CONTRIBUTED PAPERS

DETERMINATION OF POTASSIUM IN PLANT MATERIALS WITH A FLAME PHOTOMETER[1]

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A number of different makes of flame photometers have recently been placed on the market, and because of convenience and speed there has been increasing interest in their use for the determination of potassium in a variety of materials. Several papers dealing with their use in the analysis of plant materials have been published recently. Attoe (1) extracted the exchangeable potassium from plant materials with an aqueous solution 2 *N* with respect to ammonium acetate and 0.2 *N* with respect to magnesium acetate, and then evaluated potassium in the filtered extract directly. Results obtained compared favorably with those by the cobaltinitrite procedure. Toth *et al.* (5) used a flame photometer with lithium as an internal standard for evaluating potassium in wet-ashed samples of plant materials. Values agreed well with those by the platinic chloride method. Meyers *et al.* (2) dry-ashed samples of plant materials, extracted the ash with 0.1 *N* hydrochloric acid, and evaluated the potassium content of the extracts with both a flame photometer and the cobaltinitrite procedure. Results obtained by the two methods were again in good agreement.

Potassium is determined routinely in this laboratory in a large variety of plant materials, and it seemed desirable to investigate the possibility of substituting the flame photometric method for the more time-consuming platinic chloride method. Before complete confidence could be placed in the values obtained with the flame photometer, answers to the following questions seemed to be of considerable importance:

1. How closely will the values for potassium obtained by use of a flame photometer agree with those obtained by the A.O.A.C. platinic chloride method (3), customarily used in this laboratory?
2. How should the samples be treated to insure the best agreement between results obtained from the two procedures?
3. Should the direct intensity procedure be used or will it be necessary to use an internal standard to obtain satisfactory agreement of results?
4. Will the over-all manipulation, including requisite sample preparation, result in an appreciable saving of time?

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To answer the above questions, flame photometric determinations employing various types of sample preparation were made on a variety of plant materials. For comparison, potassium also was determined in all samples by the platinic chloride method. This paper reports the results obtained.

EXPERIMENTAL

A Perkin-Elmer flame photometer, Model 52A, with acetylene as fuel, was used in this study. The platinic chloride method as described in Methods of Analysis, A.O.A.C. (3) was taken as the basis of comparison of the potassium values obtained.

The first procedure involved ashing the samples in porcelain crucibles with sulfuric acid as a fixative for potassium. Depending upon the potassium content, from 0.5 to 2 g of the sample was weighed into a porcelain crucible of appropriate size. One ml of 1:1 sulfuric acid and enough distilled water to insure thorough wetting of the sample were added to the crucible, which was placed on a steam bath for an initial drying period and in an air oven at about 105° C. for 1 hour to remove any remaining moisture. (If too much heat was applied to the crucible at first, there was some tendency for the contents to spatter and creep over the sides; hence the preliminary use of the steam bath.) After drying in the oven, the sample was ashed at 500° to 550°C. for approximately 16 hours or overnight. After ashing was complete 3 ml of concentrated hydrochloric acid was added to the crucible, and the ash was digested by boiling gently for 1 minute. The acid extract was transferred quantitatively with hot distilled water to a 200-ml volumetric flask and made to volume. To remove any solid matter which might plug the atomizer of the flame photometer, a portion of the solution was filtered through Whatman No. 42 filter paper and analyzed for potassium by the direct intensity method. Standard solutions of potassium chloride in distilled water were used to calibrate the instrument. The values obtained for potassium in a number of plant materials, together with corresponding values by the platinic chloride method, are given in Table 1.

To determine the effect of calcium in the extract upon the potassium values ob-

TABLE 1.—*Percentages of potassium in plant materials by different methods*[1]

PLANT MATERIAL	FLAME PHOTOMETER-DIRECT INTENSITY METHOD						PLATINIC CHLORIDE METHOD	
	ASHED WITH H_2SO_4 AND ASH DIGESTED WITH HCl				EXTRACTED WITH ACETATE SOLUTION[2]			
	(Ca PRESENT)		(Ca REMOVED)					
Celery	3.79	3.72	4.04	4.18	4.20	4.24	3.87	3.80
Corn	0.35	0.30	0.38	0.32	0.32	0.33	0.34	0.33
Hay #2	0.95	1.01	1.09	1.09	1.17	1.15	1.14	1.13
Hay #6	2.42	2.42	2.62	2.62	2.63	2.58	2.65	2.61
Oats	0.48	0.47	0.53	0.52	0.50	0.50	0.51	0.52
Onion bulbs								
(a) White globe	1.39	1.38	1.49	1.49	1.42	1.44	1.57	1.51
(b) Yellow globe	1.76	1.77	1.84	1.92	1.86	1.88	1.96	1.98
Soybean oil meal	2.17	2.02	2.26	2.14	2.20	2.19	2.13	2.14

[1] Results from duplicate determinations.
[2] According to Attoe (1).

tained by the flame photometer, it was removed from a portion of the extract and potassium again evaluated. Calcium was removed as follows: 100 ml of the filtered solution described above was pipetted into a 200-ml pyrex beaker and 1 drop of methyl red indicator solution and 10 ml of saturated ammonium oxalate solution were added. The solution was heated to boiling, ammonium hydroxide solution was added dropwise with stirring until only a faint pink color remained, and slow heating was continued until the volume was reduced to about 20 ml. After standing overnight to insure complete precipitation of the calcium, the solution was filtered through Whatman No. 42 filter paper into a 100-ml volumetric flask. The paper was washed with cold water and the contents of the flask were made to volume with distilled water. Potassium in the solution was evaluated with the flame photometer, which was calibrated with aqueous solutions of potassium chloride that contained 50 ml of saturated ammonium oxalate solution in 500 ml. The results are given in Table 1.

TABLE 2.—*Percentages of potassium in plant materials by the platinic chloride method and by a flame photometer with lithium as internal standard*[1]

KIND OF PLANT MATERIAL ANALYZED	FLAME PHOTOMETER VALUES	PLATINIC CHLORIDE VALUES
Beet pulp	0.21	0.15
Corn cobs	0.66	0.60
Corn grain	0.32	0.33
Corn silage	1.11	1.09
Hay #2	1.06	1.13
Hay #6	2.46	2.63
Gladiolus leaves	3.42	3.49
Oat grain	0.52	0.52
Wheat grain	0.43	0.41

[1] Averages of results from duplicate determinations.

To test Attoe's extraction procedure (1) a second portion of sample was placed in a 250-ml Erlenmeyer flask together with 100 ml of acetate extracting solution prepared according to Attoe's directions. The flask was stoppered, shaken for 4 hours and allowed to stand overnight. The contents were filtered through Whatman No. 42 filter paper and potassium in the filtrate was determined with the flame photometer, which had been calibrated for this use with standard solutions prepared by adding potassium chloride to appropriate volumes of the acetate extracting solution.

To determine whether use of an internal standard would give greater accuracy, the flame photometer was calibrated with standard solutions of potassium chloride containing 15 p.p.m. of lithium as internal standard, 100 p.p.m. of magnesium, and 50 p.p.m. of calcium. Samples of a number of plant materials were treated with sulfuric acid, ashed, and extracted with hydrochloric acid as previously described. Enough lithium was added to each sample solution to give a final concentration of 15 p.p.m., and the potassium in the extracts was evaluated without removing calcium. The results obtained, together with those by the platinic chloride method, are given in Table 2.

DISCUSSION

The results of the flame photometric evaluation of potassium in solutions prepared as described above agree well with values obtained with the A.O.A.C. platinic chloride method, in which separately ashed samples

were used. In general, values for solutions obtained by digesting the ash with acid, filtering, and diluting to volume without the removal of calcium or addition of lithium were lower than those by the other procedures and by the platinic chloride method. Seay *et al.* (4) investigated the effect of calcium on the flame photometric determination of sodium and potassium in acetate extracts of plant material and concluded that the amount of exchangeable calcium was too low to interfere with the evaluation of potassium. The values obtained in this investigation appear to be in general agreement. The potassium values obtained by the acetate extraction procedure are slightly higher than those obtained from the solutions prepared from the ashed samples without the removal of calcium. However, the acetate extracted solutions filtered rather slowly, and in some instances it was necessary to leave the solutions in the funnels overnight to obtain enough extract to work with. This practice is objectionable because of the effect of evaporation.

The direct intensity method of evaluation seems to be satisfactory unless a high degree of accuracy is desired. In this case the indirect method is preferable. Compared to the platinic chloride method, the use of the flame photometer affords a considerable saving of time even when the lithium internal standard method is employed.

SUMMARY

Potassium concentrations were determined in 13 different plant materials by flame photometric methods and compared with the values obtained by the platinic chloride method. Three different procedures were used to prepare solutions of plant tissues for flame photometric evaluation by direct intensity readings and by the use of an internal standard. The percentages of potassium obtained by the use of a flame photometer were in good agreement with those obtained by the platinic chloride procedure.

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IDENTIFICATION OF CEREAL INSECTS IN STORED GRAIN BY THEIR MANDIBLE CHARACTERISTICS

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Various methods are available for the extraction of insect fragments from food products (1, 2). Such methods involve the microscopic examination of the extracted material and the identification of those particles which are from insects (3).

Increased attention is now being given to the identification of insect fragments, both in respect to their identification as insect fragments and the determination of the specific insects which yielded the fragments. Harris and Nicholson (3) and Harris (4) discussed the problem of insect fragment identification and determined insect types from which these fragments were derived. Gier, Wilbur, and Miller (5) investigated the use of biological stains as an aid in distinguishing between plant and insect fragments. It is often important to identify the type of insect from which fragments are derived so the original source of infestation can be determined. This requires a study of the morphological characters of the fragments.

Identification of the insects which contribute insect fragments to cereal products is difficult because of the comminuted condition of the product and the fragments. This makes the commonly used taxonomic characters of little value. Fragments with characters that will survive grinding and can be extracted from the food product in a comparatively intact condition are needed. Insect mandibles best fulfill these conditions in that they are relatively resistant to grinding, more readily recovered in substantial numbers in proportion to the total fragments, and moreover are easily examined microscopically.

During a recent survey (6) on the relationship between insect infestation in wheat and the fragment content of milled products, two of the authors of this paper (N. A. C. and H. C. V.) recognized, independently, the possibilities of using mandible morphology to identify and distinguish between the common stored cereal insects.

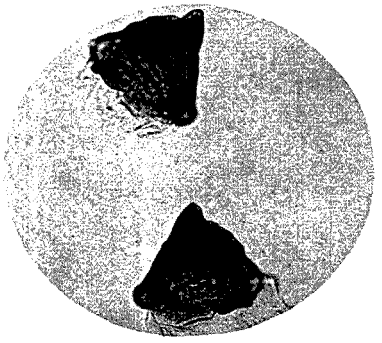
The mandible key described herein has been designed primarily to aid the analyst in the identification of those insects commonly found as storage or mill contaminants of wheat and corn. Inasmuch as time is not available to the analyst for a detailed study of each mandible under high magnification, characters or features that are reasonably clear with the wide-field microscope have been selected. In order to obtain a more workable approach to identification, the characters have been divided, first, into two primary groups, indicated by Roman numerals I and II. These two divisions are subdivided into major groups A and B. For the

* At Washington, D.C., St. Louis, Mo., and Cincinnati, Ohio, respectively.

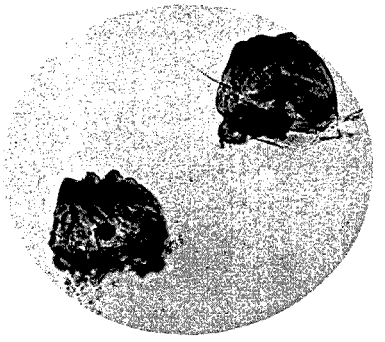
most part each mandible will be restricted to its respective group with little difficulty. However, until the analyst becomes better acquainted with the mandible characters, and because of the presence of an occasional non-conforming mandible, there may be borderline cases where positive identification will be difficult. Therefore, it seems advisable to discuss briefly some of the major key characters and mention several cases where two species are so closely related that mandible differences may not be detected. The figure designations refer to two series: 1a, 2a, etc., are photomicrographs; 1b, 2b, etc., are line drawings.

1a

2a



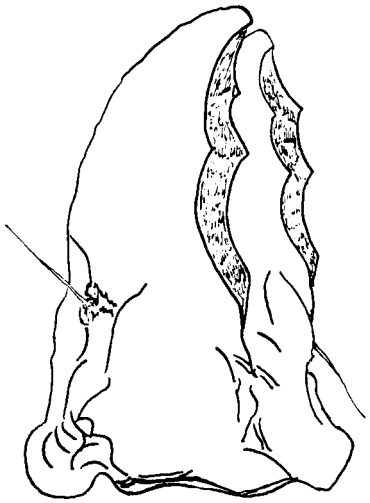
3a



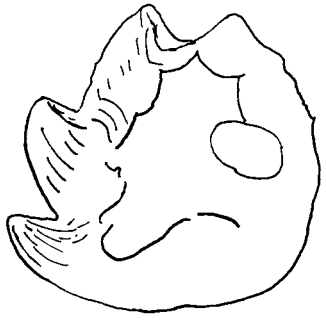
4a

FIG. 1a.—Cadelle beetle larva (×140).
FIG. 3a.—Rice weevil larva (×140).

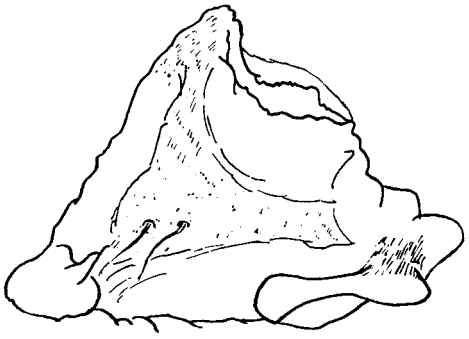
FIG. 2a. Rice or granary weevil adult (×375).
FIG. 4a.—Angoumois moth larva (×140).



1b



2b



3b

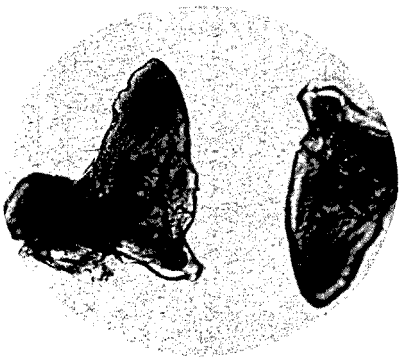
4b

FIG. 1b.—Cadelle beetle larva (×145).
FIG. 3b.—Rice weevil larva (×370).

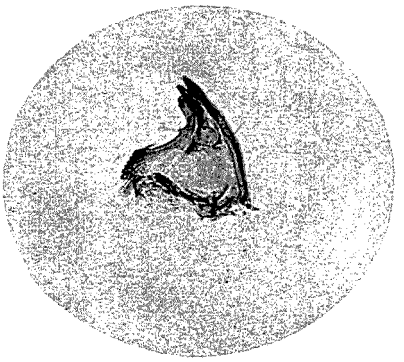
FIG. 2b.—Rice or granary weevil adult (×250).
FIG. 4b.—Angoumois moth larva (×350).

The first primary group of the key considers those mandibles with four teeth. This group is restricted to only three species, and as several teeth may be missing the characteristic shape will suffice for positive identification (Figs. 2 and 4). Insofar as there were no distinct features to differentiate between the adult mandibles of the rice and granary weevils, both species are identified together. The larval mandibles are similarly grouped.

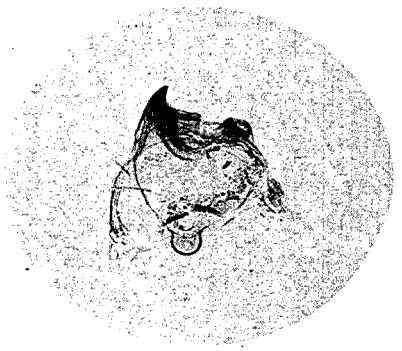
The second primary group considers those mandibles without four teeth. The group is further divided into section "A" in which the mandibles have a prominent or slight but distinct protuberance on the inner surface (Figs. 6, 7, 9, 10, 12) and section "B" in which the mandibles have



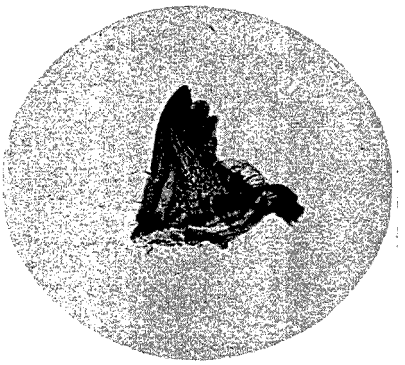
5a



6a



7a



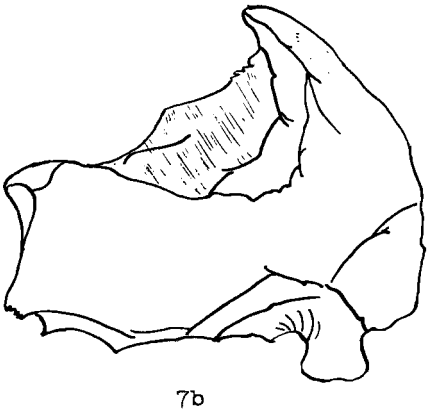
8a

FIG. 5a.—Lesser grain borer adult (×140).

FIG. 7a.—Confused flour beetle or rust-red larva (×140).

FIG. 6a.—Sawtooth grain beetle larva (×140).

FIG. 8a.—Lesser grain borer larva (×140).



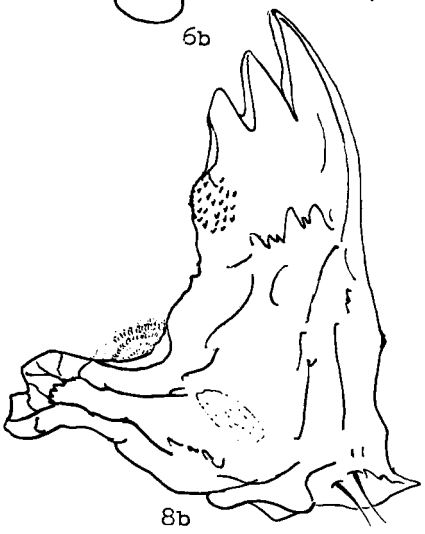


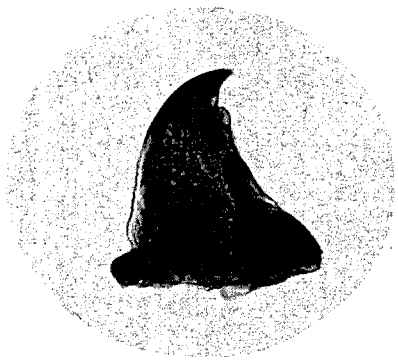
FIG. 5b.—Lesser grain borer adult (×270).
FIG. 7b.—Confused flour beetle or rust-red larva (×350).

FIG. 6b.—Sawtooth grain beetle larva (×350).
FIG. 8b.—Lesser grain borer larva (×400).

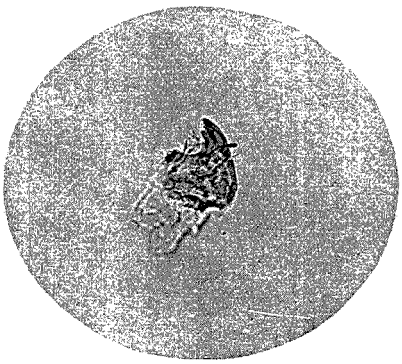
no protuberance on the inner surface (Figs. 1, 3, 5, 8, 11). Since the mandible morphology of the *Tribolium* species (confused and rust-red flour beetles) is identical, they are combined as one in section "A." In section "B" of the key, the adult and larva of the cadelle beetle appeared to have no well-defined mandible characters with which they could be separated. However, a separation might be made on the basis of the two setae present in the larval stage (Fig. 1), although it is not certain that this feature is always absent in the adult form. This protuberance on the inner surface described in section "A" will appear nearly triangular regardless of size. The inner surface will be considered that side of the man-

9a

10a



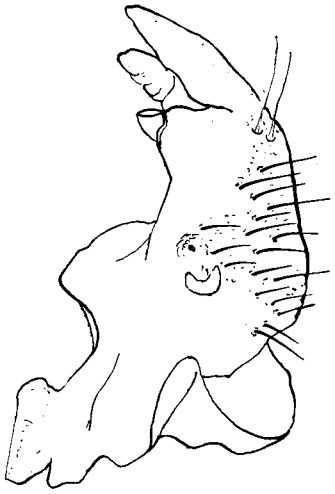
11a



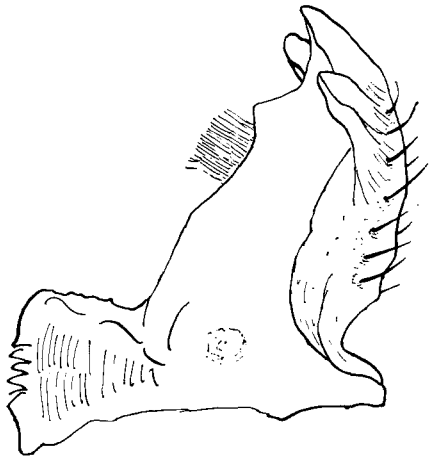
12a

FIG. 9a.—Flat grain beetle adult (×140).
FIG. 11a.—Confused flour beetle or rust-red adult (×140).

