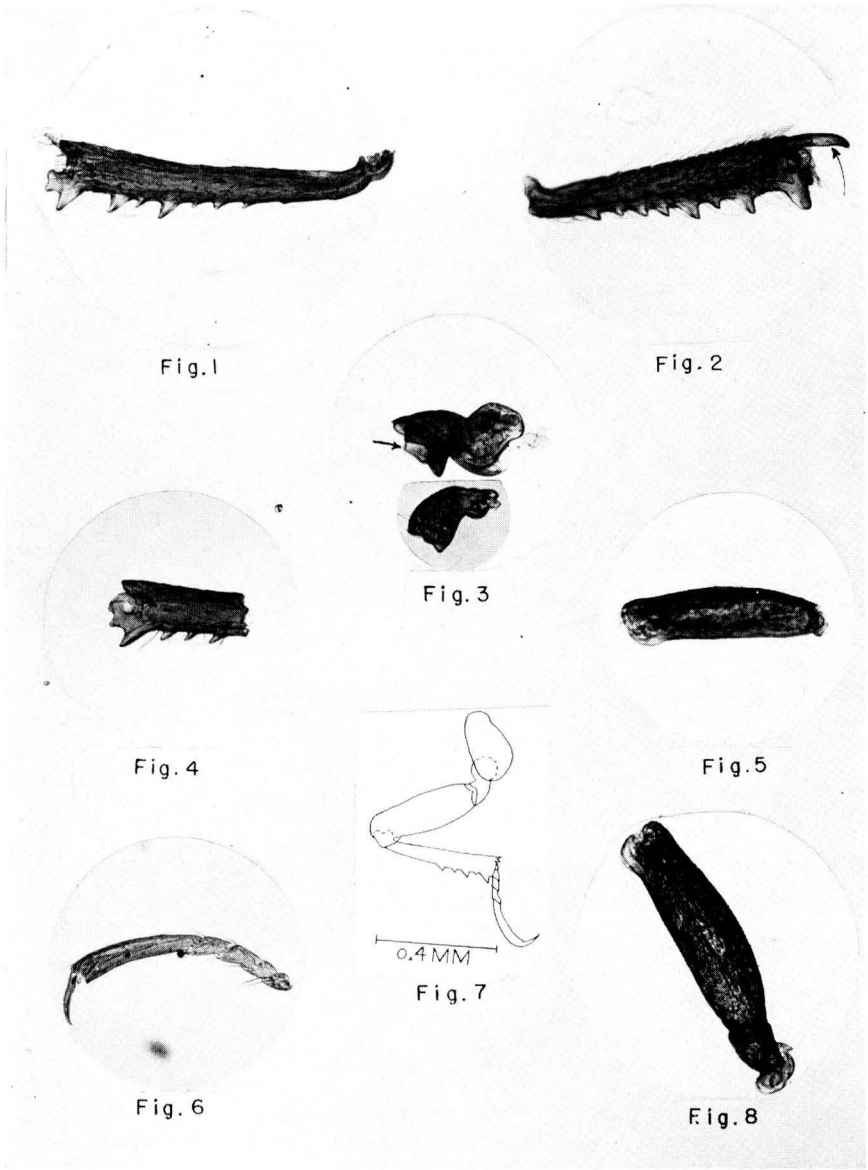

PLATE VIII.—Fig. 1: Meso- or metathoracic tibia. Fig. 2: Prothoracic tibia. Fig. 3: Trochanters. Fig. 4: Tibial fragment. Fig. 5: Femur. Fig. 6: Tarsus. Fig. 7: Intact leg (drawing). Fig. 8: Femur with trochanter attached.

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obliquely margined; longitudinal margin opposite the grooved margin is slightly convex (*see* arrow). *Length in millimeters:* pro-, 0.64; meso-, 0.65; meta-, 0.65.

Larder Beetle: Fig. 8. Similar in shape to the Black Carpet Beetle femora, but with a conical-shaped core (*see* arrow) at the proximal end; this core is

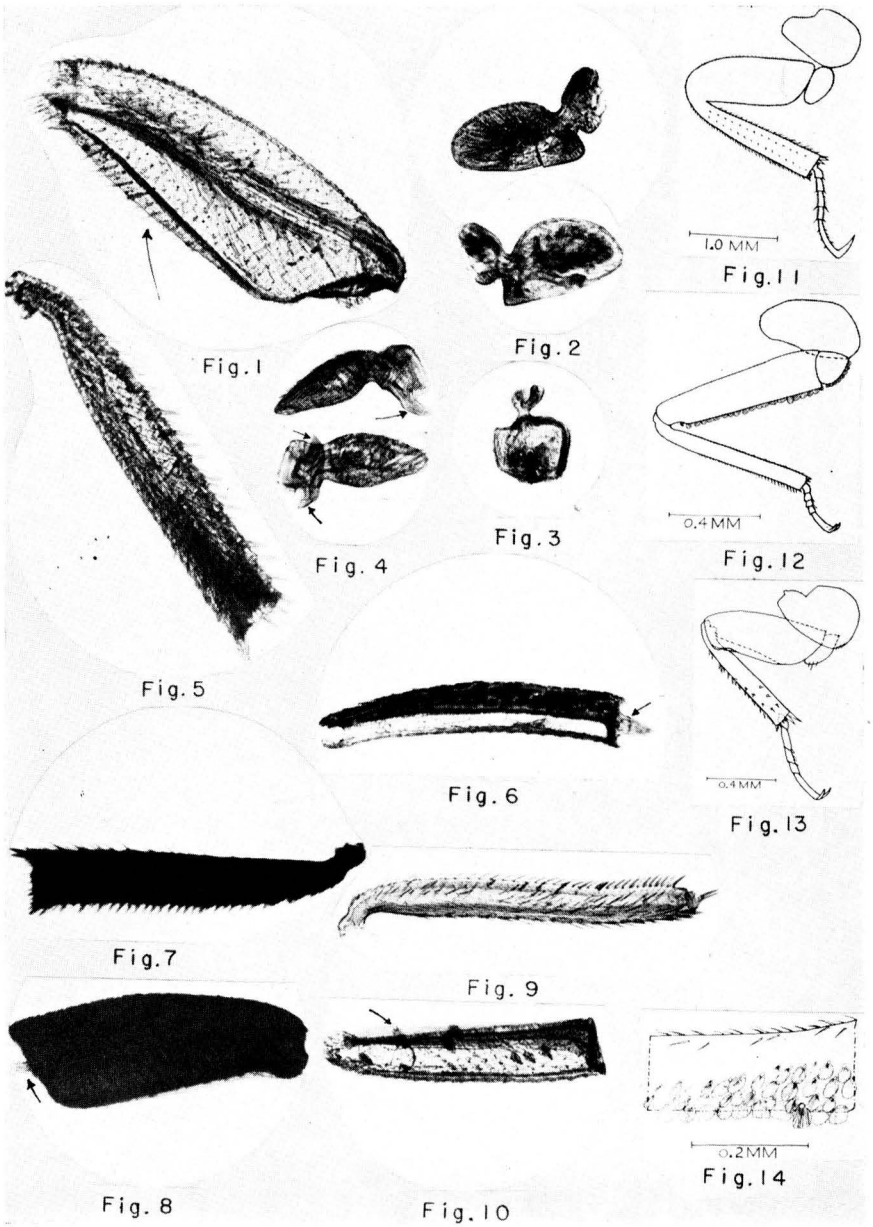

PLATE IX.—Fig. 1: Black Carpet Beetle femur. Fig. 2: Black Carpet Beetle metathoracic trochanters. Fig. 3: Buffalo Carpet Beetle trochanter. Fig. 4. Black Carpet Beetle, pro- or mesothoracic trochanters. Fig. 5: Black Carpet Beetle tibia. Fig. 6: Buffalo Carpet Beetle femur (positioned to show the longitudinal groove). Fig. 7: Larder Beetle tibia. Fig. 8: Larder Beetle femur. Fig. 9: Buffalo Carpet Beetle tibia. Fig. 10: Buffalo Carpet Beetle femur. Fig. 11: Larder Beetle intact leg (drawing). Fig. 12: Buffalo Carpet Beetle intact leg (drawing). Fig. 13: Black Carpet Beetle intact leg (drawing). Fig. 14: Buffalo Carpet Beetle, section of femur with characteristic setae.

associated with the cavity of the trochanter. The segment is almost black and is thus represented in the photograph by a silhouette. *Length in millimeters:* pro-, 1.26; meso-, 1.26; meta-, 1.30.