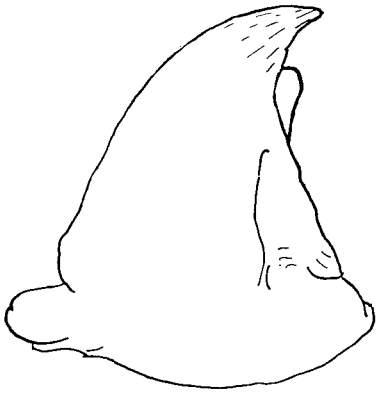
FIG. 10a.—Sawtooth grain beetle adult (×140).
FIG. 12a.—Flat grain beetle larva (×375).



9b



10b



11b

12b

FIG. 9b.—Flat grain beetle adult (×420).

FIG. 11b.—Confused flour beetle or rust-red adult (×250).

FIG. 10b.—Sawtooth grain beetle adult (×320).

FIG. 12b.—Flat grain beetle larva (×450).

dible that touches the opposite mandible when attached to the head. As noted in the key the characteristic protuberance may be greatly enlarged or very minute and usually so located that it will not be confused with the mandibular teeth or a saw-like inner surface. If the protuberance is obstructed because of the size and position of teeth, a saw-like edge on the inner surface, or its own minuteness, as may be the case with the flat grain beetle and confused flour beetle larvae, identification can be made in the section of the key where the protuberance is absent.

The "concavity" is the concave surface on the inner side of the mandible. The depth is measured to a line drawn from the point of articulation to the tip of the nearest tooth. The depth of concavity will be at least half the distance of the line for the confused flour beetle and flat grain beetle larvae. With the exception of several mandibles which are well indicated by their specific characters, all others will be more elongate in appearance, and the depth of concavity, therefore, will be less than half the distance extending from the tooth tip to the articulation point.

Consideration should be given to the possible wear of the mandibles which may leave certain characters useless, particularly under low magnification. If the mandible features seem inadequate due to possible wear, positive identification might be made on the basis of over-all shape by comparing with the diagrams.

CLASSIFICATION OF INSECTS BY MANDIBLE CHARACTERISTICS

I. Mandible with four distinct teeth, usually short or curved and appearing as a saw-like edge of the mandible.
   - A. All four teeth projecting from outside curve or crown of mandible; entire mandible appearing as a chicken comb or fan (Figs. 2a & b). *Rice or Granary Weevil Adult.*
   - B. Teeth projecting from the narrower width of the nearly rectangular (Trapezoid) mandible (Figs. 4a & b). *Angoumois Moth Larva.*

II. Mandible with fewer than four teeth.
   - A. Mandible with prominent or slight but distinct pointed (triangular) protuberance on the inner surface between teeth and articulation point.
     1. Translucent, membranous, appendage posterior to protuberance in "A"; protuberance adjacent to teeth; dark spot between two points of articulation (Figs. 10a & b). *Sawtooth Grain Beetle Adult.*
     2. Mandible without membranous appendage.
        - a. Protuberance in "A" greatly enlarged, occupying much of the space between the articulation point and teeth. Three teeth are visible (Figs. 9a & b). *Flat Grain Beetle Adult.*
        - b. Protuberance very small—occupying very little area between teeth and articulation point.
          - (1) Three prominent teeth with each extending nearly the same distance and appearing as the toe end of a foot; inside tooth with a notch; large articulation point scalular in appearance (Figs. 6a & b). *Sawtooth Grain Beetle Larva.*
          - (2) Difficult to determine number of teeth; the depth of the concavity on the inner surface is at least half the distance from articulation point to the tooth tip.

(a) Protuberance in "A" is adjacent to the teeth and is the smallest protuberance present; one or two large setae on crown or outside surface (Figs. 12a & b). *Flat Grain Beetle Larva.*

(b) Protuberance approximately midway between teeth and articulation point. A jagged edge may appear on either side, in which case it may be difficult to determine (Figs. 7a & b). *Confused Flour Beetle or Rust-Red Larva.*

B. Protuberance absent on the inner surface.

1. Translucent, fleshy appendage (half moon) in the concavity and adjacent to the articulation point.
   a. Entire mandible appearing as one tooth; inner surface forming a saw-like edge. One serration of this edge may be *so* prominent that it appears as an individual tooth (Figs. 5a & b). *Lesser Grain Borer Adult.*
   b. Three prominent teeth visible and so located and extended that a straight line will touch the tip of each and extend back to the very enlarged articulation point. Scalular patch posterior to teeth, but will appear as a slightly raised area if scales are not visible. (Figs. 8a & b). *Lesser Grain Borer Larva.*
2. Fleshy appendage in the concavity absent.
   a. Mandible with two teeth equally large; sides and base line approaching an isosceles triangle. One seta projecting near each articulation point (Figs. 1a & b). *Cadelle Beetle Larva or Adult.*
   b. Mandible with fewer or more than two teeth; seta near each articulation point absent.
      (1) Sides of mandible forming a very evident equilateral triangle (pyramid); probably only one tooth present appearing as a short, curved tip of the mandible. If two are present the appearance will be the same. Two setae present at one point of articulation (Figs. 3a & b). *Rice Weevil Larva.*
         (a) Mandible elongate; the depth of the concavity on the inner surface is less than half the distance from articulation point to the tooth tip; probably three teeth (at least two are distinct); a structure which may be a tooth is located independently of and posterior to the prominent curved tooth; a distinct bulge posterior to this structure (Figs. 11a & b). *Confused Flour Beetle or Rust-Red Adult.*
         (b) Mandible more compact and circular; the depth of the concavity on the inner surface is at least half the distance from articulation point to the tooth tip.
            (i) The concavity of the inner surface is enclosed on one side by a chitinized shield which may bear one minute triangular protuberance or a series of serrations appearing as a saw-like edge (Figs. 7a & b). *Confused Flour Beetle or Rust-Red Larva.*
            (ii) The concavity of the inner surface is not enclosed on either side by a chitinized shield; a very minute, triangular protuberance may appear adjacent to the teeth; a very pronounced protuberance posterior to and between the two points of articulation (Figs. 12a & b). *Flat Grain Beetle Larva.*

It is hoped that this technique will develop into a useful tool for the

identification of the common storage insects which in turn may help to determine the original source of infestation.

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RAPID DETERMINATION OF CAROTENE IN ALFALFA

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Usual methods for the carotene assay of alfalfa meal require either a one hour extraction with hot solvent or an overnight extraction with unheated solvent (3, 4) for quantitative removal of the carotene from the dried material. Occasionally, quality control at a commercial dehydrator or blending plant requires a carotene assay in the shortest possible time. The following procedure involves rehydration of the meal, and a complete carotene assay can be performed in less than one-half hour.

DETERMINATION OF CAROTENE IN ALFALFA MEAL

A 2.00 g sample of meal is placed on a No. 1 Whatman filter paper and washed with hot water (500 ml at 50°C.). Vacuum is used to move the water through the meal (1). The volume of water absorbed by the filter paper and the rehydrated meal is ascertained. The filter paper containing the sample is transferred to an electrical blendor and disintegrated at high speed for one minute in the presence of 100 ml of acetone. A 5-ml aliquot of the acetone extract is taken (2) and added to 5 ml of hexane and 3 ml of water in a 50-ml separatory funnel. Gently swirling the funnel for a minute transfers most of the acetone into the lower water layer which is then withdrawn and discarded.

The entire hyperphase is chromatographed on a column 12 mm in diameter, packed to 70 mm in height with an equal-parts mixture by weight of magnesium oxide (No. 2642, Westvaco) and filter aid (4). The carotene is eluted with a mixture of 1 part acetone and 9 parts hexane until 25 ml is collected in a volumetric flask. The optical density (absorbance) of the solution is read at 440 mμ in a suitably standardized colorimeter or spectrophotometer. The carotene content in the original alfalfa-meal sample is determined from the aliquot of the acetone extract taken, allowing for the water absorbed by the sample and the filter paper.

Table 1 presents the results of 12 replicate analyses of a commercial sample of dehydrated alfalfa meal. These samples were assayed by the proposed method involving rehydration (rapid method) as well as by

[1] Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture. Report of a study made under the Research and Marketing Act of 1946.

TABLE 1.—*Precision of rapid method and of overnight soaking procedure*

CAROTENE CONTENT	
RAPID METHOD	OVERNIGHT SOAKING
p.p.m.	*p.p.m.*
155	156
163	157
161	161
157	157
156	154
157	157
160	159
156	156
158	153
154	156
155	159
156	156
Average 157	157
σ* 2.7	2.2

* Standard deviation from the mean.

the method routinely employed in this Laboratory which involves soaking the meal overnight with 30% acetone in hexane to extract the carotene (4). Since the mean values by the two methods are within the experimental error of the reference method it appears that the rehydration method does not lead to systematic errors.

TABLE 2.—*Comparison of rapid method with overnight soaking procedure*

MEAL SAMPLES	CAROTENE CONTENT	
	RAPID METHOD	OVERNIGHT SOAKING
	p.p.m.	*p.p.m.*
Dehydrated (sample 1)	168	177
Dehydrated (sample 2)	180	184
Dehydrated (sample 3)	193	192
Dehydrated (sample 4)	123	114
Sun cured	17	17
Leaf meal (sample 1)	250	250
Leaf meal (sample 2)	402	400

Table 2 presents the carotene assays, as obtained by the two methods, of a number of commercial meals which vary widely in their initial carotene contents.

DETERMINATION OF CAROTENE IN FRESH ALFALFA

A similar procedure is used for analysis of fresh tissue. If the analysis is to be performed immediately, the freshly harvested material is sampled and a 10-g portion is weighed out and disintegrated with 100 ml of ace-

tone. Following the procedure described for alfalfa meal a 5-ml aliquot is then taken for assay. If it is not convenient to run the analysis at once, the freshly harvested material must be frozen rapidly to prevent carotene loss; this is conveniently done by packing in powdered dry ice. The frozen samples can be stored in tightly stoppered bottles for long periods before assay.

SUMMARY

A method is described which permits a carotene assay of fresh or dehydrated alfalfa in less than a half hour, and which is comparable in precision and accuracy to the established method which involves overnight soaking of the sample.

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THE DETERMINATION OF AUREOMYCIN IN FEEDS

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When the animal feed industry became aware of the advantage of adding aureomycin in conjunction with Vitamin B-12 (the "animal protein factor") to feed, a study was initiated to devise a method for assaying the aureomycin in feeds. The standard procedure for similar samples had been to extract them with an acidified, acetone-water solution and to assay the extract by the regular microbio (turbidimetric) method. However, two difficulties were apparent when this method was considered: (1) the small quantities of aureomycin in the feed samples (about 0.03 mg /g) and (2) the fats and oils which the acetone would inevitably extract from the feed.

After many trials of simpler methods, the following procedure was found to give results with acceptable precision and accuracy, although there was still room for improvement. Petroleum ether is used to remove the fats and oils before making the extraction for aureomycin.

METHOD

EXTRACTION PROCEDURE

Weigh 10 g of the feed to be tested into a sintered glass funnel (medium porosity) and set up for vacuum filtration. Add 75 ml petroleum ether (boiling range 30–65°C.) and, after 5 minutes standing, filter off. Repeat this operation five times, using 30 ml petroleum ether, with 10 minutes standing each time. Dry the feed by allowing air to be sucked through the funnel for a few minutes. Discard the petroleum ether

extract and substitute a clean suction flask. Add 30 ml acetone (reagent grade) and filter off after 3–5 minutes standing.

The actual extraction of aureomycin is begun at this point, with an acid-acetone solution prepared as follows: mix together 50 ml 4.0 *N*. hydrochloric acid (reagent grade), 650 ml acetone and 300 ml distilled water. First, use 50 ml acid-acetone solution, and filter off after 20 minutes standing. Repeat this operation four times using 30 ml acid-acetone solution with 5 minutes standing each time. After all possible wash is sucked out of the feed, mix the filtrate well, measure, and record the volume.

If a precipitate appears at this point, allow the filtrate to stand for a period to flocculate and then filter by gravity through Schleicher and Schuell #576 filter paper (or equivalent). About 20–30 ml clarified filtrate will be sufficient for the microbio assay.

ASSAY PROCEDURE

The turbidimetric assay of aureomycin is based upon the fact that graded dilutions of aureomycin produce inversely proportional inhibition of growth of *Staphylococcus aureus* in nutrient broth. The inhibitory effect of various dilutions of aureomycin solutions of unknown potency inoculated with *Staphylococcus aureus* is measured turbidimetrically and compared with a standard of known potency that is run simultaneously. The method used is that described by Dornbush and Pelcak (1).

PRECAUTIONS AND SPECIAL CONSIDERATIONS

1) In the extraction procedure the volume of the combined washes is measured. This volume is used for calculating the total aureomycin content of the sample weighed. Hence it is no longer necessary to preserve *all* of the solution but it is neces-

FEED MIX NO.	KNOWN POTENCY	VOL. OF FILTRATE	MICROBIO ASSAY OF FILTRATE	AVERAGE POTENCY FOUND	APPARENT ERROR
	γ/g	ml	γ/ml	γ/g	per cent
Blank	0	165	0 0	0	0
1	18.5	150	1.1 1.4	19	+3
Blank	0	169	0 0	0	0
2	9.3	172	0.6 0.6	10	+7
3	18.5	170	1.0 1.1	18	−3
4	27.8	166	1.8 1.8	30	+8
5	37.0	166	2.0 1.8	32	−13

sary to prevent evaporation of the acetone. The gravity filtration is therefore carried out as rapidly as possible. The filter is covered and only as much filtrate as will be necessary to provide an adequate sample is collected for the microbio assay. This sample must be immediately stoppered and placed in the freezer until assay time.

2) It was found, by working with known mixtures, that certain feeds give a pseudo antibiotic assay. However, this is always very low if, in fact, measurable at all. At any rate, it is best to use dilutions on blank feed samples (i.e. those containing no aureomycin) corresponding to the dilution level used on the feed containing aureomycin.

3) Sometimes a precipitate appears after freezing a filtrate sample. In this case the solution should be filtered again by gravity immediately before assay.

TYPICAL RESULTS OF AUREOMYCIN FOUND IN EXPERIMENTAL FEED MIXES

Feed mixes of known aureomycin potency were prepared in the laboratory. A 10-g aliquot was weighed out for each of the following mixes. Each extraction filtrate was assayed in duplicate. The average assay and the filtrate volume were used to calculate the average potency of the feed mix.

SUMMARY

A method is described for the determination of aureomycin in feeds by extracting with an acid-acetone solution and assaying with the standard *Staphylococcus aureus* turbidimetric procedure.

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POLAROGRAPHIC DETERMINATION OF BENZALDEHYDE IN WINE

ACTION OF A FERMENTATION MEDIUM ON BENZALDEHYDE

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The presence of amygdalin in cherry pits and its conversion into benzaldehyde is well known. There is, however, little published data concerning the disappearance of benzaldehyde during the course of alcoholic fermentation in the manufacture of wine.

The conversion of sugars to ethyl alcohol has been studied extensively. Ethyl alcohol is produced in the final step by the reduction of acetaldehyde in the presence of phosphate, adenosine, and cozymase glyceric-aldehyde (1). During the period of fermentation, acetaldehyde is always present as an intermediate between sugar and ethyl alcohol. Riberau-Gayon (2), investigated the effect of acetaldehyde on the course of yeast

fern entations and found that 0.2 g per liter of acetaldehyde did not alter the course of fermentation but that fermentation was stopped by 1.0 g per liter. As shown below, benzaldehyde in the amount of 1.0 g per liter will also prevent fermentation unless it is effectively tied up by some chemical compound.

Tomiyasu (3) reports the formation of acetoin from acetaldehyde in glycogen-free yeast preparations by the action of carboligase. Dirscherl and Schollig (4) find acyloins formed in fermentation from pyruvic acid, and acetoin formed by direct condensation of acetaldehyde, combined with carboxylase or after liberation of the acetaldehyde from the enzyme. The existence of carboligase is denied by the latter (5). Acetylphenylcarbinol and benzoylmethylcarbinol have both been ascribed to the condensation of acetaldehyde and benzaldehyde by carboligase (6).

Neuberg and Hirsch (7, 8) describe a procedure for the production of 1-acetylphenylcarbinol, based on the biological condensation of benzaldehyde and acetaldehyde through the agency of a yeast fermentation. From 100 g of benzoic acid-free benzaldehyde, 25 g of 1-acetylphenylcarbinol, 45 g of benzyl alcohol, and 4.5 g of benzoic acid are obtained.

Lintner (9, 10) finds that furfural present in a fermenting media completely disappears during the conversion of sugar to alcohol. The furfural is reduced to furfural alcohol and an unidentified crystalline material melting at 50.5°C.

Mohler and Hammerle (11, 12) report that authentic cherry brandy contains not less than 20 mg per liter of benzyl alcohol. The determination is made from ultraviolet absorption measurements in benzene. Benzyl alcohol in bitter almond water is determined, with 95 per cent accuracy, by ultraviolet absorption measurements in hexane.

Stainer (13) found that fluorescent pseudomonads can utilize certain aromatic compounds as substrates. Attack is by adaptive enyzmes. Benzyl alcohol appears to participate in the reaction chain, via benzaldehyde. An example is given of the conversion of *d,l*-mandelic acid to benzoic acid:

$$C_6H_5CHOHCOOH \rightarrow C_6H_5COCOOH \rightarrow C_6H_5CHO \rightarrow C_6H_5COOH.$$

Enzymatic oxidizing systems are found almost universally in living material (14). Yeast contains a number of enzyme systems, and some of the enzymes are present in large amount (15). An oxidizing enzyme "meta oxidase" (16) is found in cherries. Peroxidase (17) will catalyze the oxidation of mesitol (2,4,6-trimethylphenol) to yield 4-hydroxy-3,5-dimethyl benzaldehyde and 2,6-dimethylbenzoquinone. The aldehyde is further oxidized to yield the quinone. Other functional groups may also be attacked.

The benzoic acid found in fermented media which contained benzaldehyde may have been produced by an enzymatic oxidizing system or

may have been produced directly from oxidation by dissolved air. The experimental work described below gives some indication of the course of the reactions of benzaldehyde in a fermenting medium. The references cited above offer a certain amount of substantiating evidence for the production of 1-acetylphenylcarbinol, benzyl alcohol, benzoic acid, benzoin, and benzil which are found in the fermented media.

The polarographic method was employed in this work to follow the disappearance of benzaldehyde during the course of fermentation. The fermentation media were steam distilled at periodic intervals to obtain the benzaldehyde in suitable condition for polarographic determination. This method was considered more desirable than the use of chemical reagents. Those generally used react with other carbonyl groups which may be produced during fermentation. The experimental conditions for the polarography of benzaldehyde are essentially those employed by Norton (18) in studying this compound. Benzaldehyde shows two waves between *p*H 2.6 and 6.0 in the presence of phosphate citrate buffer (19), but only one wave at *p*H values greater than 6.2.

METHOD

APPARATUS

Polarograph.—A Heyrovsky polarograph, Model XII manufactured by E. H. Sargent and Company, was used for polarographic measurements. The drop time for the capillary was: $t = 2.5$ seconds at $E_{d.e.} = 0$ volts vs. S.C.E. in base solution. The temperature of the cell was maintained at 25°C. ±0.25° by means of a thermostatically controlled water bath (20).

Beckman Model DU Photoelectric Quartz Spectrophotometer.—Ultraviolet sensitive phototube, hydrogen lamp.

REAGENTS

Phosphate Buffer, 0.25 M KH_2PO_4 plus sufficient KOH to bring *p*H to 7.

Gelatin, 0.1% aqueous solution.