Buffalo Carpet Beetle: Figs. 6–10. The proximal end is truncate and does not narrow in width. It bears a small conical core (*see* arrow) similar to that of the Larder Beetle; one longitudinal margin is deeply grooved (Fig. 6, light portion) and has a distinct, fine, sharp margin along the same side (Fig. 10; *see* arrow). Characteristic setae (Fig. 14) profusely clothe the segment. *Length in millimeters:* pro-, 0.70; meso-, 0.73; meta-, 0.70.

Tibiae: Longitudinal margins of the three species are relatively parallel (Figs. 5, 7, and 9) except for a short, constricted section of the proximal end. One or both longitudinal margins bear coarse, stiff setae. The tibiae of the Buffalo Carpet Beetle (Fig. 9) are appreciably flattened. *Length in millimeters.—Black Carpet Beetle:* pro-, 0.55; meso-, 0.67; meta-, 0.67. *Larder Beetle:* pro-, 1.25; meso-, 1.25; meta-, 1.58. *Buffalo Carpet Beetle:* pro-, 0.67; meso-, 0.77; meta-, 0.79.

CIGARETTE BEETLE (LASIODERMA SERRICORNE) AND DRUG STORE BEETLE (STEGOBIUM PANICEUM)—PLATE X

Trochanters: The trochanters of the Drug Store Beetle closely resemble one another (Fig. 3). Their exposed portion is oval with a straight margin joined to the femur. The enclosed portions are small and rounded, and bear one or two rounded protuberances. The trochanter joins the proximal end of the femur along a straight line at a right angle to the longitudinal axis (Figs. 5, 6, and 8; *see* arrow). The metathoracic trochanter of the cigarette beetle has a spherical exposed portion and a small enclosed portion with a single "process" (Fig. 4*b*). Note its point of union with the femur (Fig. 2*a*). Note the shape of the pro- and mesothoracic trochanters (Fig. 4*a*) and the trochanter-femur union (Figs. 2*a* and 13).

Femur: Figs. 2, 5, 6, 8, and 13. In both species the apical region bends slightly to one side, especially that of the Drug Store Beetle, to produce a somewhat oblique apical margin.

Drug Store Beetle: Pro- and mesothoracic femora (Fig. 6) are minutely notched, equally on each side of the proximal end; metathoracic femur (Fig. 5) is grossly notched on one side (*see* arrow) and minutely notched on the opposite side of the proximal end. Note the cap-like trochanter and its union with the femur (Fig. 8; *see* arrow). *Length in millimeters:* pro-, 0.47; meso-, 0.49; meta-, 0.47.

Cigarette Beetle: Metathoracic femur is abruptly constricted at one side of the proximal end (Fig. 2*a* and *b; see* arrow) and only the narrow extension bears the trochanter (Fig. 2*a*). The proximal end of the pro- and mesothoracic femora is slightly oblique (Fig. 13; note attachment of trochanter). Color is light yellow in contrast to the reddish-brown femora of the Drug Store Beetle. *Length in millimeters:* pro-, 0.52–0.60; meso-, 0.52–0.59; meta-, 0.40–0.50.

Tibia: The prothoracic tibiae of the Cigarette Beetle are appreciably flattened, enlarged distally, and narrowed at the apical end by an abrupt angular cut (Fig. 1*d*). The mesothoracic tibiae may have a slight angular cut at the apical end (Fig. 1*b* and *c*). Except for the prothoracic tibiae of the Cigarette Beetle, the tibiae of the two species are slender; the longitudinal margins are relatively parallel (Figs. 1 and 11). The apical margins appear dome-shaped and lightly pigmented (*see* arrows). Close examination shows that this dome-shaped margin extends from only one rim of the apical end; the opposite apical rim is relatively straight. *Length in millimeters.—Drug Store Beetle:* pro-, 0.50; meso-, 0.50;

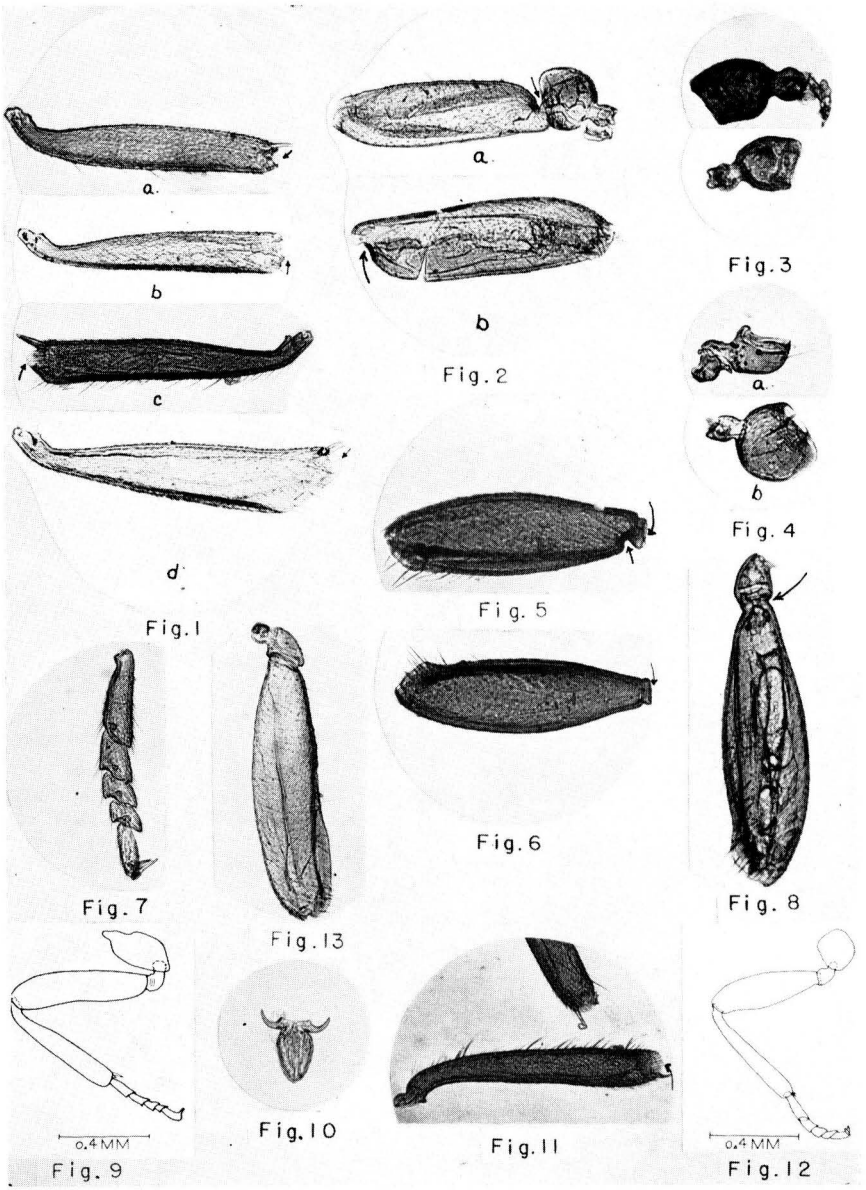

PLATE X.—Fig. 1: Cigarette Beetle tibiae; *a*—metatibia; *b* and *c*—mesotibia; *d*—protibia. Fig. 2: Cigarette Beetle metathoracic femora (2*a* with trochanter attached). Fig. 3: Drug Store Beetle trochanters. Fig. 4: Cigarette Beetle; *a*—pro- or mesothoracic trochanter; *b*—metathoracic trochanters. Fig. 5: Drug Store Beetle, metathoracic femur. Fig. 6: Drug Store Beetle, pro- or mesothoracic femur. Fig. 7: Drug Store or Cigarette Beetle tarsus. Fig. 8: Drug Store Beetle, pro- or mesothoracic femur with trochanter attached. Fig. 9: Cigarette Beetle intact leg (drawing). Fig. 10: Cigarette or Drug Store Beetle terminal tarsal segment. Fig. 11: Drug Store Beetle tibia. Fig. 12: Drug Store Beetle intact leg (drawing). Fig. 13: Cigarette Beetle, pro- or mesothoracic femur with trochanter attached.

meta-, 0.53. *Cigarette Beetle:* pro-, 0.49–0.60; meso-, 0.47–0.56; meta-, 0.40–0.50.

Tarsus: All thoracic tarsi of the two species are essentially the same (Fig. 7, side view). The individual segments assume more characteristic prominence when viewed from the intact tarsus. Note the shape of the terminal segment (Fig. 10).

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NOTES

NOTE ON DIRECT DETERMINATION OF AVAILABLE PHOSPHORUS IN FERTILIZERS*

By H. R. Allen (Kentucky Agricultural Experiment Station, Lexington, Ky.)†

In 1953 the writer reported a method for direct determination of available P_2O_5 in fertilizers (*This Journal*, **36**, 872 (1953)). This method could not be applied when calcium metaphosphate was present. Since some mixed fertilizers now contain calcium metaphosphate, the method has been modified to make it applicable to such mixtures.