PROCEDURE

To 50 ml of wine add 25 ml of water, steam distill and collect the first 50 ml of distillate. To 15 ml of distillate add 1.0 ml of gelatin and 14.0 ml of buffer. Bubble oxygen-free nitrogen through the solution for five minutes, using fritted glass disk, transfer portion of sample to electrolytic cell immersed in constant temperature bath, and continue bubbling nitrogen through solution for additional 5 minutes. Make a polarogram in the range of 0.0 to −1.5 v. vs. S.C.E. at 25°C. Determine the amount of benzaldehyde from the reduction wave at −1.31 v. vs. S.C.E. (The height in mm., using a 20 shunt ratio, divided by factor 0.525 gives the benzaldehyde concentration in mg per liter or parts per million. Other dilutions and shunt values may be employed as the occasion demands, by making appropriate calculations.)

EXPERIMENTAL

A solution of benzaldehyde 100 mg/l was prepared in 12% ethyl alcohol. A gravimetric determination of the benzaldehyde employing 2,4-dinitrophenylhydrazine gave a value of 100.5 mg per liter. This solution was diluted 1-1 with buffer and gelatin. At −1.31 v. vs. S.C.E. a polarographic wave was obtained 52.5

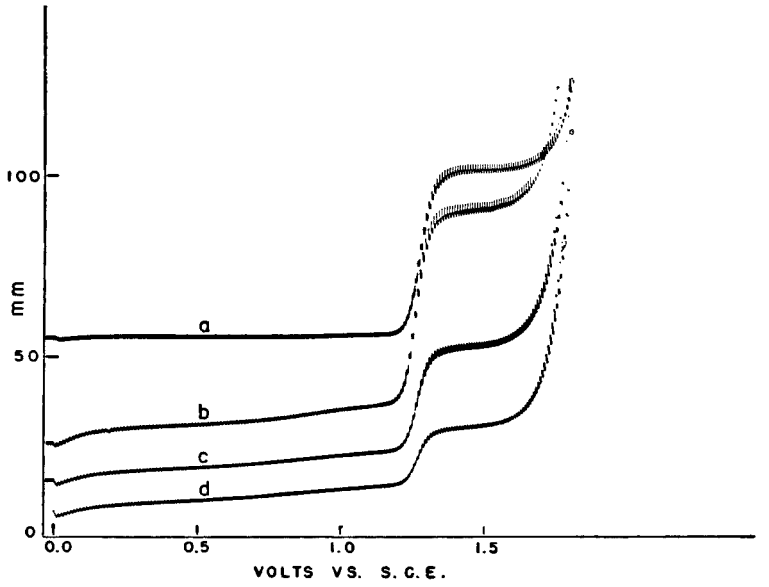

FIG. 1.—Effect of concentration on polarographic reduction wave of benzaldehyde. (a) 100 mg per liter, *p*H 7, shunt 50. (b) 50 mg per liter, *p*H 7, shunt 20. (c) 25 mg per liter, *p*H, 7, shunt 20. (d) 12.5 mg per liter, *p*H 7, shunt 20.

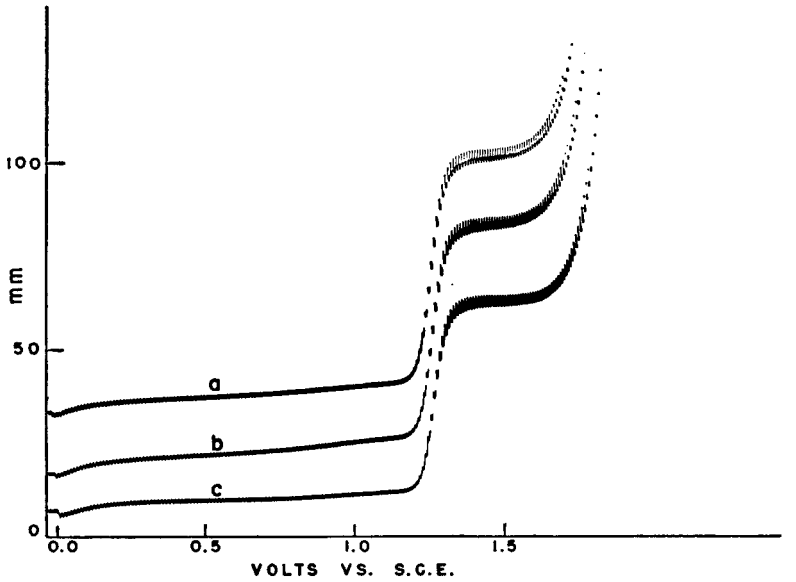

FIG. 2.—Effect of temperature on polarographic reduction wave of benzaldehyde (50 mg per liter). (a) 29°C. (b) 25°C. (c) 15°C.

mm in height. A portion of the solution treated as a wine sample under "Procedure" gave a polarographic wave at the same potential and of comparable height to the wave obtained before distillation.

Solutions were prepared in 12% alcohol containing 25.5, 51, 102 and 204 mg of benzaldehyde per liter. After addition of buffer and gelatin the solutions were polarographed and reduction waves were obtained at −1.31 v. These buffered solutions had a benzaldehyde concentration of 12.75, 25.5, 51.0, and 102.0 mg per liter and gave respective wave heights of 14, 26.5, and 53.5 on 20 shunt, and 44.5 mm on 50 shunt. Figure 1 shows these waves and demonstrates that the wave height is directly proportional to the concentration of benzaldehyde.

Figure 2 shows the polarographic reduction waves for a solution containing 5 mg of benzaldehyde per liter at 15, 25, and 29°C. Wave heights of 49.5 mm, 54.5 mm, and 59.0 mm, respectively, indicate that the wave height is not a straight line function of the temperature and therefore the temperature must be controlled quite closely for accurate results.

FERMENTATION MEDIA

To study the effect of fermentation on benzaldehyde several different types of media were prepared. Table 1 shows the basic materials employed and the amounts of added benzaldehyde, sulfur dioxide, and glycine. The sulfur dioxide was added as potassium metabisulphite. Sulfur dioxide or one of its salts is often used in wine making, and since the acid sulphite is known to form addition compounds with aldehydes it was thought that this material might serve to protect benzaldehyde from reaction during

TABLE 1.—*Benzaldehyde concentration (mg per liter) after varying fermentation intervals (Time in hours)*

SAMPLE NO.	BASIC MATERIAL	TIME	φCHO	TIME	φCHO	TIME	φCHO	TIME	φCHO	TIME	φCHO
1	Morello Cherries (juice)	0.0	102.1	168	0.0						
2	Morello Cherries (Pulp & Pits)	0.0	100.0	168	0.0						
3	Mont. Cherries (pitted)	0.0	3.0	24	0.0						
4	Mont. Cherries (no yeast added)	0.0	100.0	24	95	48	85	72	80		
5	Mont. Cherries	0.0	100.0	24	72	48	2	72	0		
6	Mont. Cherries (150 mg/l SO_2)	0.0	100.0	24	95	48	10	72	2		
7	Mont. Cherries	0.0	1000.0	24	980	48	980	144	960		
8	Blackberries	0.0	0.0	24	0.0						
9	Blackberries	0.0	100	20	12	44	0				
10	Blackberries (150 mg/l SO_2)	0.0	100	20	62	44	18	72	0		
11	Black Strap Molasses	0.0	0.0	24	0.0						
12	Molasses	0.0	100	24	31	48	2	72	0		
13	Molasses (150 mg/l SO_2)	0.0	100	24	91	48	11	72	2	96	0.0
14	Molasses	0.0	1000	96	960						
15	Concord Grapes	0.0	0.0	24	0.0						
16	Concord Grapes	0.0	1000	48	750	96	4	144	0		
17	Concord Grapes (1.4 g/l of glycine)	0.0	1000	48	6						

fer ıentation. Since amino acids react with aldehydes, glycine as a representative amino acid was added so that its effect on benzaldehyde during fermentation might be noted.

Samples of the fermenting material were taken at frequent intervals and the benzaldehyde content was determined by the polarographic method. Results are shown in Table 1. Fermentation did not commence in the same length of time in the different media, and it did not proceed in all cases with the same vigor. The various factors affecting fermentation

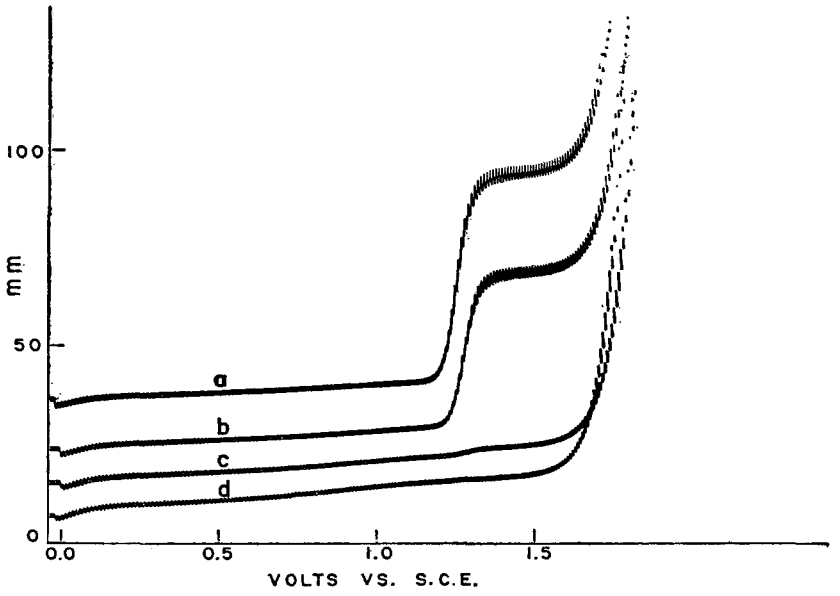

FIG. 3.—Disappearance of benzaldehyde from a cherry medium during fermentation. (Sample No. 5, Table 1.) (a) Zero hours. (b) 24 hours. (c) 48 hours. (d) 72 hours.

were not rigorously controlled, so it is not possible to show the relationship between the amount of fermentation and the disappearance of the benzaldehyde.

The sugar content of the various fermentation media was adjusted to 15 to 20 per cent by weight by addition of sugar or water. Morello cherries containing pits were crushed thoroughly and the material filtered. The filtrate was the juice used as Sample No. 1. It contained 102.1 mg/l of benzaldehyde by the polarographic method. The residue contained 175 mg per 1000 g but for use as Sample No. 2 was diluted with sugar-water solution until the benzaldehyde content was 100 mg/l. The benzaldehyde in Samples No. 1 and No. 2 was produced from the amygdalin naturally present in the pits. The benzaldehyde in the other samples was added as

such, with the exception of Sample No. 3 which initially contained 3 mg/l.

Figure 3 demonstrates the typical disappearance of benzaldehyde 100 mg/l from a cherry medium during fermentation. Figure 4 gives similar data during fermentation of a molasses medium containing an added 100 mg/l of benzaldehyde.

Fermentation was retarded slightly by sulfur dioxide, and this probably accounted for the slight lag in the disappearnace of benzaldehyde in those samples to which sulphite was added. No fermentation was noted in

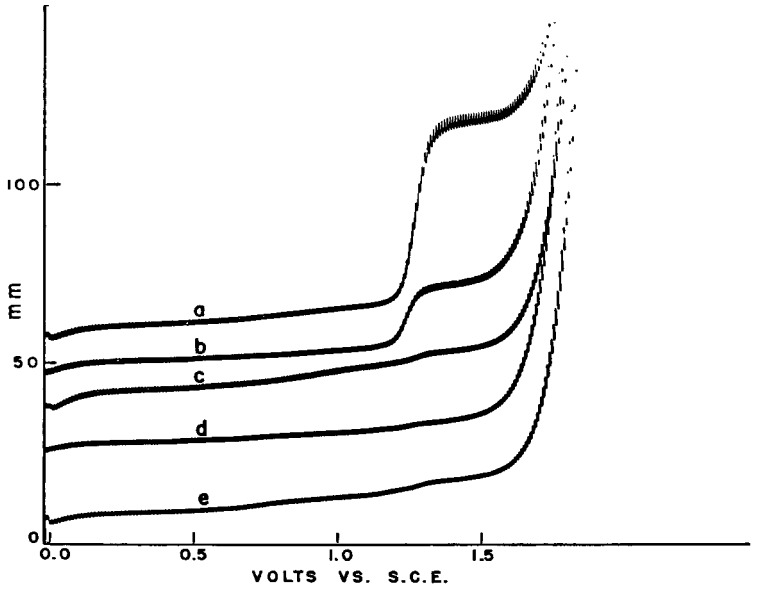

FIG. 4—Disappearance of benzaldehyde during the fermentation of a molasses medium. (Sample No. 12, Table 1.) (a) Zero hours. (b) 24 hours. (c) 48 hours. (d) 72 hours. (e) Molasses medium before addition of benzaldehyde.

cherry Sample No. 7 nor in molasses Sample No. 14, both of which contained 1000 mg/l of benzaldehyde. This concentration of benzaldehyde apparently will effectively inhibit yeast growth. The slight loss of benzaldehyde was probably due to oxidation by dissolved air. On the other hand Sample No. 16, Concord grape juice containing 1000 mg/l of benzaldehyde, showed some evidence of fermentation in 32 hours and was fermenting moderately at the end of 48 hours. From this point onward the fermentation appeared normal. Sample No. 17, Concord grape juice containing 1000 mg/l of benzaldehyde plus 1400 mg/l of glycine, fermented normally from the outset, thus showing that glycine effectively removes the inhibiting effect of benzaldehyde on yeast growth. The compound responsible for tying up the benzaldehyde in Sample No. 16 was

TABLE 2.—*Effect of fermentation on benzaldehyde added to fruit and sugar solutions*

FERMENTATION MEDIA	BENZALDEHYDE ADDED	ANALYSIS AFTER COMPLETE FERMENTATION IN MG. PER LITER					
		BENZALDEHYDE	BENZYL ALCOHOL	BENZOIC ACID	BENZOIN	BENZIL	1-ACETYL-PHENYLCARBINOL
Montmorency Cherries	3000	0	1980	125	Present	Present	Not Determined
Wild Cherries	3015	0	1870	140	Present	Present	Not Determined
Molasses	1800	0	1010	80	Present	Present	Not Determined
Concord Grapes	3385	0	1917	140	Present	Present	Not Determined
Dextrose USP	3000	0	1917	150	Present	Absent*	Not Determined

* Both benzoin and benzil were present 48 hours after addition of the final increment of benzaldehyde, but benzil had disappeared and benzoin had almost disappeared by time fermentation ceased.

not identified but possibly may have been methyl anthranilate, which is known to be present in Concord grapes.

To secure a sufficient concentration of the final products formed from benzaldehyde during fermentation, media were prepared from Montmorency cherries, wild cherries, molasses, Concord grapes, and U.S.P. dextrose plus added mineral salts. After yeasting, increments of benzaldehyde were added in the amount of 100 mg/l to the fermenting solutions as rapidly as possible without appreciably slowing fermentation. Additions were continued as long as fermentation was active. The time

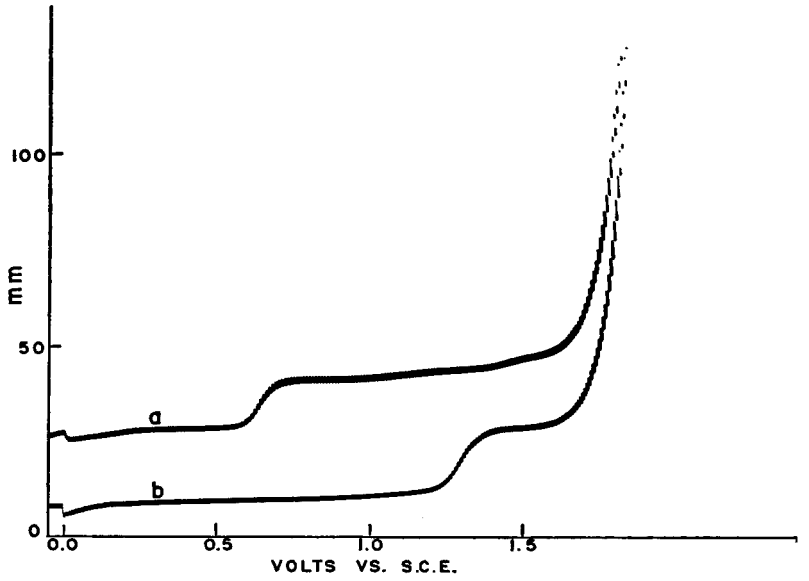

FIG. 5.—Polarograms of benzil and benzoin. Shunt 20, *p*H 7. (a) Benzil, saturated solution. (b) Benzoin, saturated solution.

intervals between additions of benzaldehyde varied between one-half and three hours, depending on the observed evolution of carbon dioxide. The benzaldehyde was added only during an 8½ hour period each day.

Table 2 gives the basic fermentation media and some analyses of the fermented products. A number of procedures were employed for both qualitative and quantitative work, but only the polarographic and spectrophotometric data are discussed in detail.

Figure 5 shows the polarographic waves obtained on benzoin and benzil in *p*H 7 phosphate buffer. The two compounds were dissolved in alcohol and diluted with water so that upon addition of buffer the concentration was 150 mg per liter. The solution immediately clouded and a precipitate formed. After filtration the benzil solution continued to form a suspension, so additional alcohol was added before the polarograms were made. The half-wave potentials of the benzil and benzoin are −0.64 and −1.31 v. vs. S.C.E., respectively. In aqueous solution, a much smaller wave is obtained with benzil (half-wave potential −0.55 v).

Figure 6 shows the polarograms of distillates from fermented cherry juice, Concord grape juice, and U.S.P. dextrose to which minerals were added. Polarogram "c" in this figure is a reproduction of a wave obtained 48 hours after the addition to the dextrose solution of the final increment

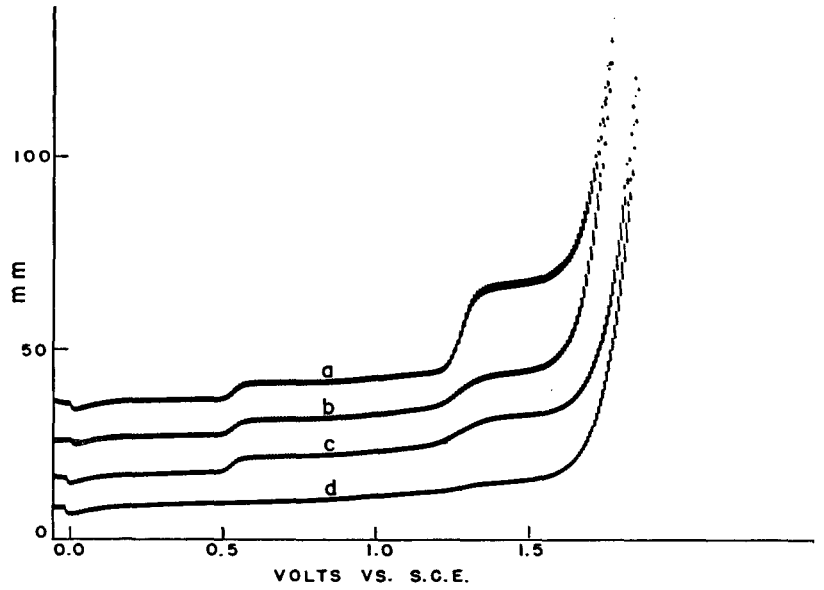

FIG. 6.—Polarograms of distillates of fermented media. (a) Montmorency Cherry, 1500 mg. per liter of benzaldehyde. (b) Concord, 3385 mg. per liter of benzaldehyde. (c) Dextrose (plus minerals). 3000 mg per liter of benzaldehyde. (d) Same as (c) after six weeks fermentation.

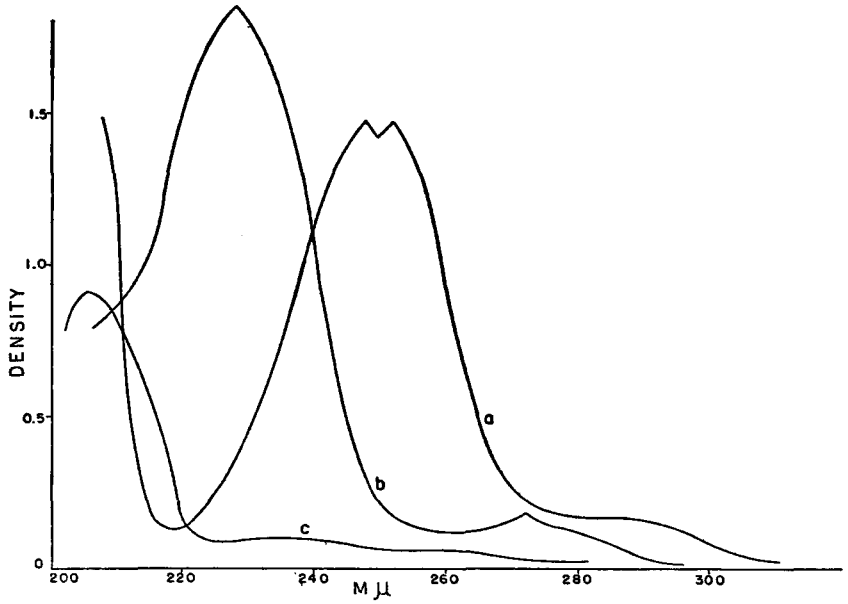

FIG. 7.—Ultraviolet absorption data. (a) Benzaldehyde, 12.5 mg per liter. (b) Benzoic acid, 12.5 mg per liter. (c) Benzyl alcohol, 12.5 mg per liter.