The modification is as follows:

After transferring the 50 ml aliquots of the combined water-soluble and citrate-soluble portions to Erlenmeyer flasks, add 10 ml (1+1) HNO_3 to each flask and

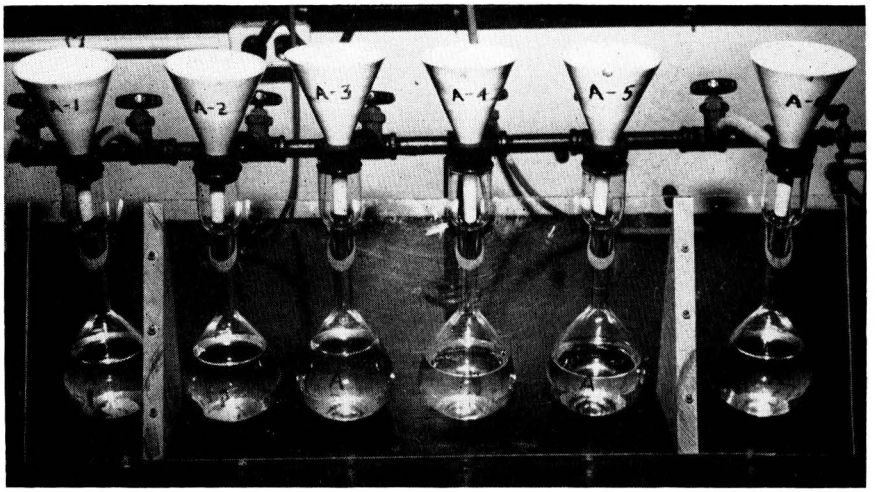

Fig. 1.—Modified filtering apparatus.

heat solutions to boiling on a hot plate. Boil gently for 10 minutes (or as soon as boiling begins, turn off heat and leave flask on hot plate over the noon hour or overnight). Cool, add the molybdate solution, and proceed as in the method.

The filtering apparatus has also been modified to eliminate transfer of the solution from the filter flask to the volumetric flask. The water-soluble and citrate-soluble portions are filtered by suction through a Hirsh funnel into a 500 ml Kohlrausch flask (Kimble Glass Co. No. 28100 was used in this work).

Figure 1 shows the filter arrangement in a 6-flask unit. The plexiglass shield protects the operator in case of flask collapse. The neck of each flask fits into a notched groove in the plexiglass; this holds the flask firmly.

J. A. Shrader of this Department designed and built the flask holder and funnel connector. The funnel connector is a No. 9 rubber stopper with a hole in the side

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† The investigation reported in this paper is in connection with a project of the Kentucky Agricultural Experiment Station and is published by permission of the Director.

to admit the brass tube that connects with the suction source. This tube is soldered to a brass collar that fits inside a larger hole through the top of the rubber stopper. The stem of the filter funnel is placed in position in this hole and suction is applied to give an air-tight fit.

NOTE ON INHIBITION OF GROWTH RESPONSE BY HEAVY INOCULA IN THE ASSAY OF VITAMIN B_{12} WITH *LACTOBACILLUS LEICHMANNII*

By HAROLD LICHTENSTEIN and H. REYNOLDS (Human Nutrition Research Division, Agricultural Research Service, U. S. Department of Agriculture, Beltsville, Md.)

Review of the directions for the preparation of inoculum as they evolved through various revisions of the U.S.P. and A.O.A.C. vitamin B_{12} assay methods shows an increasing awareness of the importance of properly adjusting the density of inoculum to attain adequate growth response.

The U.S.P. method as originally published (1) specified the use of inoculum that was centrifuged and washed but not diluted. This was followed by an interim revision (2) in which a 1+10 dilution of the washed culture was designated as inoculum. The A.O.A.C. method of 1952 (3) specified a similar inoculum. In a subsequent A.O.A.C. revision (4) the specification for density of inoculum was more precisely defined; namely, in terms of a series of percentage transmittance limits, one for each of the various instruments used for turbidimetric measurement. In more recent modifications of both the U.S.P. (5) and A.O.A.C. methods (6) the inoculum is diluted to a transmittance, determined with any suitable photometer, corresponding to a cell concentration in the range of 0.5–0.75 mg of dry cells in 10 ml of suspension medium.

It has been observed in studies carried out in this laboratory and elsewhere (7), that growth response in B_{12} assays with *Lactobacillus leichmannii* was affected by density of inoculum used. Data reported here show that maximum growth response may not be attained unless dilution of inoculum is greater than specified in current U.S.P. and A.O.A.C. methods.

The methods and materials used were essentially those of the 1955 A.O.A.C. B_{12} assay method (6) with modifications as noted. The culture of *L. leichmannii* ATCC 7830 was activated by daily transfers for a period of two weeks when first received in this laboratory and had been in continuous use, as specified in *Official Methods of Analysis*, up to the time these experiments were initiated.

To prepare inocula, *L. leichmannii* was cultivated for 20 hours in inoculum broth, centrifuged and washed twice with suspension medium, and resuspended in the same volume of suspension medium as the original culture. A series of dilutions were prepared from the undiluted inoculum, turbidimetric readings were taken on these dilutions against suspension medium, and the readings were then converted into dry cell weights by interpolation on a calibration curve established as described (5, 6). Where cell weights could not be read from the calibration curve, as is the case for extremely heavy and extremely light inocula, estimates were made by applying appropriate dilution or concentration factors.

Using 1 ml milk dilution pipets, single drops (0.05–0.06 ml) of the various suspensions were then used to inoculate triplicate assay tubes containing 0.10 milligamma B_{12} per tube. Inoculated blanks were also prepared for each inoculum dilution. The assay tubes were incubated for 24 hours; then their contents were

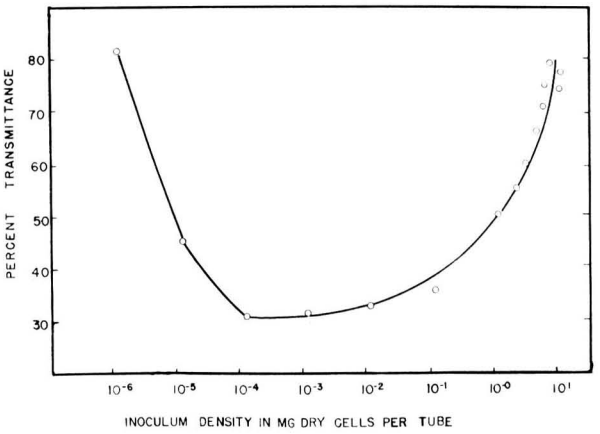

FIG. 1. Response of *Lactobacillus leichmannii* to 0.1 milligamma vitamin B_{12} with varying density of inocula.

read turbidimetrically in cuvettes in a Beckman Model B spectrophotometer at 620 mμ against corresponding inoculated blanks. Growth response values in percentage transmittance units were then plotted against dry cell weights of inoculum (Fig. 1).

It can be seen from Figure 1 that, for an inoculum having a dry cell weight of 11 mg per tube, growth response in the assay was meager, as indicated by a transmittance of approximately 77 per cent. Progressive dilution of this inoculum resulted in regular improvement of growth response; optimal values were observed when inocula were diluted to contain 0.11–0.0001 mg of dry cells per tube. This range of inocula elicited growth responses of 31–36 per cent transmittance.

Interpolation on the curve indicated that, under the conditions of these tests, values between 45 and 47 per cent transmittance could be expected with the use of inocula containing 0.5–0.75 mg per 10 ml of suspension medium, the limits specified by the U.S.P. and A.O.A.C. methods (5, 6). When the inoculum was diluted to 0.00001 mg per tube or less, the response diminished, as has often been observed with the use of minute inocula.

With longer incubation periods (40–50 hours) the growth following use of inocula containing approximately 0.002 mmg of dry cells per 10 ml of suspension medium was comparable to that attained in 24 hours with inocula containing 0.11–0.0001 mg of cells per 10 ml. However, with inocula containing 2 mg or more of dry cells per tube, comparable cell densities were not attained even during incubation periods of 50 hours.

The observation made in other laboratories (8), that *L. leichmannii* takes up from the medium and binds appreciable quantities of vitamin B_{12}, has been confirmed in this laboratory. The observed inhibition of growth by heavy inocula of *L. leichmannii* may be related to the B_{12} binding capacity of cells of this organism. If so, however, there is no apparent explanation of the failure of descendants of dilute inocula to similarly remove B_{12} from their medium and thus limit subsequent growth after they have multiplied to numbers comparable to those initially present when heavy inocula were used.