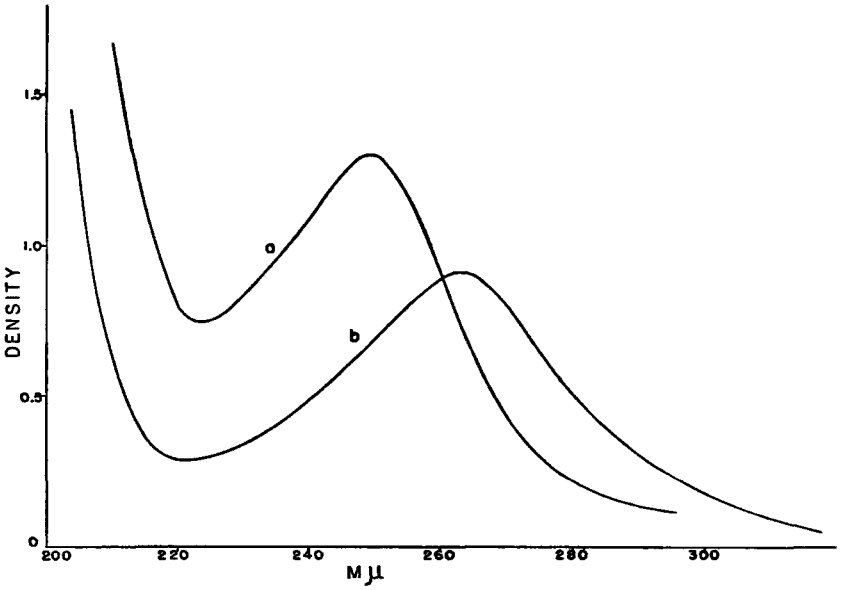

FIG. 8.—Ultraviolet absorption data. (a) Benzoin, 23.8 mg per liter. (b) Benzil, 11.5 mg per liter.

of benzaldehyde. Slow fermentation continued for approximately six weeks, and at the end of this time polarogram "d" was obtained. Reduction waves, obtained in all cases at −0.55 and −1.31 v. vs. S.C.E., substantiate the presence of benzil and benzoin.

Figure 7 presents the spectrophotometric curves obtained on 12.5 mg, per liter of benzaldehyde, benzoic acid, and benzyl alcohol in 99 per cent water and 1 per cent ethyl alcohol. Benzaldehyde gives a double absorption peak at 248 and 252 mμ; benzoic acid a major absorption peak at 228 mμ and a minor peak at 272 mμ; benzyl alcohol a single absorption peak at 206 mμ. Not shown in this diagram is the absorption curve for benzoic acid from 200 to 210 mμ. The absorption at 210 is a minimum and increases sharply with decreasing wave length.

Figure 8 shows ultraviolet absorption data on benzoin, 23.8 mg per liter, and benzil, 11.5 mg per liter. The base solution is 90 per cent water and 10 per cent ethyl alcohol. Absorption maxima are noted at 250 and 263 mμ and absorption minima at 223 and 220 mμ, respectively.

The ultraviolet absorption data on distillates from fermented Concord juice appear in Figure 9. Benzyl alcohol is the dominant component absorbing light. Absorption peaks in the three samples at 206 mμ of optical

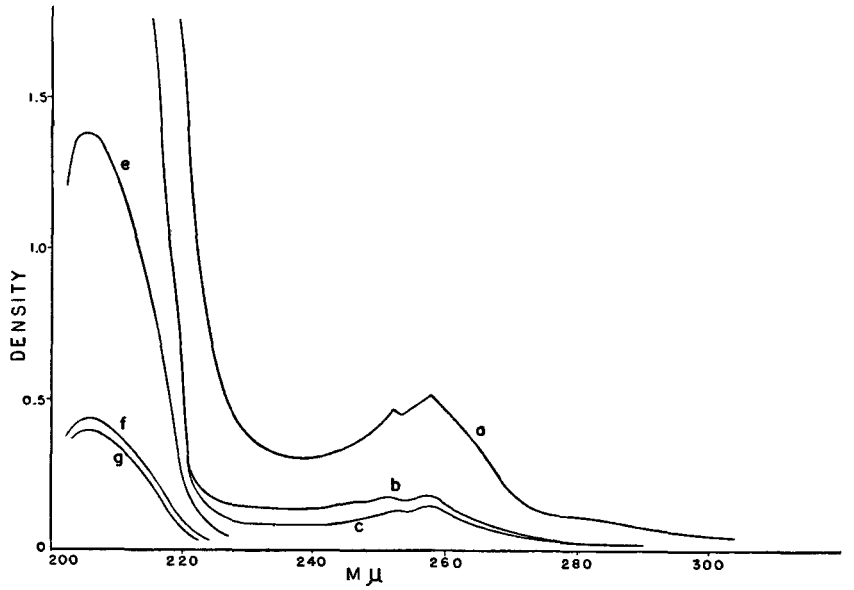

FIG. 9.—Ultraviolet absorption data on distillates from fermented Concord juice. (a) Concord, 3385 mg per liter of benzaldehyde, diluted 1:10. (b) Concord, 1000 mg per liter of benzaldehyde, diluted 1:10. (c) Concord, 1000 mg per liter of benzaldehyde plus 1400 mg per liter of glycine, diluted 1:10. (e) Same as (a), diluted 1:100. (f) Same as (b), diluted 1:100. (g) Same as (c), diluted 1:100.

d nsity 1.38, 0.43, and 0.39 indicate 1917, 597, and 542 mg per liter of b nzyl alcohol, which would correspond to a theoretical yield of 55.6 per cent, 58.6 per cent, and 53.2 per cent from the added benzaldehyde. No 1-acetylphenylcarbinol was available for spectrophotometric or polarographic study, but it might be expected that a portion of the absorption both at 206 and about 260 mμ is due to this compound.

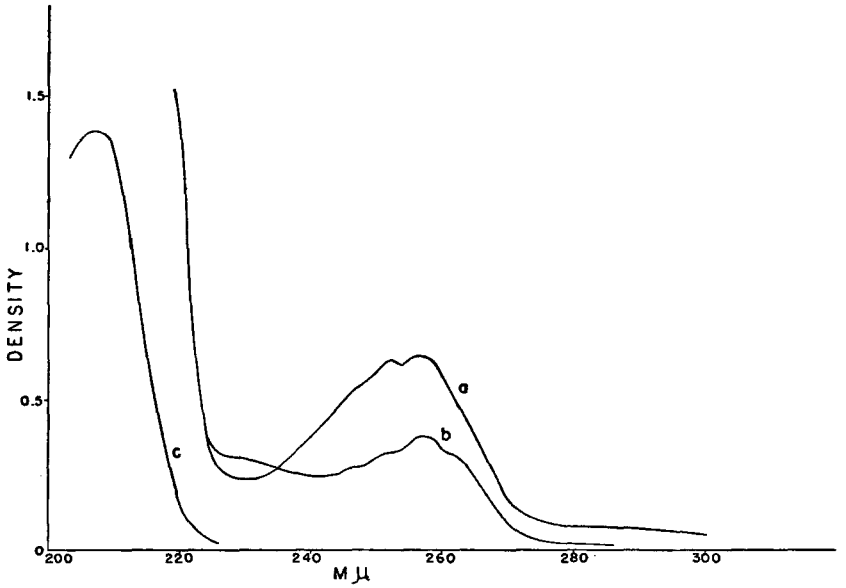

FIG. 10—Ultraviolet absorption data on distillates from fermented dextrose. (3000 mg per liter of benzaldehyde.) (a) 48 hours after adding final increment of benzaldehyde, diluted 1:10. (b) After completion of fermentation—6 weeks after (a), diluted 1:10. (c) Same as (b), diluted 1:100.

The ultraviolet absorption data on distillates from fermented U.S.P. dextrose are presented in Figure 10. The absorption waves are almost identical with those from the Concord grape juice to which benzaldehyde was added. The optical density, 1.38 at 206 mμ on a sample diluted 100 times with water, gives a benzyl alcohol concentration of 1917 mg per liter which would represent a 62.7 per cent yield from the added benzaldehyde. The absorption peaks about 260 mμ are likewise slightly higher for the dextrose solution than for the grape juice forty-eight hours after addition of the final increment of benzaldehyde. This may be explained by the fact that fermentation was not as vigorous in the dextrose solution and had not proceeded as far toward completion. Six weeks later, after the dextrose fermentation was complete, the absorption peak at 260 mμ was of comparable height to that of the grape juice to which a similar

amount of benzaldehyde had been added. At about 230 mμ it is seen that curve "b" is above curve "a". An explanation of this fact would be a conversion of some of the constituents into benzoic acid during the slow six weeks fermentation.

DISCUSSION

In Figure 4, "e," it may be noted that a distillate from unfermented molasses gave a very slight polarographic wave about −1.30 v. This wave is due to furfural or a closely allied product. An examination of the distillate by ultraviolet absorption shows an optical density of 1.60 at 277 mμ. This value corresponds with a furfural content of 9 mg per liter but probably represents only about half this amount as the background absorption was about 0.80 optical density. After fermentation, the optical density of the distillate had dropped to 0.80, demonstrating that the furfural had disappeared. (Furfural formation from superheating must be avoided during distillation of a wine as this compound will interfere with the polarographic benzaldehyde determination.)

In *p*H 7 phosphate buffer both benzaldehyde and benzoin give polarographic reduction waves at −1.31 v., thus making it impossible to determine one in the presence of the other by this means. However, it was noted that during fermentation benzaldehyde disappears at a much more rapid rate than benzoin. After addition of considerable benzaldehyde, several hundred mg per liter, to a fermenting medium, the reduction wave at −1.31 v. no longer rapidly disappears. Additional benzaldehyde added to the medium gives a distillate with the expected increase in wave height, but after a few hours the wave has dropped to its previous height or perhaps slightly below. This is fairly strong evidence for the production of benzoin during fermentation. Spectrophotometric examination of the distillates strengthens the evidence for the disappearance of benzaldehyde and the appearance of small amounts of benzoin.

The small polarographic wave appearing at −0.55 v. indicates the formation of benzil. The amount of benzil is only a few mg per liter in the distillate, but this may be due to lack of solubility and may not be a measure of the quantity produced. No examination was made of the residue which was filtered off before distillation. If diacetyl were produced and underwent no further change during fermentation it might be expected to give a polarographic wave at the same or at a slightly higher voltage than the benzil. No evidence of such wave was found in fermented solutions to which no benzaldehyde was added.

Neither acetoin nor acetylphenylcarbinol would be expected to give a polarographic wave under the conditions employed. Benzoylmethylcarbinol might be expected to give a polarographic wave, and likely some of this material was formed, but it was not identified in the fermented media.

The benzoic acid content of the fermented material was usually about 4 per cent of the theoretical yield from benzaldehyde. An analysis of Sample No. 17, Table 1, to which both glycine and benzaldehyde were added, gave a benzoic acid content of 11 mg per liter, which is about 1 per cent of the theoretical yield from benzaldehyde.

SUMMARY

A polarographic method is presented for the determination of benzaldehyde in alcoholic beverages.

Benzaldehyde is shown to disappear during a fermentation process with the production of benzyl alcohol as the main product.

Benzaldehyde in a concentration of 1000 mg per liter can effectively inhibit fermentation.

Evidence for the formation of benzil and benzoin in small amounts from benzaldehyde during fermentation is presented.

Ultraviolet absorption data for benzaldehyde, benzoic acid, benzyl alcohol, benzoin, and benzil, in aqueous or weakly alcoholic solutions, are presented.

The determination of benzyl alcohol in aqueous distillates at 206 mμ with an ultraviolet spectrophotometer is shown to be feasible.

Attention is directed to a possible similarity in the conversion of acetaldehyde, benzaldehyde, and furfuraldehyde into the corresponding alcohols through the agency of fermentation.

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WATER INSOLUBLE FATTY ACIDS AND BUTYRIC ACID IN CREAM STORED AT FOUR DEGREES CENTIGRADE

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In the course of studies on water insoluble fatty acids (WIA) and butyric acid in butter as indices of the condition of the cream from which it was made (1), a study of progressive decomposition of cream was included (2). Work which involved storage of cream at 32°, 25°, and 20°C. has been extended to include the effect of holding at 4°C. for varying periods, followed by holding at approximately 25°C. until the cream was considered decomposed.

The official AOAC methods (3) for WIA and butyric acid were employed. The course of bacterial development during the aging of the cream into the decomposed state was also studied, using the following methods:

BACTERIOLOGICAL METHODS

The method for the estimation of the numbers of lipolytic microorganisms in the cream at various stages of the experiments was basically that devised by G. M. Eisenberg (4). Before any method was adopted, however, considerable investigational work was carried out to find a procedure by which plate counts of lipolytic colonies could readily be made. Some methods gave only a fraction of the true number. Difficulty also arose because of the presence of nonlipolytic blue colonies. G. Knaysi (5) studied the use of water insoluble basic dyes for the demonstration of the hydrolysis of fat. The Nile blue oil indicator was prepared by incorporating the water insoluble, fat soluble Nile blue into the Eisenberg formula. This resulted in the confirming of a much higher percentage of the total bacteria as lipolytic. The plate counting of lipolytic bacteria was greatly facilitated by the elimination of non-lipolytic blue colonies which did not appear on the plate because of the removal of the water soluble portion of the dye.

PROCEDURE

A saturated aqueous solution of Nile blue A (National Anilin) was precipitated with *N*.NaOH, and the water insoluble precipitate was washed with buffered water (3 g Na_2HPO_4 plus 2 g KH_2PO_4, both anhydrous, per liter) on the filter paper until nearly freed from a tinge of blue. The dried precipitate was then dissolved in fresh cotton seed oil in the proportion of 200 mg to 100 ml, and further washed with water in a separatory funnel until practically free from soluble dye. From this point the dye-oil indicator method of Eisenberg was followed, i.e., the stained fat was added to an equal amount of warm 4 per cent gelatin (Difco) in distilled water and put through a hand homogenizer a sufficient number of times so that no oil separated

upon autoclaving. The resulting hot mixture had a pink to pink-yellow color. The indicator solution was held in screw-capped bottles in the refrigerator until used. It was then placed in the Arnold sterilizer along with the agar. The preparation did not show appreciable separation after repeated heating.

For total counts tryptone-glucose extract agar was used.

For lipolytic counts the following agar was prepared: yeast extract 3.0 g, proteose-peptone 10.0 g, NaCl 5.0 g, Na_2HPO_4 (Anhyd) 3.0 g, KH_2PO_4 (Sorensen's) 2.0 g, agar (Difco) 15.0 g, plus distilled water to 1000 ml. The agar was dissolved in one-half the water before mixing with the other ingredients. After combining, the medium was brought to a boil on a hot plate or heated sufficiently in the Arnold sterilizer to bring down the insoluble phosphates. The medium was then filtered through paper pulp. No adjustment in reaction was required.

The medium fulfilled the requirements prescribed by Knaysi in that it was highly buffered, slightly alkaline and free from fermentable carbohydrate.

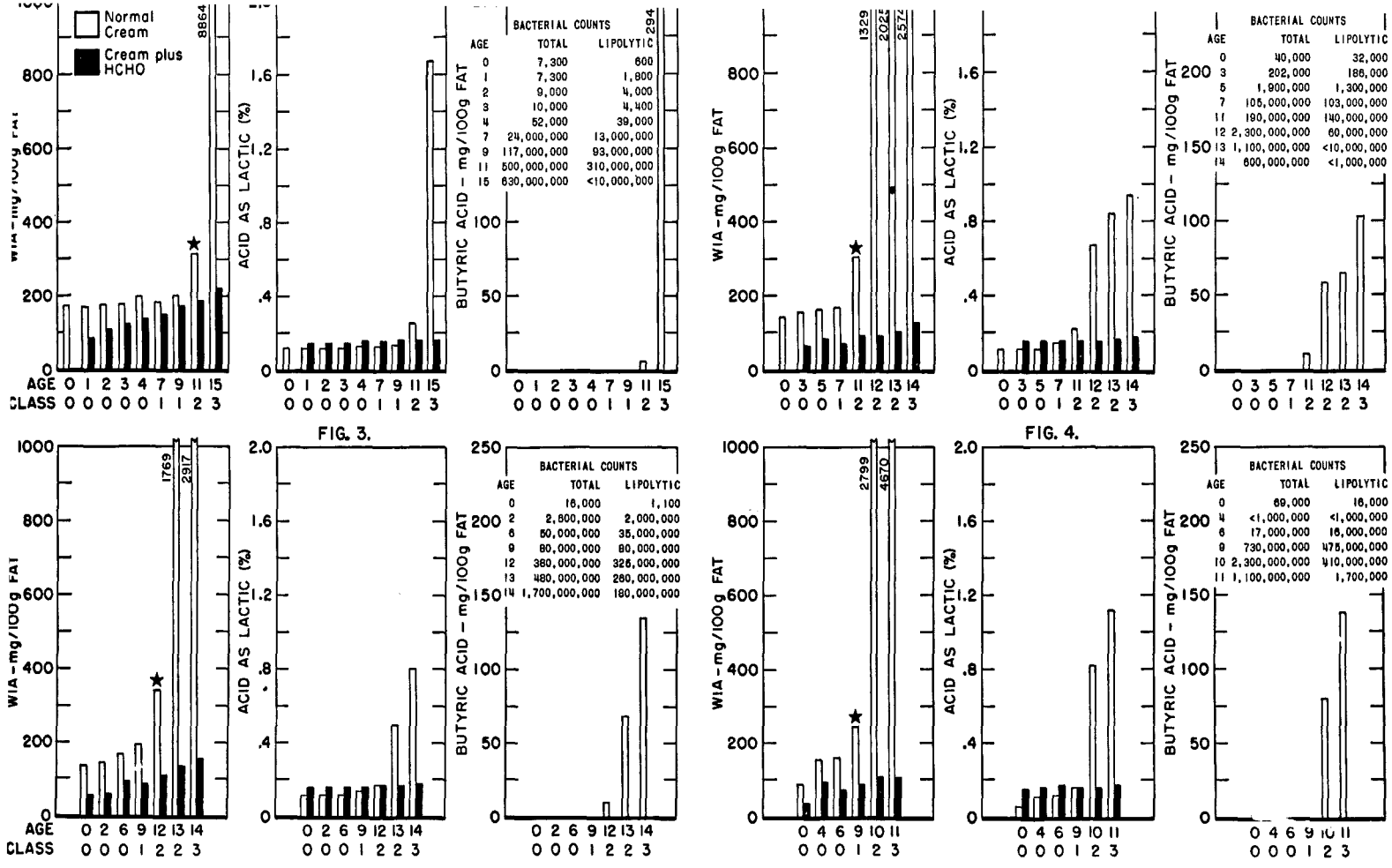
Preparation of Plates: Serial dilutions in duplicate were prepared for both total counts and estimation of lipolytic microorganisms. For lipolytic plates 5.0 ml of the indicator mixture was added to 80 ml of the yeast extract buffered agar in screw-capped bottles and mixed by rotation just before pouring at approximately 45°C. The plates were incubated inverted at 30°C. for 4 to 5 days.

By this method all blue colonies on the plates were counted as lipolytic. No attempt was made to identify the species of the lipolytic organisms except that a sufficient number of the colonies representing different colonial characteristics were examined further to confirm them as lipolytic types.