A number of treatments tested for influence on the growth inhibition caused by heavy inocula were without effect. Heavy inocula were inhibitory even after repeated washing. It does not seem probable, therefore, that the inhibition was caused

by carry-over of inhibitory material with the inoculum. In tests of the effect of age of culture on inhibition, heavy inocula of 6-hour and 20-hour cultures were both inhibitory. Inocula prepared from slow-growing and fast-growing cells, as determined by the time required for appearance of macroscopic colonies on plates, reacted similarly. Inoculum grown in A.O.A.C. inoculum culture broth was no different from that grown in assay medium. Heavy inocula prepared from a culture of strain ATCC 7830, newly obtained from the American Type Culture Collection, also resulted in growth inhibition.

Data presented indicate that, in the U.S.P.-A.O.A.C. assay for vitamin B_{12} with *L. leichmannii*, dilution of inoculum to a concentration of less than 0.5–0.75 mg of dry cells per 10 ml may be necessary for optimal growth when the inoculum is dispensed one drop (0.05–0.06 ml) per assay tube by pipet.

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Amino Acid Handbook. By RICHARD J. BLOCK and KATHRYN W. WEISS. Charles C Thomas, Springfield, Ill., 1956. xiii+386 pp. Illus., index, bibl. Price $10.50.

For the biochemist who works with amino acids, *Amino Acid Handbook* provides a foundation for the three important methods of analysis: chemical, microbiological, and chromatographic. The contents of this handbook also include the preparation of protein samples for analysis, the requirements for amino acids, and protein and amino acid consumption in the United States.

Fully one-fourth of the book is given over to tables of the amino acid composition of various proteins; an extensive bibliography, in addition to an author index and a subject index, rounds out the contents. Moderately complete and detailed instructions for preparing the sample for analysis include methods of determining nitrogen and removal of lipids, nucleic acids, and carbohydrates, in addition to methods for hydrolysis of the proteins. The amino acids are divided into eight groups, each of which is provided with a separate method of chemical analysis.

The microbiological methods for the determination of amino acids are well treated: a short discussion of the general aspects introduces the subject and is followed by a careful description of the details of the analysis. Suggestions about the type of glassware and even the name of the detergents used to prepare the apparatus are also given in detail.

The two chapters devoted to chromatographic analysis include a wealth of material. The methods described include paper chromatography and column chromatography on ion-exchange resins. Little space is devoted to partition column chromatography except for the listing of several reviews in this field.

As may be expected (see Block, Durrum, and Zweig: *A Manual of Paper Chromatography and Paper Electrophoresis*, New York, Academic Press, 1955) the chapter devoted to paper chromatography is exceptionally well handled. Solvent systems, types of paper, R_F values, length of run, and color reagents are all given for the amino acids; in addition, a long list of useful solvent systems is included separately.

It should not be assumed that this handbook includes all the systems used to determine amino acids nor all the color tests that may be applied to such procedures. However, it does compile, in a lucid and exact manner, a wealth of data that makes this a valuable aid to the analyst.

S. M. HESS

***p*H Measurements: Their Theory and Practice.** By VICTOR GOLD. John Wiley and Sons, Inc., New York; Methuen and Co., Ltd., London; 1956. 125 pp. Illus., index, bibl. Price $2.25.

Those who still define *p*H as the negative logarithm of the hydrogen ion concentration will find this short treatise of assistance in obtaining a correct interpretation. A simultaneous review of the electromotive force chapter in any physical chemistry text will also be helpful.

The modern concept of *p*H is practical, not theoretical. It is based, in the National Bureau of Standards system, on the assignment of reasonable values to a number of convenient reference standards, such as phthallate, phosphate, and borate. The lack of correlation with theory lies in the uncertainty of evaluating the activity coefficients of the ions involved.

The concept of *p*H, the e.m.f. and indicator methods of measurements, and some of the most important applications to aqueous and nonaqueous systems are treated as adequately as possible within the 104 pages of text. An appendix lists the *p*H

values at various temperatures of the N.B.S. standards and the methods of preparing these buffer solutions, as well as a number of subsidiary standards that cover the range from 1.10 (0.1*M* hydrochloric acid) to 12.88 (0.1*M* sodium hydroxide). A list of references rounds out each chapter.

WILLIAM HORWITZ

The World Fertilizer Economy. By MIRKO LAMER. Stanford University Press, Stanford, California, 1957. xvi+715 pp. Illus., index, bibl. Price $12.50.