CHEMICAL DATA

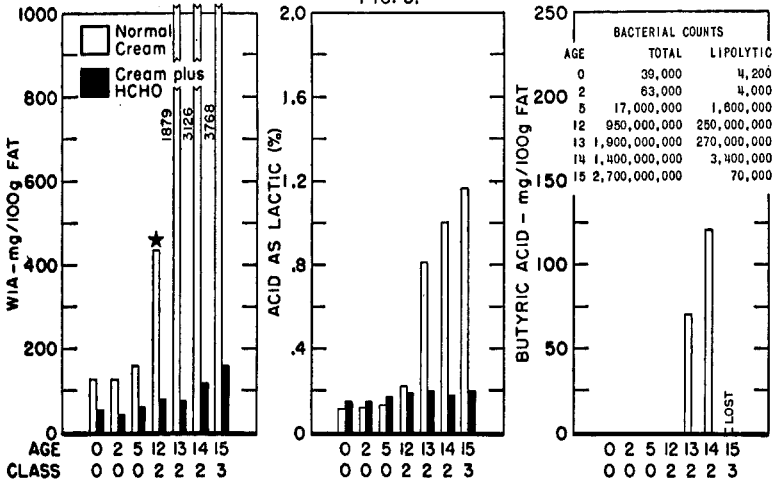
The source of the cream used in this work is described in the succeeding paper (6). The cream separated from each milk was divided into two portions of approximately one gallon each. Formaldehyde equivalent to 0.1 per cent was added to one portion and both were held at 4°C. The stored cans were sampled at the intervals shown in the figures. Determinations of titratable acidity, WIA and butyric acid as well as total and lipolytic counts were made. The analytical data are presented in the figures. In the case of each cream the number of days elapsing between examinations is shown at the base of each figure. The ★ in one column, in connection with the WIA results, indicates the age of the cream when it was removed from 4°C. to 25°C. The organoleptic classification of the cream at each sampling period is given in the line titled "class" on each figure. The description of the classification system used has been given previously (7).

The figures show the same general pattern. WIA in the normal cream (not treated with formaldehyde) did not show any pronounced increase until the cream reached that stage of deterioration (class 2) where the organoleptic classification would condemn it for use in the manufacture of butter. The titratable acidities likewise did not show a pronounced increase until the cream reached class 2. Butyric acid in most cases did not appear in the cream until class 2 had been reached. When small quantities of butyric acid were found in class 1 cream (borderline) some increase in WIA was noted; this shows that appreciable decomposition was taking



FIGS. 1–4.—Progressive decomposition studies.

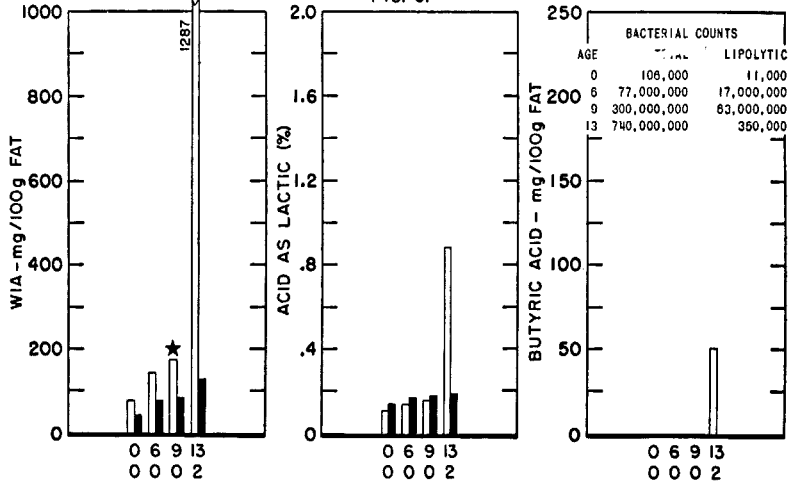
FIG. 5.



BACTERIAL COUNTS

AGE	TOTAL	LIPOLYTIC
0	39,000	4,200
2	63,000	4,000
5	17,000,000	1,600,000
12	950,000,000	250,000,000
13	1,900,000,000	270,000,000
14	1,400,000,000	3,400,000
15	2,700,000,000	70,000

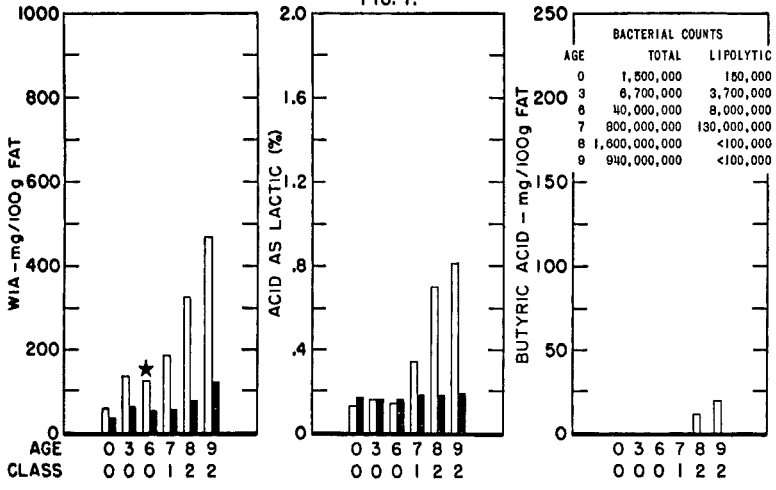
FIG. 6.



BACTERIAL COUNTS

AGE	TOTAL	LIPOLYTIC
0	106,000	11,000
6	77,000,000	17,000,000
9	300,000,000	63,000,000
13	740,000,000	350,000

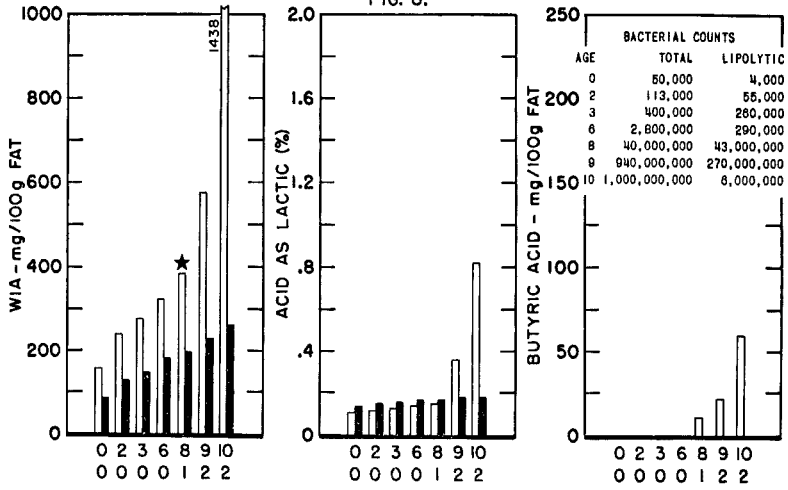
FIG. 7.



BACTERIAL COUNTS

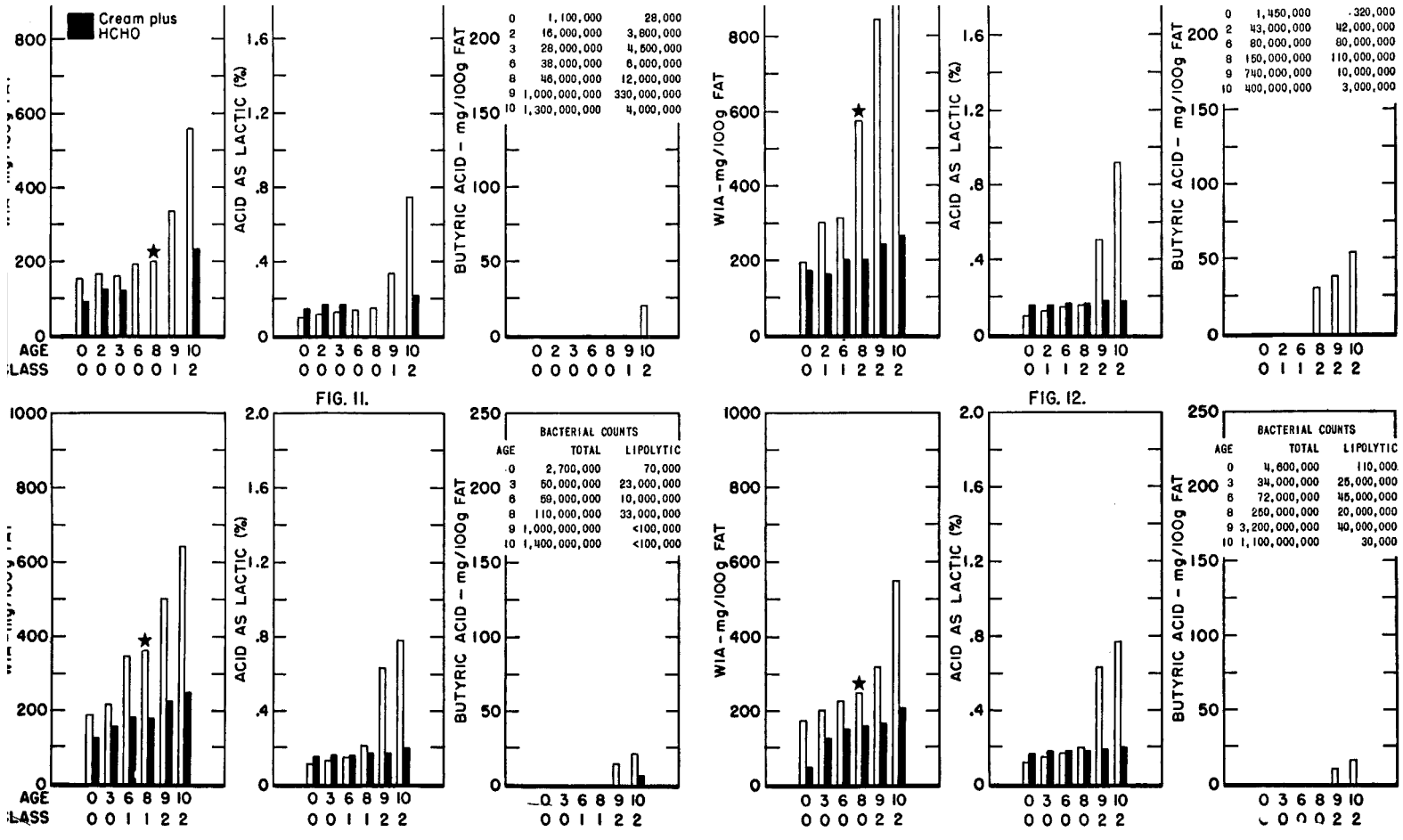
AGE	TOTAL	LIPOLYTIC
0	1,500,000	150,000
3	6,700,000	3,700,000
6	40,000,000	8,000,000
7	800,000,000	130,000,000
8	1,600,000,000	<100,000
9	940,000,000	<100,000

FIG. 8.



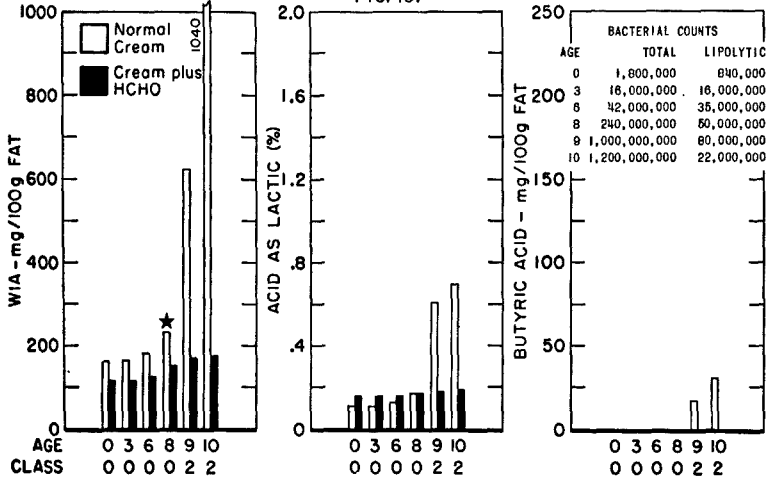
BACTERIAL COUNTS

AGE	TOTAL	LIPOLYTIC
0	50,000	4,000
2	113,000	55,000
3	400,000	260,000
6	2,800,000	290,000
8	40,000,000	43,000,000
9	940,000,000	270,000,000
10	1,000,000,000	6,000,000



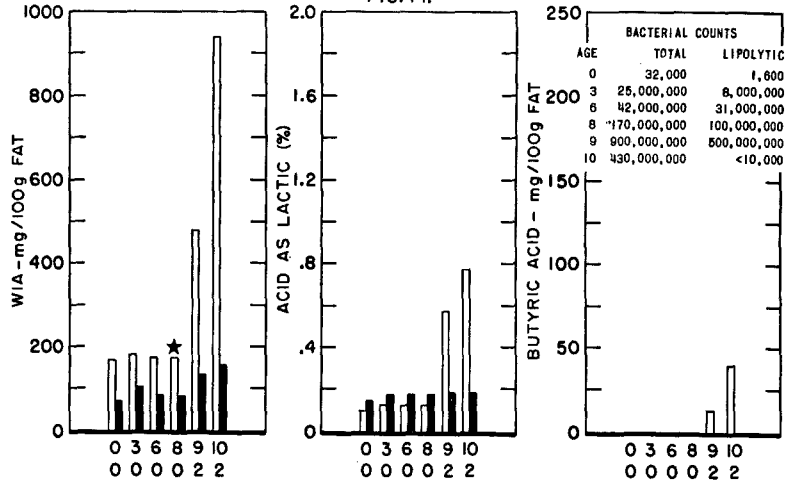
FIGS. 9–12.—Progressive decomposition studies.

FIG. 13.



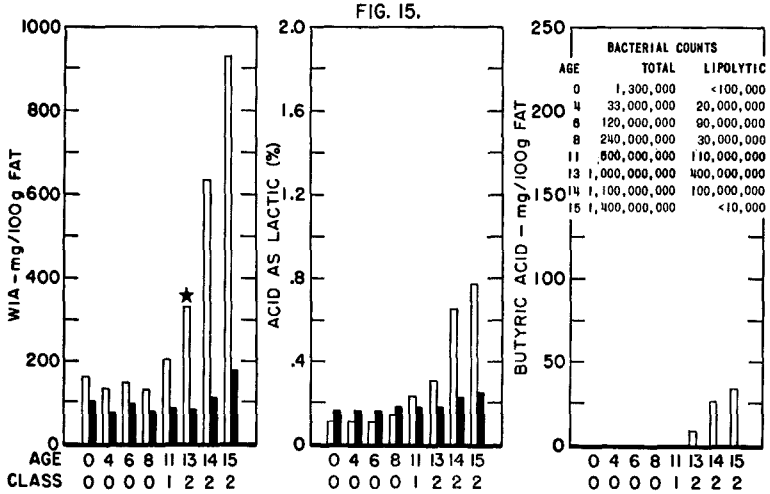
BACTERIAL COUNTS		
AGE	TOTAL	LIPOLYTIC
0	1,800,000	840,000
3	16,000,000	16,000,000
6	42,000,000	35,000,000
8	240,000,000	50,000,000
9	1,000,000,000	80,000,000
10	1,200,000,000	22,000,000

FIG. 14.



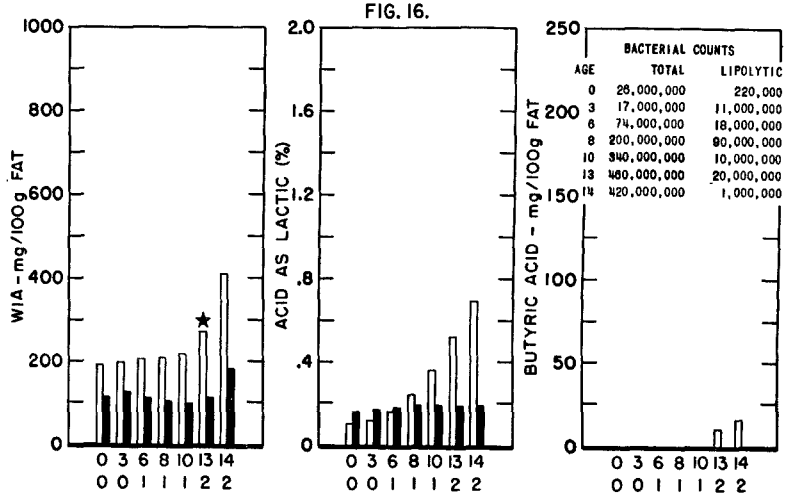
BACTERIAL COUNTS		
AGE	TOTAL	LIPOLYTIC
0	32,000	1,600
3	25,000,000	8,000,000
6	42,000,000	31,000,000
8	170,000,000	100,000,000
9	900,000,000	500,000,000
10	430,000,000	<10,000

FIG. 15.



BACTERIAL COUNTS		
AGE	TOTAL	LIPOLYTIC
0	1,300,000	<100,000
4	33,000,000	20,000,000
6	120,000,000	90,000,000
8	240,000,000	30,000,000
11	800,000,000	110,000,000
13	1,000,000,000	400,000,000
14	1,100,000,000	100,000,000
15	1,400,000,000	<10,000

FIG. 16.



BACTERIAL COUNTS		
AGE	TOTAL	LIPOLYTIC
0	26,000,000	220,000
3	17,000,000	11,000,000
6	74,000,000	18,000,000
8	200,000,000	90,000,000
10	340,000,000	10,000,000
13	480,000,000	20,000,000
14	420,000,000	1,000,000

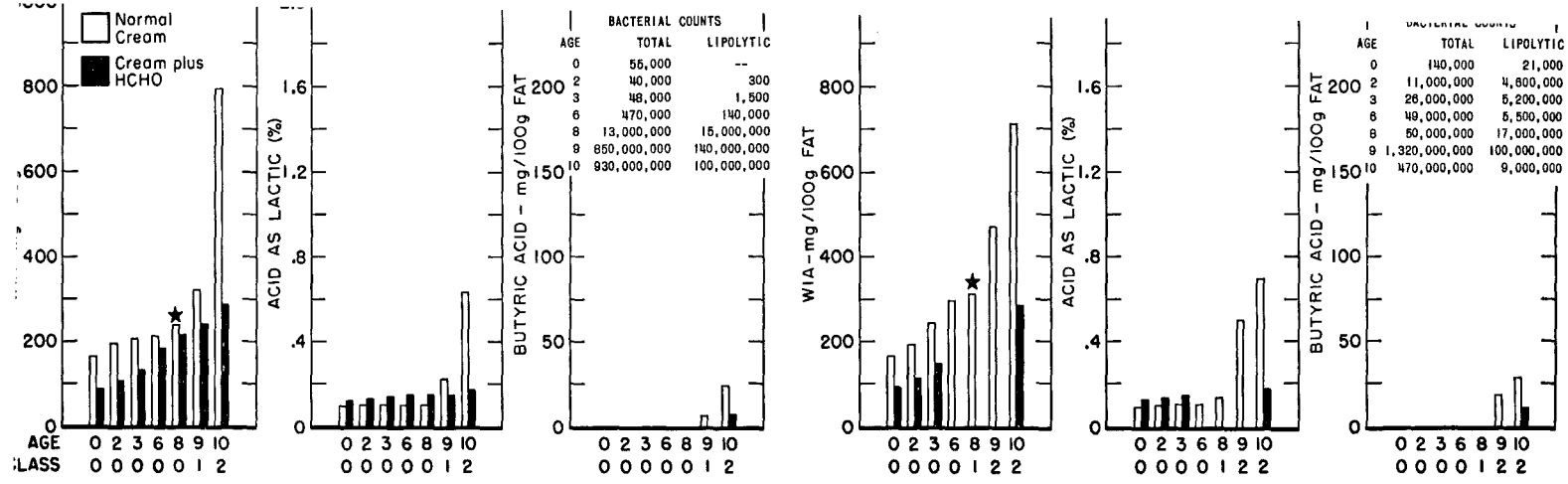
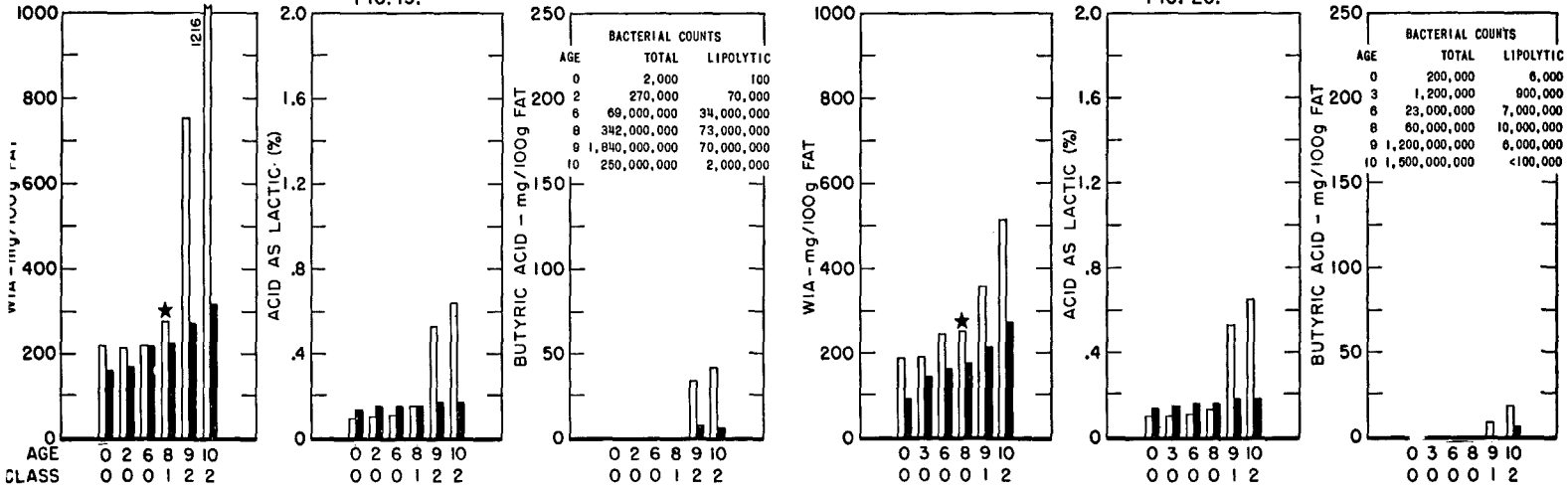


FIG. 19.

FIG. 20.



FIGS. 17–20.—Progressive decomposition studies.

place even though it could not be definitely detected by organoleptic examination. It is interesting to note that in cases where a small quantity of butyric acid was found in class 1 cream, that on the succeeding day materially larger quantities of the acid as well as WIA were found, and that organoleptically, the cream had reached class 2.

When cream was removed from storage at 4°C. and subsequently held at 25°C. deterioration took place very rapidly. This is shown in Table 1.

TABLE 1.—*Cream deterioration at 25° C. after storage at 4° C.*

FIGURE NO.	ORGANOLEPTIC CLASS AND AGE AT TIME CREAM WAS REMOVED FROM 4°C. TO BE HELD AT 25°C.		ADDITIONAL DAYS HELD AT 25°C. TO BECOME ORGANOLEPTIC CLASS 2
	CLASS	AGE	
4	1	9	1
6	0	9	4*
7	0	6	2
8	1	8	1
9	0	8	2
11	1	8	1
12	0	8	2
13	0	8	1
14	0	8	1
A	0	8	2
B	1	8	1
C	1	8	1
D	0	8	2

* No determination made between 9th and 13th days.

Eight samples were class 0 when removed from storage at 4°C.; 5 of these required 2 additional days at 25°C. to become class 2, and 2 required but one day. One sample was not analyzed until 4 days after removal from storage at 4°C. Five samples were class 1 when removed from storage at 4°C and they required but 1 additional day at 25°C. to reach class 2.

Seven other samples (not included in the Table) reached class 2 while being held at 4°C. Six of these required 11, 11, 12, 12, 13 and 13 days, respectively, and one 8 days to reach class 2.

In most of the experiments there was little or no increase in WIA or titratable acidity in the samples to which formaldehyde had been added, even after long periods of storage at 4°C. followed by 1 to 4 days at 25°C. In the case of some creams (Figs. 11, 18, 19, 20) small quantities of butyric acid were found in the formaldehyde treated samples after the same storage period during which the normal creams reached class 2.

Detailed discussion of the bacteriological data seems unnecessary, since it is believed that they are self-explanatory. It will be noted that in instances where the lipolytic count at the end of the 4° storage period rep-

resented a high percentage of the total organisms present, that the WIA content rose to a high level after the samples were then held at 25°C. for 1 day.

SUMMARY

Data are presented to show that cream can be held at 4°C. for a reasonable period of time without undergoing marked deterioration. However when cream is removed from storage at 4°C. and subsequently held at 25°C., deterioration may take place at a rapid rate and render the cream unfit for the manufacture of butter.

ACKNOWLEDGMENT

Grateful appreciation is extended to J. C. Palmer, of the Division of Food, for his assistance in the chemical examinations, and to H. Leininger, of the Microbiological Division, for making many of the bacteriological examinations.

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EFFECT OF FEED ON WATER INSOLUBLE FATTY ACIDS IN CREAM

By FRED HILLIG and J. C. PALMER (Division of Food, Food and Drug Administration, Federal Security Agency, Washington 25, D. C.)

The quantity of water-insoluble fatty acids (WIA) in butter is a good index of the quality of the cream from which it is made (1). Most data have been obtained on commercial butters churned from vat creams representing the production of more than one herd of cows. Other workers have reported (2) that milk from three individual cows on dry feed showed a high WIA content (182, 371, and 550 mg/100 g fat) and that when the cows were returned to pasture the WIA dropped to lower figures (63, 104 and 133 mg. respectively). The experiment reported in this paper was designed to verify whether milk produced by cows on dry feed contains higher quantities of WIA than milk produced by the same cows on pasture.

Commercial butter, or the cream entering into its production, is never that of a single cow. Accordingly this study was conducted on herd milk.

Arrangements were made with the Bureau of Dairy Industry of the U. S. Department of Agriculture, at Beltsville, Maryland, to furnish milk from a herd of 8 cows. The herd remained intact during the course of the experiment, from October 29 to December 11, 1951. Afternoon and following morning milkings were combined to furnish sufficient cream for the determination of WIA, as well as for the experiments which were discussed in the preceding paper. The separated cream was adjusted to 35 per cent fat. The first sample was obtained while the cows were still on pasture, the second on the day the cows were removed from pasture and placed in the barn on dry feed, and 5 additional samples were obtained while the cows were on various types of dry feed and silage. Table 1 gives types of feed, titratable acidities, and WIA on these samples.

TABLE 1.—*Effect of feed on WIA in cream from herd of 8 cows*

CREAM NUMBER	DATE COLLECTED	TYPE OF FEED	WIA	TITRATABLE ACIDITY
			mg/100 g fat	*per cent*
1	10/29/51	Pasture, Plus dry grains, alfalfa	173	0.12
2	11/ 2/51	Pasture (Cows removed from pasture)	141	0.11
3	11/ 7/51	Grass silage, dry grains, alfalfa	139	0.12
4	11/ 9/51	Grass silage, dry grains, alfalfa	83	0.05
5	11/13/51	Grass silage, dry grains, alfalfa	126	0.11
6	11/20/51	Grass silage, dry grains, alfalfa	76	0.11
7	12/11/51	Dry grains, alfalfa (from 11/20/51)	55	0.13

There was no increase in WIA when the cows were removed from pasture and put on dry feed, nor after they had been on dry feed for approximately 5 weeks.

In a second experiment beginning January 22, 1952, a study was made on milk from a different herd of 4 cows, then on dry grains, silage, and hay, and later going to pasture. Milkings from 2 days were combined in order to furnish sufficient cream. In Table 2 data are presented on types of feed, titratable acidities, and WIA.

Here again there is no indication that the type of feed had any influence on the WIA content of the cream.

To supplement the above data some creams (adjusted to 35% fat), from 20 gallons of commercial milk in each case, were examined during January and February, a time when the cows would be on barn feed. Each sample represented mixtures of milk from several Maryland and Virginia herds. The titratable acidities and WIA content of these creams are presented in Table 3.

The results are well within the normal range reported for sound butter making cream.

TABLE 2.—*Effect of feed on WIA in cream from herd of 4 cows*

CREAM NUMBER	DATE COLLECTED	TYPE OF FEED	WIA	TITRATABLE ACIDITY
			mg/100 g fat	*per cent*
8	1/22/52	Alfalfa silage plus dry grains	153 165	0.11
9	2/ 5/52	Alfalfa silage plus dry grains	150 153	0.10
10	2/19/52	Alfalfa silage plus dry grains	205 186	0.10
11	3/ 4/52	Alfalfa hay plus dry grains	190 186	0.11
12	3/18/52	Alfalfa hay plus dry grains	179 169	0.12
13	4/ 1/52	Alfalfa hay plus dry grains	167 157	0.11
14	4/15/52	Pasture plus dry grains and hay	164 171	0.10
15	5/ 1/52	Pasture plus dry grains and hay	150 173	0.11
16	5/13/52	Pasture plus dry grains and hay	191 190	0.10

TABLE 3.—*Effect of feed on WIA*

CREAM SAMPLES	TITRATABLE ACIDITY	WIA
A	0.09	167 160
B	0.09	169 164
C	0.09	217 216
D	0.10	187 188

SUMMARY

Data are presented to show that milk produced by cows on dry feed does not contain larger quantities of WIA than milk produced by the same cows on pasture.

ACKNOWLEDGEMENT

Appreciation is expressed for the cooperation of the Bureau of Dairy Industry, U. S. Department of Agriculture, and the assistance offered by C. F. Hufnagel and N. W. Hooven, Jr., of that Bureau.

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FREEZING POINTS OF SOUR MILKS

BY HERMANN C. LYTHGOE*

The official cryoscopic method (1) for the detection of added water in milk states that the method is "applicable only to samples with titratable acidity not exceeding 0.18% when determined as directed under 15.4." This stipulation is based upon the work of Keister (2) and Bailey (3), who point out that the freezing point of milk decreases as acidity increases; if this factor is not recognized it is possible that samples of sour milks containing added water might be reported as pure milks. Both of these investigators have noted an average freezing point lowering of about 0.003°C. for each 0.01 per cent increase in acidity. However, because of variability from this figure in the case of individual milks, this average lowering has not been applied as a correction figure in the cryoscopy of sour milks.

This note is intended to show that if data showing the correlation between acidity and freezing point lowering are properly considered it should be possible to make proper corrections for acidity and thus to determine with reasonable certainty whether or not a sample of sour milk has been watered.

The reported data are very meager; Keister (2) has reported 12 observations of the acidity-freezing point relationship, Bailey (3) has reported 15, and the writer has supplied 10 figures.

It was noted that when acidities are low the freezing point lowering per 0.01 per cent increase in acidity tends to be considerably higher than the average. This is shown in Table 1 for the 12 samples of lowest acidity.

It was further noted that when freezing points are plotted on the arithmetic scale of semi-log paper and acidities are plotted on the log scale, a nearly straight line results. Using the above-mentioned data, the equation of this line was calculated, but further study revealed that

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TABLE 1.—*Freezing point decrease with acid increase—Low acid samples*

SAMPLE NO.	FREEZING POINT (INITIAL)	ACIDITY (% INCREASE)	FREEZING POINT (DECREASE)	Δ F. P. PER 0.01 ACIDITY
1	−0.539	0.02	−0.022	−0.0110
2	.496	0.03	.019	.0063
3	.546	0.04	.018	.0045
4	.537	0.05	.023	.0046
5	.533	0.06	.023	.0038
6	.538	0.06	.023	.0038
7	.515	0.06	.023	.0038
8	.541	0.06	.023	.0038
9	.530	0.07	.025	.0036
10	.541	0.075	.026	.0035
11	.550	0.095	.037	.0039
12	.560	0.120	.037	.0031

several samples were either higher in acidity or in freezing point than is recognized (for normal milks) in the official method. Hence, a new computation, excluding these, was made. The data finally used are taken from observations on 10 milks (Table 2) and they are plotted in Figure 1 on

TABLE 2.—*Freezing point—acidity data*

SAMPLE NO.*	OBSERVATIONS							
	INITIAL		SECOND		THIRD		FOURTH	
	FREEZING POINT	ACIDITY	FREEZING POINT	ACIDITY	FREEZING POINT	ACIDITY	FREEZING POINT	ACIDITY
L3	−0.539	0.13	−0.562	0.19	0.687	0.79		
K2	.539	.15	.548	.18	.602	.34		
B 18707	.540	.15	.570	.25	.660	.55		
B 18708	.541	.15	.567	.22				
B 17834	.541	.15	.590	.31				
K1	.545	.15	.548	.18	.637	.42		
K4	.552	.15	.555	.17	.558	.20	.636	.46
K5	.541	.16	.546	.18	.564	.22		
L4	.544	.17	.650	.51				
L1	.539	.18	.561	.20	.713	.92		

* (B = Bailey, K = Keister, L = Lythgoe).

the semi-log basis. The theoretical relationship is shown (dotted line). In the calculations, acidities are expressed as parts per thousand to avoid negative characteristics. The relationships are:

$$\text{Log X (acidity in p.p.t.)} = -4.41624\ Y - 2.16553$$
$$Y\ \text{(freezing point)} = -0.226437 \log X - 0.490356.$$

(The value of Y is carried to an impractical number of places merely for purposes of calculation.)

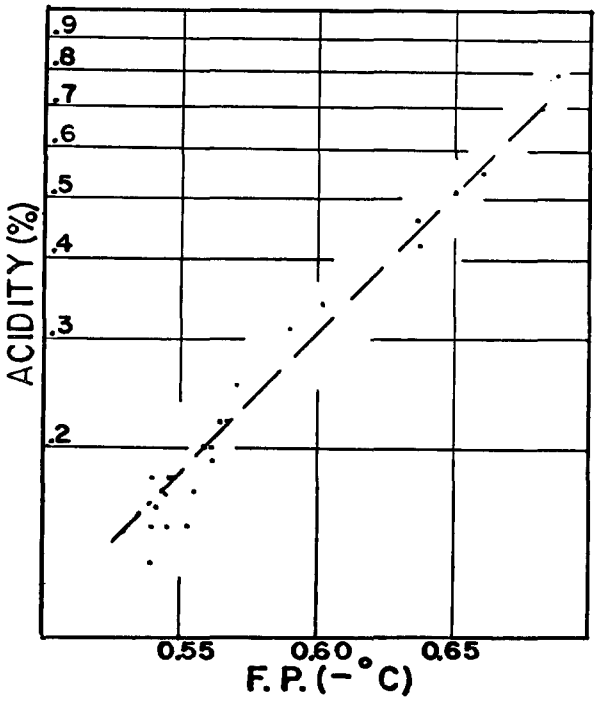

FIG. 1.—Freezing point—acidity relationship.

In illustration, a few values calculated from the foregoing equations are given in Table 3.

TABLE 3.—*Freezing points calculated for various acidities*

ACIDITY	FREEZING POINT	ACIDITY	FREEZING POINT
per cent	°C.	*per cent*	°C.
.18	−0.548		
.20	.559	.60	−0.667
.30	.598	.70	.682
.40	.627	.80	.695
.50	.649	.90	.706

The relationship between freezing point and acidity is best applied as follows: (Milk with acidity of 0.18 per cent and a freezing point of −0.548 is considered "normal"). This freezing point is subtracted from the

theoretical freezing points of "sour" samples and the difference is divided by the difference in acidities. A series of *adjusted* factors result (similar to the 0.003 factor of Bailey and Keister) which can be used to compute the freezing point of sour samples back to an acidity of 0.18 per cent. A series of these calculated factors with directions for their application is given in Table 4.

TABLE 4.—*Factors for estimating the freezing points of sour milks at (a theoretically restored) 0.18% acidity**

ACIDITY	FACTOR	ACIDITY	FACTOR	ACIDITY	FACTOR
.20	0.518	.50	0.314	.80	0.236
.25	.416	.55	.297	.85	.228
.30	.419	.60	.282	.90	.220
.35	.385	.65	.269	.95	.213
.40	.357	.70	.256	.99	.208
.45	.332	.75	.246		

* From the per cent acidity of the sour milk subtract 0.18 and multiply the difference by the factor corresponding to the acidity of the sour sample. Add the product to the freezing point of the sour sample.

Two examples are given below. (Neither set of data was used in the computations.)

Lythgoe sample—Observed F.P. at 0.79% acid = −0.684; 0.79−0.18 =0.61. Factor for 0.79% acid (not shown in condensed Table 4) =0.238; 0.61×0.238=0.145; −0.684+0.145=−0.539° (found at 0.16% acid, −0.533°).

Keister sample—(According to Winter's table (4) this sample contained 9.8 per cent added water.) Observed F.P. at 0.27% acid =−0.536°. 0.27−0.18=0.09. Factor for .27% acid =0.443. 0.09×0.443 =0.0399; −0.536+.040=0.496° (found at 0.18% acid, −0.496°).

DISCUSSION

The samples of milk from which these calculations were derived were not of known purity. Their average freezing point, −0.542°, corresponds, according to Winter's table, to milk containing 1.45 per cent added water. Still, the figures listed in Table 3, approximate as they are, have value. If a sample of sour milk freezes at a point materially above that corresponding to its acidity, the presence of added water is to be suspected and the suspicion approaches certainty as the difference increases.

SUMMARY

Preliminary studies have brought out a more exact relationship between the freezing point and acidity of milk. This relationship could be more precisely derived from the analysis of many more samples of milks of known purity. It is suggested that the A.O.A.C. re-investigate the subject.

The problem is intrinsically a simple one and its solution should add to the value of the cryoscopic test for the detection of added water in milk.

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DETERMINATION OF DEXTROSE AND LEVULOSE IN HONEY

COMPARISON OF METHODS[1]

By JONATHAN W. WHITE, JR., C. RICCIUTI, and JEANNE MAHER (Eastern Regional Research Laboratory[2] Philadelphia 18, Pennsylvania)

Although levulose is the most important sugar of honey, both quantitatively and because of its effect upon the physical properties, relatively little attention has been given in recent years to its determination in this product. The method of low- and high-temperature polarization was introduced by Wiley (1) in 1896 and used by Browne in his classical analyses of honey reported in 1908 (2). Although included since the first edition of the "*Methods of Analysis*," A.O.A.C., it is still designated first action in the 1950 edition. The method is strictly applicable only when the rotation of all other substances in a mixture is unaffected by temperature change. It was recognized that this is not strictly true with honey (2).

The fundamental constant upon which this method is based, i.e. the change of rotation per gram of levulose in 100 ml solution in a 2.00 dm tube between 20° and 87°C., was found by Wiley to be 0.0357°V. for the interval 0–88°C. He also found the change to be uniform over this temperature range. Browne and Zerban (3) listed the values for this constant calculated from the data of five investigators previous to Wiley; the average was 0.0362. The value of 0.036 was confirmed by Jackson and Silsbee (4) who reported it to vary somewhat with concentration and details of manipulation. However, later study by Jackson and Mathews (5) using very pure levulose and improved methods, yielded a value of 0.03441 over a concentration range of 3 to 18 per cent and a temperature range of 20° to 70°C. This figure is the average of 30 observations at six concentrations. Lothrop (6) reported an average value of 0.03415 between 20° and 70° for two concentrations.

Tsuzuki and co-workers (7) have determined the specific rotation of

[1] Report of work carried out under the Research and Marketing Act of 1946.
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levulose over the temperature range 10–90°C. and for concentrations of 5 to 40 per cent by weight. Calculations from their values for 10 per cent levulose give, for the change in rotation per gram levulose per degree, the values 0.03449 for 20–90°C. and 0.03454 for 20°–70°.

Jackson and Mathews (5) also reported their determination of the mean expansion coefficient for levulose solutions between 20° and 70° to be 0.00044 ml per ml per degree, rather than the 0.00047 value used by Browne (2) and subsequently by the A.O.A.C. This latter value is the expansion of water over the range.

The polarimetric determination specified by the A.O.A.C. requires the determination of rotation at 20° and 87°C. Jackson and Mathews recommended that readings be made at 20° and 70° when their revised constants are used. It is implied (3) that since the change of rotation of levulose is uniform, readings may be made above 20° for the low temperature value. This would permit somewhat simpler apparatus.

A number of chemical methods for selective determination of dextrose or levulose in honey were studied by Lothrop and Holmes (8). A modification of the Hinton and Macara method for dextrose was developed and applied to honey. In their method dextrose is quantitatively oxidized by hypoiodite under strictly controlled conditions; correction is made for a 1.2 per cent oxidation of levulose, and levulose is calculated by difference from values for total reducing sugar by the Munson and Walker or Lane and Eynon methods. They reported the analysis of 33 floral types of honey by their method and included a comparison of levulose values for ten samples by both oxidation and polarization methods. Consistently higher values (average 1.33 per cent levulose) were obtained by the chemical method. In 1938 Lothrop (6) pointed out that if instead of the A.O.A.C. value, the Jackson-Mathews polarimetric constant of 0.03441 were used to calculate levulose, the average difference in levulose content found by the two methods became 0.19 per cent.