This book is the tenth publication by the Food Research Institute in a series of studies on "Food, Agriculture, and World War II." The author declares his purpose is "to show the changes during World War II in the magnitudes and forms of commercial fertilizers produced and used in various countries of the world." In achieving this objective, he examines the underlying principles of fertilizer economics in relation to the developments effected by wartime necessities.

The first four chapters are devoted to concise discussions on the history and classification of fertilizers and the technical aspects of fertilizer science; treatments of plant physiology, soil science, chemistry, and manufacturing processes are necessarily limited. These discussions apparently are intended to furnish only the minimum information needed to understand the economic roles of the various fertilizer materials.

The general framework of fertilizer economics in each of the affected countries before and during World War II is thoroughly analyzed. Developments due to wartime expediency are particularly emphasized. Of considerable historical interest are passages describing the division, among the belligerents, of the natural reserves of materials used in the production of fertilizers (Chapter 11) and the subsequent military operations motivated at least partially by the desire to relieve critical shortages of phosphate rock. It is revealed that practically all of the phosphate reserves were in the hands of the Allies, while the potash sources were within the Axis orbit.

The author, of Yugoslavian birth, has published several previous reports on other industries within the Soviet Union. He authoritatively discusses the commercial fertilizer situation in U.S.S.R. in Chapters 18 and 25. These two chapters should be a valuable contribution to the literature because of the previous lack of information on the Soviet fertilizer industry.

The book, said to be the first study on the economics of commercial fertilizers that is worldwide in scope, contains impressive statistics on fertilizer production, consumption, and trade, and an extensive bibliography. It should be a valuable reference work for the agricultural economist and those in the fertilizer industry.

J. G. CUMMINGS

Cereal Laboratory Methods. 6th Ed. EMERY G. SWANSON, Editor. Compiled by Committee on Revision, American Association of Cereal Chemists, Inc., University Farm, St. Paul 1, Minn., 1957. viii+528 pp. 25 Tables, 2 Indices. Price, $11.00.

This book is already well known through its previous editions. The 6th edition is presented in a new style. It is divided into 100 major categories, each subdivided into specific methods. For example, the first major category, Acids, contains the method for acetic, butyric, and lactic acids in rye flour; arsanilic acid in feeds; ascorbic acid; and so on, through the acids usually of interest in this field. The inclusion of a "method index with numbers" and a "materials index" facilitates finding a specific method.

Technical personnel will find the standard methods required in the cereal field

Numerous new methods have been added, such as those for nitrophenide, nitrosal, phenothiazine, sulfaquinoxaline in feeds, additional firmness methods and sensory perception tests for bread, monoglycerides in fats, sedimentation test for flour and wheat, physical dough tests, methods for prepared mix ingredients, etc.

The value of many of the methods is enhanced by the fact that they were developed and tested by other associations. There are relatively few methods based on personal communications.

The statement on p. 280, 'the standards of U. S. Department of Agriculture . . . ," apparently refers to the old advisory standard which is no longer in effect.

This reviewer agrees with the editor that the book is useful. It is especially valuable and almost a "must" for those with special interest in cereal sciences.

V. E. MUNSEY

Official Publication. Association of American Feed Control Officials, Incorporated (1957). Obtained from the Executive Secretary, L. E. Bopst, College Park, Maryland. 151 pp. Price $5.00.

This annual bulletin represents the latest action of the Association on its 19 regulations, 28 resolutions, and 247 official definitions of utmost importance to the feed control official and the feed industry.

This year 33 drugs are listed for use in animal feeds and model labels are given for two types of feeds containing drugs: one for feeds containing drugs for growth promotion or disease prevention, the other for feeds containing drugs at a treatment level.

Many of the latest methods for the analysis of feeds that are contained in the Eighth Edition of *Official Methods of Analysis* of the Association of Official Agricultural Chemists may also be found in this bulletin. Methods for total arsenic, nitrophenide, and furazolidone have been added to the determinations of F, Co, Cu, Mn, vitamins, carotene, gossypol, sulfaguanidine, Enheptin, *p*-arsanilic acid, and sulfaquinoxaline, and the usual feed analysis methods.

The names and addresses of the various feed control officials in the U. S. and Canada are given, and feed tax legislation in the various states is summarized.

Two of the reproduced speeches at the annual meeting, "The Relationship of Disease to Nutrition," by Morris Erdheim, and "The Flexible Meal Mixture Formula," by E. W. Crampton, may be of special interest to the feed industry.

V. E. MUNSEY