For a selective levulose method, Jackson and Mathews (5) took advantage of the difference in reducing power of levulose against a modified Ost's solution, and against another reagent (Quisumbing and Thomas, or Lane and Eynon). Two simultaneous equations were solved for dextrose and levulose content. This was applied to ten honey samples by Jackson, Mathews, and Chase (9). Expressing a desire for a more accurate, reproducible procedure for the determination of dextrose and levulose in honey, Marshall and Norman (10) preferred a method in which each sugar is determined directly, rather than determination of one sugar directly and the other by difference from a total reducing sugar value. They pointed out that in indirect methods any error in the determination of one sugar affects the other in the opposite direction, resulting in a value for levulose to dextrose ratio that may be far from correct. Determination of levulose by a copper-reduction method after destruction of dextrose was the principle

adopted. They selected the Lothrop-Holmes procedure, with slight modification, for the determination of dextrose, and also for its destruction prior to the determination of residual sugars as levulose by the Shaffer-Somogyi method. They studied mutual interference of the two sugars and presented equations for calculation of levulose and dextrose contents. Results of the application of this method to 13 British honeys are listed. Subsequently Ugarte and Karman (11) reported the analyses of 58 Argentine honey samples determined by this procedure.

Hurd *et al.* (12) described the application to honey of their method of determination of sugars by distillation of the propionates. By this procedure the sugars are grouped as mono-, di-, and trisaccharides and no distinction is made between dextrose and levulose. They reported, however, the analysis of six samples of honey by the Jackson and Mathews copper method as well as by the Becker and Englis (13) procedure in which levulose is oxidized by ferricyanide. The former procedure gave levulose to dextrose ratios of less than 1 for three samples, and lower in all cases than by the Becker-Englis method. They suggested that their distillation procedure indicated the presence of a reducing disaccharide as a general component of the samples.

In their description of a colorimetric method for the determination of reducing sugars using triphenyltetrazolium chloride, Mattson and Jensen (14) list analyses of seven honey samples for levulose and dextrose but do not report comparison data by any other method.

Of these publications, that of Lothrop and Holmes gives results of analyzing ten honey samples by more than one procedure, as does that of Hurd *et al.* for six samples. We have applied most of the methods outlined above to the determination of levulose and dextrose in fifteen domestic honey samples representing fourteen floral sources. The A.O.A.C. and Jackson-Mathews polarimetric procedures have been compared; the Lothrop-Holmes method, Jackson-Mathews modified Nyns method, and the Marshall-Norman procedure were also used on the same samples. Several other procedures were given preliminary study. One of them was a combination of the diphenylamine method of Rolf, Surtshin and White (15) with the Shaffer-Somogyi method; another was a combination of the diphenylamine method at 75°C. in which the levulose to dextrose color-production ratio is 64 (15) with a similar procedure at 104°C. in which the color ratio is about 8 (15, 16).

EXPERIMENTAL

PREPARATION OF SAMPLES

Fifteen authentic unheated honey samples in 60-pound containers had been procured by the Kansas Agricultural Experiment Station for a study of the role of honey in baking. These were carefully heated for 30 minutes at 160°F., strained, and sampled. The samples were shipped to this laboratory for analysis. Table 1 shows the floral source, area of production and color classification of the samples.

ANALYTICAL METHODS

1. *High- and low-temperature polarization.*—Each sample was analyzed in duplicate by the A.O.A.C. method outlined for honey (17). A silver-lined 2 dm tube was used, with temperature control to ±0.1°C. In addition to readings at 20° and 87°C., values were obtained at 25° and 70°. The higher temperature readings were taken on different aliquots to avoid the effects of decomposition by heat. Levulose was calculated from these results, using: (a) the Wiley coefficient of 0.0357, the expansion coefficient of 0.00047, and the temperatures 20° and 87°, and (b) the Jackson-Mathews coefficient of 0.0344 and expansion coefficient of 0.00044. Dextrose was calculated by the A.O.A.C. procedure by difference between the levulose values and the total reducing sugar values obtained below.

TABLE 1.—*Honey samples*

NO.	FLORAL SOURCE	LOCALE	MOISTURE[1]	COLOR
			per cent	mm. Pfund
1	Yellow Sweet Clover	Kansas	15.12	25
2	Mesquite	Texas	16.60	32
3	Alfalfa	Arizona	14.92	44
4	Star Thistle	California	15.96	49
5	Tupelo	Florida	18.24	54
6	Eucalyptus	California	17.00	64
7	White Clover	California	15.60	22
8	Orange	California	14.76	21
9	Heartsease	Iowa	16.68	50
10	Horsemint	Texas	15.32	40
11	Spanish-needle	Kansas	17.80	73
12	Buckwheat	New York	15.44	119
13	Fall Flower	New York	17.24	111
14	Alfalfa	California	14.28	53
15	Cotton	Texas	16.04	26

[1] *By refractometer.*

2. *Lothrop-Holmes method.*—Each sample was analyzed in duplicate, using aliquots from the solutions clarified for polarization. Total reducing sugars were determined* by a modified Luft-Schoorl method and levulose was calculated by the procedure of Lothrop and Holmes.

3. *Jackson-Mathews modified Nyns method.*—Each sample was analyzed in duplicate by this procedure. After filtration the precipitated cuprous oxide was determined by the volumetric dichromate method of Jackson and Mathews (5). Direct determination without filtration was not found applicable because of excessive loss of iodine caused by CO_2 evolution upon acidification of the reaction mixture. Total reducing sugars were determined by the Munson and Walker method.

4. *Marshall-Norman method.*—Each sample was analyzed in duplicate by this method.

5. *Other procedures.*—Preliminary studies using known solutions indicated that the diphenylamine procedures previously mentioned were not sufficiently accurate to permit application to the levulose-dextrose system.

* We are indebted to Mrs. P. D. Harper of the Analytical, Physical-Chemical and Physics Division for these analyses.

TABLE 2.—*Determination of dextrose in honey*

NO.	BY DIFFERENCE			DIRECTLY	
	AOAC	JACKSON-MATHEWS (POLARIMETRIC)	JACKSON-MATHEWS (OXIDATION)	LOTHROP-HOLMES	MARSHALL-NORMAN
1	35.15	33.21	40.87	35.68	36.81
	35.22	32.91	40.95	35.70	36.67
2	36.72	34.66	42.96	37.41	37.10
	37.03	35.95	43.21	37.39	37.49
3	37.84	36.03	41.66	37.87	36.99
	37.98	35.97	41.02	37.97	36.43
4	37.32	35.82	40.05	37.10	35.46
	37.03	35.27	39.97	37.12	35.75
5	29.97	28.11	31.66	29.11	29.37
	30.01	28.27	31.53	28.99	29.30
6	33.92	32.51	38.51	33.87	33.08
	33.73	32.12	38.09	33.64	33.63
7	37.56	35.32	39.74	37.61	37.35
	37.71	35.80	39.14	37.55	37.04
8	34.77	33.23	38.98	35.06	34.15
	34.69	33.38	40.31	35.15	34.48
9	35.74	34.12	41.97	36.24	35.89
	35.79	33.74	41.80	36.22	36.22
10	36.48	36.53	37.37	36.19	36.21
	38.66	36.84	39.58	36.21	36.12
11	33.60	32.01	35.61	32.17	28.30
	33.48	32.19	33.94	32.44	28.18
12	36.46	36.06	33.19	36.34	34.91
	37.32	35.78	32.55	36.70	34.81
13	38.76	37.15	39.73	37.06	36.32
	39.02	37.47	38.47	36.82	36.16
14	35.70	33.65	41.34	37.88	37.35
	35.92	33.84	40.15	37.87	37.99
15	40.04	38.86	39.70	37.89	37.90
	40.02	39.40	39.27	37.64	37.58

TABLE 3.—*Determination of levulose in honey*

NO.	DIRECTLY				BY DIFFERENCE
	AOAC	JACKSON-MATHEWS (POLARIMETRIC)	MARSHALL NORMAN	JACKSON-MATHEWS (OXIDATION)	LOTHROP-HOLMES
1	37.61	39.73	40.83	36.30	39.76
	37.53	40.06	40.89	35.55	39.74
2	38.37	39.62	41.83	36.17	39.61
	38.03	39.21	42.32	36.13	39.63
3	38.32	40.30	40.57	35.78	40.32
	38.17	40.36	40.05	36.57	40.41
4	35.63	37.27	37.85	35.01	37.81
	35.95	37.87	39.32	34.83	37.79
5	41.66	43.74	46.45	39.36	44.46
	41.71	43.56	45.86	39.48	44.59
6	37.90	39.44	41.46	35.98	39.83
	38.11	39.87	41.13	35.93	40.08
7	38.21	40.66	41.31	35.77	40.15
	38.05	40.13	41.78	36.17	40.21
8	38.74	40.43	41.03	38.46	40.36
	38.83	40.36	41.06	38.01	40.27
9	38.16	39.94	42.09	36.38	39.58
	38.11	40.35	42.47	35.97	39.60
10	35.54	37.67	38.94	34.43	36.06
	35.34	37.33	39.06	33.88	36.04
11	40.44	42.18	43.41	37.73	41.65
	40.57	41.98	43.07	37.87	41.36
12	36.44	36.87	36.16	39.32	36.17
	35.50	37.18	36.15	38.92	36.00
13	37.42	39.18	41.09	34.63	39.07
	37.14	38.83	40.72	35.29	39.33
14	38.04	40.28	41.97	36.81	37.63
	37.80	40.08	41.86	37.00	37.64
15	37.77	39.06	41.04	36.65	39.79
	37.79	38.47	41.00	37.25	40.06

RESULTS

The results of the application of these analytical methods to the fifteen honey samples are shown in Tables 2 and 3.

When applied to honey the Wiley method for levulose presupposes not only that the change of rotation for levulose with temperature is constant over the range involved, but also that no other component of honey shows significant change of rotation with temperature. Browne and Zerban (3) state that 1.5 g arabinose, 3.0 g galactose, 7.0 g maltose or 9.0 g lactose show about the same variation of rotation with temperature as 1 g of levulose. Of these sugars, only maltose has been demonstrated in honey (18); Hurd *et al.* (12) state that maltose or another reducing disaccharide is a general component of honey.

The polarimetric data at the four temperatures have been used to calculate the change of rotation (°S) per degree C per g levulose in each

TABLE 4.—*Change in polarization per degree per gram levulose in clarified honey solutions*[a]

TEMPERATURE INTERVAL	$\frac{\Delta P}{ATg}$
20–25°C.	0.0159
25–70	0.0374
70–87	0.0324
20–70	0.0346
20–87	0.0340
20–70[b]	0.0344
20–70[c]	0.0345
20–87[d]	0.0357
20–90[c]	0.0345

[a] Degrees S for solutions of 26 g. honey per 100 ml. in 2 dm. tubes.
[b] Value for levulose by Jackson-Mathews (5).
[c] Values for levulose by Tsuzuki et al. (7).
[d] Value for levulose by Wiley (1).

of the clarified solutions from the 30 samples. To provide a value for levulose independent of the saccharimetric data, levulose was calculated for each sample from the Lothrop-Holmes results. Although the results have no absolute significance, the relationships among them are of interest. Table 4 shows the averages of all samples calculated in this manner. It is significant that the change over the 20–25° range is only about 40 per cent of that for the 25–70° interval. The lower value for the 70–87° interval might be ascribed to heat destruction of levulose, but the low value in the 20–25° range must be due to other substances in the honey that do not show a uniform temperature-rotation relationship over the entire range. It can be seen that if the intervals 25–70 or 25–87 are selected for analysis for levulose, considerably higher values will be obtained, since the change over this temperature range is considerably

greater than either the Wiley or Jackson-Mathews constants. The agreement of the value for 20–70° in Table 4 with the Jackson-Mathews constant is as expected, since the levulose as determined by the Lothrop-Holmes method and used in this calculation is in general agreement with levulose values calculated from the Jackson-Mathews constant.

Lothrop (6) has compared the average levulose values for ten honeys as determined by the Lothrop-Holmes iodometric method and by low- and high-temperature polarization. In the latter procedure he compared use of the Wiley constant of 0.0357 with the Jackson-Mathews value of 0.0344. Close agreement was demonstrated between levulose values by the iodometric method and those from the optical method using the Jackson-Mathews constant. His results are shown in Table 5. Also shown in this table are similar average values for the 15 honeys analyzed in this study. The agreement between values obtained by these two methods

TABLE 5.—*Average levulose content of honey as found by different methods (per cent)*

	NO. OF HONEYS ANALYZED	LOTHROP-HOLMES IODOMETRIC	POLARIMETRIC		
			AOAC	JACKSON-MATHEWS	DIFFERENCE
		(*A*)		(*B*)	(*B*–*A*)
Lothrop-Holmes (6)	10	40.03	38.70	40.22	0.19
This Research	15	39.58	38.00	39.73	0.15

appears to be similar to that found by Lothrop. It will be shown, however, that the difference is statistically significant.

The polarimetric data and the Lothrop-Holmes analyses were carried out on the same solutions. Results by the other methods were obtained intermittently over the following four months. It is doubted whether any significant change took place in total reducing sugar values by enzymatic action on sucrose, or in the dextrose and levulose content of the samples. Auerbach and Bodlander (19) state that the proportion of levulose in honey apparently increases on storage. Later work by Boer (20) failed to substantiate this for honeys having levulose to dextrose ratios greater than 1.06.

The Jackson-Mathews copper reduction method, as previously noted, seems frequently to give higher dextrose and lower levulose values than other methods (Tables 6 and 7). This method has been included as alternative to the polarimetric method for levulose in honey by the A.O.A.C. since the fifth (1940) edition of *Methods of Analysis*. No data have been found in the literature comparing results of levulose analysis by these two methods.

TABLE 6.—*Analysis of variance for the determination of dextrose in honey by five methods*

NO.	A.O.A.C.	JACKSON-MATHEWS (POLARIMETRIC)	JACKSON-MATHEWS (OXIDATION)	LOTHROP-HOLMES	MARSHALL-NORMAN	TOTALS
1	35.2	33.1	40.9	35.7	36.7	181.6
2	36.9	35.3	43.1	37.4	37.2	189.9
3	37.9	36.0	41.3	37.9	36.7	189.8
4	37.2	35.6	40.0	37.1	35.6	185.5
5	30.0	28.2	31.6	29.0	29.3	148.1
6	33.8	32.3	38.3	33.8	33.3	171.5
7	37.6	35.6	39.4	37.6	37.2	187.4
8	34.7	33.3	39.5	35.1	34.3	176.9
9	35.8	33.9	41.9	36.2	36.0	183.8
10	38.6	36.7	38.5	36.2	36.2	186.2
11	33.5	32.1	34.7	32.3	28.2	160.8
12	36.9	35.9	32.8	36.6	34.9	177.1
13	38.9	37.3	39.1	36.9	36.2	188.4
14	35.8	33.7	40.7	37.8	37.7	185.7
15	40.0	39.1	39.5	37.8	37.7	194.1
Totals	542.8	518.1	581.3	537.4	527.2	2706.8

Calculations for the analysis of variance

	75 ENTRIES	METHODS TOTALS	SAMPLE TOTALS	GRAND TOTAL
Sum of squares	98,383.7	1,467,707.7	490,560.9	7,326,766.2
Divisor	1	15	5	75
Quotient	98,383.7	97,847.2	98,112.2	97,690.2
Subtract	97,690.2	97,690.2	97,690.2	
Sum of squares	693.5	157.0	422.0	

Analysis of variance

VARIANCE ASSOCIATED WITH	BASED ON DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE OR VARIANCE	F	F (5% LEVEL)
Methods	4	157.0	39.25	19.24	2.54
Samples	14	422.0	30.14	14.77	1.88
Experimental error	56	114.4	2.04		
Whole set of 75 measurements	74	693.4			

STATISTICAL ANALYSIS OF DATA

Dextrose.—An analysis of variance was made to determine the extent of the contribution of the five dextrose methods to the total variance, which is the sum of the variances due to both samples and methods. Since the samples were from fourteen different floral sources, differences in their dextrose contents were expected. The averages of duplicate values

TABLE 7.—*Analysis of variance for the determination of levulose in honey by five methods*

NO.	A.O.A.C.	JACKSON-MATHEWS (POLARIMETRIC)	MARSHALL-NORMAN	JACKSON-MATHEWS (OXIDATION)	LOTHROP-HOLMES	TOTALS
1	37.6	39.9	40.9	35.9	39.7	194.0
2	38.2	39.9	42.1	36.1	39.6	195.9
3	38.2	40.3	40.3	36.2	40.4	195.4
4	35.8	37.6	38.6	34.9	37.8	184.7
5	41.7	43.6	46.2	39.4	44.5	215.4
6	38.0	39.7	41.3	36.0	39.9	194.9
7	38.1	40.4	41.5	35.9	40.2	196.1
8	38.8	40.9	41.0	38.2	40.3	199.2
9	38.1	39.1	42.3	36.2	39.6	195.3
10	35.4	37.5	39.0	34.1	36.0	182.0
11	40.5	42.1	43.2	37.8	41.5	205.1
12	36.0	37.0	36.2	39.1	36.1	184.4
13	37.3	39.0	40.9	34.9	39.2	191.3
14	37.9	40.2	41.9	36.9	37.6	194.5
15	37.8	38.8	41.0	36.9	39.9	194.4
Totals	569.4	596.0	616.4	548.5	592.3	2922.6

Calculations for the Analysis of Variance

	75 ENTRIES	METHODS TOTALS	SAMPLE TOTALS	GRAND TOTAL
Sum of Squares	114,311.9	1,711,052.9	570,379.8	8,541,590.8
Divisor	1	15	5	75
Quotient	114,311.9	114,070.2	114,075.9	113,887.9
Subtract	113,887.9	113,887.9	113,887.9	
Sum of squares	424.0	182.3	188.0	

Analysis of Variance

VARIANCE ASSOCIATED WITH	BASED ON DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE OR VARIANCE	F	F (5% LEVEL)
Methods	4	182.3	45.58	47.48	2.54
Samples	14	188.0	13.43	13.99	1.88
Experimental error	56	53.7	0.96		
Whole set of 75 measurements	74	424.0			

(Table 2) for the five methods and the fifteen samples were, therefore, treated as a block experiment to provide a means of calculating the samples and methods variances. The average dextrose values, calculations for the analysis of variance, and the analysis of variance are given in Table 6. This table shows that the variance is about equally divided between the methods and the samples. Both the F values of 19.24 for methods and

14.77 for samples are highly significant when compared to their respective critical 5 per cent F values of 2.54 and 1.88. This shows not only that appreciable differences exist among the individual samples, as expected, but also that the dextrose values found for any given honey sample are dependent upon the method used.

To compare the relative precisions of the five methods, Youden's (21) treatment was used, in which the sum of the squares of the differences between duplicate values for each sample for any one method is compared to that of another method chosen as reference. Although the A.O.A.C. method may be considered the standard method, the Lothrop-Holmes values were used as reference, since the following summations were smaller, and hence always in the denominator in the equation:

$$F = \frac{\Sigma d_a^2}{\Sigma d_b^2}, \text{ where } \Sigma d_a^2 > \Sigma d_b^2.$$

Comparison of each of these F values with the critical F value from statistical tables (21) gives the relative precision of the dextrose methods to be as shown in Table 8, where they are ranked in descending order of precision.

Levulose.—As in the case of the dextrose values, Table 7 shows the average percentage levulose values, the calculations for the analysis of variance, and the analysis of variance. In this treatment, the methods account for a greater proportion of the total variance than they do in the

TABLE 8.—*Relative precision of methods for the determination of sugars in honey*

DEXTROSE

RANK	METHOD	d^2	F VALUE[1]
1	Lothrop-Holmes	0.4137	—
2	A.O.A.C.	1.1766	2.84[2]
3	Marshall-Norman	1.5248	3.68[2]
4	Jackson-Mathews Polarization	3.8282	9.25[2]
5	Jackson-Mathews Oxidation	14.1072	34.10[2]

LEVULOSE

RANK	METHOD	d^2	F VALUE[1]
1	Lothrop-Holmes	0.3548	—
2	A.O.A.C.	1.4066	3.96[2]
3	Jackson-Mathews Oxidation	3.0819	8.69[2]
4	Marshall-Norman	3.7789	10.65[2]
5	Jackson-Mathews Polarization	4.7541	13.40[2]

[1] $F = \Sigma d_a^2 / \Sigma d_b^2$ where d_a is difference between duplicates for method under test and d_b is difference between duplicates for Lothrop-Holmes method.
[2] Greater than the critical 5% level F value of 2.48 (21) and hence significantly less precise.

case of the dextrose values which were obtained by the same methods on the same samples. The F values of 47.48 for methods and 13.99 for samples are highly significant when compared with the respective critical 5% level F values of 2.54 and 1.88. This indicates that the samples do not have the same levulose content and that the levulose values found for a given honey sample are dependent upon the method used.

The duplicate levulose values were used to determine the relative precision of the methods. Table 8 lists the methods in order of precision. As was found for dextrose, the Lothrop-Holmes gave significantly more precise values than any of the four other methods, with the A.O.A.C. method ranking next.

A *t*-test comparison (21) was made between the dextrose and levulose values obtained by the Lothrop-Holmes method and those obtained by the A.O.A.C. method. In this test the differences, d, between the dextrose values obtained by each method for each honey sample are squared, and the standard deviation of the differences is found by the following equation:

$$s_d^2 = \frac{1}{(n-1)} (d_1^2 + d_2^2 + d_3^2 + \cdots + d_n^2 - n\bar{d}^2)$$

where d_1, d_2 etc. = differences between dextrose values obtained for each honey sample, $\bar{d}$ = average difference, and n = the number of samples = 15. Then substitution of the numerical values gives: $s_d^2 = (1/15-1)(22.7801 - 11.6160) = (1/14)(11.1641)$, or $s_d = 0.8929$. This value of the standard deviation of the differences was then substituted in the following equation:

$$t = \frac{\bar{d}\sqrt{n}}{s_d} = \frac{0.88 \times \sqrt{15}}{0.8929}, \text{ or } t = 3.81.$$

Since the value of *t* of 3.81 is higher than the 5% critical *t* value of 2.145 for 14 degrees of freedom, it can be concluded that the dextrose values obtained by the Lothrop-Holmes method were significantly different from those obtained by the A.O.A.C. method.

The same test was applied to the levulose values obtained by the Lothrop-Holmes and by the A.O.A.C. methods. Here the calculated *t* value of 4.53 was again significantly higher than the 5% critical *t* value of 2.145, showing that the levulose values obtained by these two methods were also significantly different. The fact that the Lothrop-Holmes method gave higher levulose values for 14 of the 15 honey samples may also be regarded as significant.

Since it has been stated (6) that the Lothrop-Holmes and the Jackson-Mathews polarimetric method gave comparable results for dextrose and levulose values in honey, the *t*-test, as described above, was applied to the dextrose values obtained by these methods for the fifteen honey samples.

The calculated t value of 3.04 was higher than the 5% critical t value of 2.514; therefore, the dextrose values obtained by two methods were significantly different. Application of the t-test to the levulose values obtained by these two methods yielded a value of 5.74 (critical 5% t value $=2.514$), showing that the levulose values obtained by these two methods were also significantly different. Thus, the apparently close agreement in levulose results by the two methods shown in Table 7 may not signify that the values obtained are the true levulose contents of the samples, even though the principles of the methods differ.

DISCUSSION

The reason for the lower precision obtained with the polarimetric method recommended by Jackson and Mathews must lie in the 70°C. reading since the value for the 20°C. reading was used for both this method and for the A.O.A.C. method.

A possible reason for the lower precision found for the dextrose determination by the Marshall-Norman method when compared with the Lothrop-Holmes procedure may be temperature variation during the oxidation. The sole difference in the dextrose determination by these two methods is that Lothrop and Holmes require 20°C. while Marshall and Norman specify 15 to 18°C. Accordingly the temperatures employed in this study were 20 ± 0.05°C. and 17 ± 1°, respectively.

We cannot determine from these data which of the several methods gives results closest to the actual composition of the sample. Since the determinations are empirical, the superior precision of the Lothrop-Holmes method over the A.O.A.C. method, considered with the smaller equipment requirement and the simplicity of the procedure, indicate the desirability of future comparative work on the determination of dextrose and levulose by these two methods.

SUMMARY

In a comparative study of methods for the determination of sugars in honey, fifteen samples have been analyzed for dextrose and levulose by five methods. Statistical treatment of the results ranks the methods as follows in order of decreasing precision: for dextrose, Lothrop-Holmes, A.O.A.C., Marshall-Norman, Jackson-Mathews (polarimetric) and Jackson-Mathews (oxidation); for levulose, Lothrop-Holmes, A.O.A.C., Jackson-Mathews (oxidation), Marshall-Norman, Jackson-Mathews (polarimetric). Analysis of variance showed that variance due to methods was as great as that due to differences in dextrose and levulose content of the samples from fourteen different floral sources.

Levulose and dextrose values obtained by the Lothrop-Holmes method are significantly different from those obtained by the A.O.A.C. and by the Jackson-Mathews polarimetric methods.

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CHEMISTRY OF ANALYTICALLY IMPORTANT PECTIC ACIDS

I. CHEMICAL PROPERTIES OF EHRLICH, WICHMANN AND CARRÉ-HAYNES PECTIC ACIDS*

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Since the development of the modern concept (1) of the constitution and stability of pectic acids, certain aspects reported earlier and not sufficiently clarified have needed re-investigation.

In 1922, Wichmann (2) introduced an analytical method for the determination of pectin, based on the saponification of the alcohol precipitate from plant extracts with cold, dilute alkali and the precipitation of the resulting pectic acid with hydrochloric acid, followed by boiling with about 0.25 per cent hydrochloric acid for 10–15 min. to facilitate

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filtration. Nelson (3) identified the pectic acid with the "digalacturonic acid A" reported by Ehrlich and Sommerfeld (4), produced by long and relatively drastic, hot acid hydrolysis of pectin. Subsequently, "Official Methods of Analysis of the A.O.A.C." adopted the term "Pectic acid (digalacturonic acid)" which term still appears in the most recent (1950) edition of this handbook.

It has now been amply demonstrated that the pectic acid obtained by this procedure could not be digalacturonic acid (5). Link *et al.* (5b) suggested that the fundamental unit of citrus pectin contained a polygalacturonide chain consisting of a minimum of 8–10 units, but more recently Jansen *et al.* (5d), by relatively drastic treatment of citrus and apple pectic acid obtained a pectic acid of molecular weight of approximately 6000, equivalent to 32 galacturonic anhydride residues, in yields as high as 50 per cent. The "number" average molecular weight* of the Wichmann pectic acid prepared by us from citrus pectin was about 10,800; its "weight" average molecular weight* was about 26,000. The number average molecular weights of Ehrlich Acid C and Ehrlich Acid B prepared from the same citrus pectin were, respectively, 5,900 and 3,600; the weight average molecular weights of the two Ehrlich pectic acids were 14,000 and 2,200, respectively.

The Wichmann method has not attained wide popularity, particularly abroad, and most workers in the field prefer the Carré and Haynes (6) calcium pectate method, which with some modifications (7) has become one of the few standard methods of analysis. In this procedure the saponification of the extracted pectins is carried out by dilute cold alkali; the resulting sodium pectate solution is then acidified with acetic acid and the pectic acids precipitated as calcium pectate. This method measures as the neutral calcium salts the proportion of polygalacturonic acids above the threshold of precipitability. The molecular size of the pectic acids whose calcium salts are soluble in the reaction mixture is not known, although it is inferred in studies on the course of enzymic hydrolysis (8) that di- and tri-galacturonides are not precipitable. Carré and Haynes (6) found, rather constant calcium content, varying between the narrow limits of 7.5–7.8 per cent in the calcium pectate prepared by them from apple pectin. This value agreed with the theoretical value of 7.66 per cent calculated from the now abandoned Fellenberg (9) formula for pectic acids. Gaddum (10) found similar values for calcium pectate obtained from citrus fruits. Sinclair and Crandall (11), however, reported that the calcium content of calcium pectates from lemon peel ranged from 8.17 to 8.76 per cent, and that the values for similar preparations from lemon peel albedo ranged from 8.52 to 8.85 per cent. They attributed their

* "Number" average molecular weight is derived from physical measurements (such as of osmotic pressures) where the effect is due to the colligative or number action of the molecules; "weight" average molecular weights are derived from physical measurements (viscosity) where the effect is mainly due to their mass.

higher values to more thorough drying. Our preparation of calcium pectate from citrus pectin had a calcium content of 8.40 per cent. The average molecular weight of the pectic acid prepared from the Carré-Haynes calcium pectate was found to be 15,000, and its number average molecular weight, about 12,800.

In order to characterize these pectic acids, their chemical properties: ash content, methoxyl value, uronic anhydride content, non-uronide matter, neutralization equivalent, and acid behavior were determined. Optical rotatory power, viscosity, and osmotic pressure were also measured. The results of the measurements of chemical composition are presented here and in the following paper.

MATERIALS AND METHODS

Citrus pectin

Commercial, 183 grade, rapid set citrus pectin, obtained from the California Fruit Grower's Exchange. (It was a pale yellow powder, and had been stored at 0°C. until used in this work.)

Wichmann Pectic Acid

(In the preparation of this acid the A.O.A.C. method (2c), developed by Wichmann (2a), was modified to allow treatment of higher concentrations of pectin.) To 700 ml of a 1% pectin solution (prepared by blending pectin with water in a Waring blendor for 5 minutes at 25°C.), 37 ml. of 10% sodium hydroxide solution were added dropwise and with constant, vigorous stirring. Fifteen minutes after the addition of the alkali had been completed, 55 ml of HCl (1 part of conc. HCl+2.5 parts of water) were added dropwise to the vigorously-stirred solution. When about 40 ml of acid had been added, the amount of gelatinous precipitate which had been slowly forming increased suddenly, and the entire contents of the container became so gelatinous that very vigorous stirring caused only slight movement of the mass. Upon heating, the volume of the precipitate decreased and the solution became quite liquid. The boiling point was reached as rapidly as possible and boiling was continued for 5 minutes before the hot solution was filtered through a sintered-glass filter of medium porosity. The colorless gelatinous precipitate obtained was washed with about 1 liter of hot water, then transferred into 700 ml of cold water. It was redissolved in alkali, filtered to remove any foreign material, then re-precipitated with acid, boiled, filtered, and washed with hot water until the filtrate gave a negative test for chloride. The precipitate was dried as much as possible on the filter by suction and pressing, and was thoroughly mixed and washed three times with 95% alcohol. The alcohol-treated precipitate, sucked as dry as possible on the filter, was broken up, spread in a thin layer in a large evaporating dish, and dried in a vacuum desiccator over concentrated H_2SO_4. From 105 g of undried pectin there were obtained 63 g of powdered acid containing about 10% moisture.

The material prepared above is referred to throughout this work as Wichmann pectic acid.

Ehrlich Pectic Acids

(In 1929 Ehrlich and Schubert (5a) described three tetragalacturonic acids (A, B and C) prepared by heating pectin with 5% HCl for 6 hours on a water-bath, but the ready conversion of acid A to acid C by mild alkali treatment, and the similarity of their properties, led Ehrlich later to conclude that the acid A was a partially

esterified derivative of the acid C. The acid B, however, differed markedly from the acid C in solubility and optical rotation, and it was considered that these two acids were essentially different in structure. In the preparation of acids C and B Ehrlich's methods (5a) were followed as closely as possible, but were modified at times when his procedure was found to be unsatisfactory.)

(1) *Ehrlich Acid C.*—Two hundred grams of citrus pectin were blended with 4 liters of 5% HCl in a Waring blendor for 3–5 minutes. This solution was heated on a steam bath, with frequent stirring, for 6 hours after the temperature had risen above 80°C., the volume being maintained constant by the addition of water. After standing overnight the solid material was centrifuged out, washed five times with 2.5% HCl and then with cold water until the supernatant liquid remained cloudy after centrifuging and gave only a faint blue color with Congo-red paper. The washed material was then taken up in the minimum volume of boiling water required to give a solution readily filterable by gravity. To this filtrate, after cooling, 25 ml. of concentrated HCl per liter were added, and the flocculent precipitate which settled was removed by filtration. The precipitate was then washed with dilute HCl, redissolved in water, reprecipitated, treated with alcohol, then with 2% NaOH for 32 hours at room temperature. After saponification, the solution was acidified with concentrated HCl. The acidified suspension was diluted with 300 ml of 5% HCl and filtered through a hardened paper, and the residue was washed with dilute HCl until the filtrate was colorless, then several times with water, and finally three times by blending with 95% alcohol (1500 ml each time). The residue, light brown in color, was thoroughly broken up, spread in a thin layer and dried in vacuum over concentrated H_2SO_4 for several days. The resulting material was ground in a ball-mill to pass a 60 mesh screen; the final product was faintly brown in color and powdering reduced the color considerably. From 200 g of undried pectin, 52 g of Ehrlich Acid C (moisture content about 10%) were prepared in this way.

(2) *Ehrlich Acid B.*—The filtered HCl extract from the digestion of pectin (see Ehrlich Acid C) was concentrated under vacuum at 30°C. to about one-sixth its original volume. Six volumes of 95% alcohol were added to the concentrate with the immediate formation of a white precipitate which settled rapidly and left a light brown supernatant liquid. The precipitate was removed by filtering through a coarse-porosity sintered glass filter and was washed twice with HCl-containing alcohol and twice with 95% alcohol. After air-drying, the precipitate was dissolved in 350 ml cold water, filtered, and then reprecipitated by the addition of 2 liters of 95% alcohol. The final white precipitate was removed by filtration through sintered glass, washed twice with small amounts of HCl-containing alcohol and finally with 95% alcohol to a total volume of about 600 ml, and dried in a vacuum oven at 70°C. The yield of Ehrlich Acid B from 200 g undried pectin was only 4.6 g.

Carré-Haynes Calcium Pectate

(The method used here was essentially the Carré-Haynes (6) method modified for the precipitation of larger quantities of pectin.)

Thirty-five ml of 10% NaOH were added slowly to 700 ml of 1% pectin solution. After 30 minutes, 20 ml of glacial acetic acid were added dropwise and with vigorous stirring. Five minutes after the addition of acid, 100 ml of an aqueous solution containing 32 g of $CaCl_2 \cdot 2H_2O$ were added slowly and with rapid stirring. The amount of colorless, gelatinous precipitate which formed increased slowly until, after the addition of about 90 ml of the calcium chloride solution, the contents of the beaker set to almost a solid gel. About 250 ml of water were added to facilitate stirring, and the precipitated solution was allowed to stand at least one hour at room temperature, with frequent stirring. It was then filtered through a coarse-porosity sintered

glass filter under very slight vacuum and washed repeatedly with hot water until the filtrate gave a negative test for chloride. The residue was washed from the filter and digested with boiling water for 5 minutes, refiltered, and washed several times with boiling water. This treatment was repeated until the filtrate from the aqueous digestion give a negative chloride test. After washing three times with 95% alcohol (allowing the alcohol to remain in contact with the solid for at least 30 minutes each time), the pure white gelatinous residue was dried overnight in a vacuum oven at 70°C. The dried material was pale yellow in color (the color deepened on prolonged heating) and was ground to pass a 60 mesh screen. About 37 g (moisture content 12%) of this product, which is referred to hereafter as Carré-Haynes calcium pectate, were obtained from 42 g of undried pectin.

Humidification

The powdered samples prepared above—Wichmann pectic acid, Ehrlich Acid C, Ehrlich Acid B, Carré-Haynes calcium pectate and a 50 g. sample of the original pectin—were humidified for four days according to the method of Jansen, *et al.* (5d) and, after drying over calcium chloride for about two weeks, were stored in glass-stoppered bottles in a desiccator.

Preparation of a pectic acid from Carré-Haynes Calcium Pectate.—About 5 g of Carré-Haynes Calcium Pectate was shaken continuously with 450 ml of water and 50 ml of 0.62 *N* ammonium oxalate for one hour. The pectic material slowly dissolved and calcium oxalate was precipitated as a very fine, white powder which did not settle completely even on prolonged standing. After standing at 0°C. overnight, the solution was centrifuged for one hour and the very turbid, rather viscous supernatant liquid was carefully drawn off and adjusted to *p*H 3 with glacial acetic acid (about 8.5 ml of glacial acetic acid was required for 100 ml solution). Sufficient 95% alcohol (6 volumes) was added to give a final concentration of 80%, and the colorless gel which precipitated was removed by gravity filtration (No. 50 Whatman paper) and washed with 1500 ml of 80% alcohol by removing it from the paper, blending for 3 minutes in a Waring blendor and refiltering. The final filtrate gave a negative test for oxalate. To facilitate drying the washed precipitate was blended twice with 95% alcohol and once with ether, and a medium-porosity sintered-glass filter under slight vacuum was used for the separations. After washing three times on the filter with ether, the precipitate was removed from the filter, broken up, spread in a thin layer and left to air-dry. Finally the snow white, dry product was ground to a fine powder. In this way 4.4 g (dry weight) of product were obtained from 5.24 g of original preparation.

Precipitation of Wichmann Pectic Acid as calcium pectate and the preparation of a pectic acid from this salt.—440 mg (dry weight) of Wichmann pectic acid was converted to the calcium salt by the method already outlined for the preparation of Carré-Haynes calcium pectate. The resulting product was a white powder which became pale yellow in color on vacuum drying at 70°C. for 16 hours. The yield of calcium pectate was 470 mg. This calcium salt was then decalcified by the method described above (for the preparation of a pectic acid from Carré-Haynes Calcium Pectate), and the air-dried and powdered product was dried in vacuum at 70°C. for 20 hours. The final yield was 390 mg of a fine, white powder.

Drying Method

Pectic substances have been subjected to a wide variety of drying procedures; air and vacuum ovens and Abderhalden driers have been em-

ployed by various authors at different temperatures for different times. With the finely powdered substances used in this investigation constant weight was obtained after heating in a vacuum oven at 70°C. for 5 hours. To ensure a sufficient margin, all moisture determinations were made by heating in a vacuum oven at 70°C. for 8 hours. Except where noted later, the dried material was not used in analytical work. Each time a sample of a pectic substance was weighed out the sample analyzed was corrected for this moisture content. Although there is no evidence that the pectic substances prepared above are depolymerized or otherwise modified on heating in a powdered form, this was found to be the easiest way to handle the materials. It will be noted that each of the acids (except the Ehrlich Acid B) was ground to a fine powder. Lampitt, *et al.* (12) report that the molecular weight of pectin is decreased appreciably on grinding in a ball-mill, but, although the effect of this treatment on the above acids was not investigated, it appears unlikely that it would bring about much change especially in the Wichmann and Ehrlich acids, since the original pectin is appreciably degraded in the process of preparation.

RESULTS

Ash Content, Alkalinity of Ash, and Calcium Content.—The ash content was determined by heating the previously dried and charred sample in a platinum dish at 550°C. for 16 hours. The alkalinity of the ash was determined by heating the ash with a known excess of 0.1 *N* H_2SO_4 on a steam bath for 15 minutes, cooling and titrating the excess acid with 0.1 *N* NaOH to a methyl red end-point. The solution from this treatment was acidified and analyzed in toto, or was made to 250 ml, and two 50 ml aliquots were analyzed for calcium by the A.O.A.C. volumetric method (13).

The results of these analyses are presented in Table 1 and all figures are reported on a dry-weight basis. Throughout this work the analytical

TABLE 1.—*Ash content, alkalinity of ash, and Ca content of pectin and pectic substances*

SAMPLE	TOTAL ASH	ALKALI REQUIRED TO NEUTRALIZE THE ASH FROM 1 g MATERIAL	CALCIUM	ALKALI REQUIRED TO NEUTRALIZE CALCIUM PRESENT IN 1 g MATERIAL
	per cent	*mEq.*	*per cent*	*mEq.*
Pectin	2.52	0.410	0.53	0.264
Wichmann Pectic Acid	0.81	0.007	—	—
Ehrlich Acid C	0.20	0.002	—	—
Ehrlich Acid B	7.83	1.140	1.07	0.569
Carré-Haynes Calcium Pectate	12.93	4.190	8.40	4.192
Pectic Acid prepared from Carré-Haynes Calcium Pectate	2.45	0.753	1.50	0.748

results reported are averages of very closely agreeing or identical duplicate determinations.

According to Owens *et al.* (14), the ash of commercial citrus pectin consists mainly of silicon, calcium, aluminum, and sodium salts. From Table 1, it is seen that the calcium content of the pectin failed to account for 0.148 mEq./g of the alkali required to neutralize the ash. Since the pectin was in all probability prepared by the aluminum hydroxide method, it is not unlikely that some aluminum is present, but, in the absence of any direct evidence, the only conclusion that can be drawn is that each gram of the pectin used contains 0.146 mEq. of cations other than calcium.

The total ash content of the Wichmann pectic acid and Ehrlich Acid C was used as an index of the insoluble ash content of these two substances. The dry weights were corrected throughout the following analytical work for the indicated amounts of insoluble ash.

The Carré-Haynes calcium pectate and the pectic acid prepared from it were free from all cations other than calcium and the milliequivalents of calcium agreed well with the milliequivalents of alkali required to neutralize the ash.

Methoxyl Value.—Methoxyl values of the pectin and pectic acids used in this work were determined by Clark's modification (15) of the Zeisel method. The samples were previously humidified (16). For the purpose of comparing the Zeisel and dilute alkali saponification procedures when applied to completely de-esterified pectic acids, the Wichmann pectic acid and the Ehrlich Acid C were analyzed not only by the Zeisel method but also by the saponification procedure developed by Hills, Ogg, and Speiser (16b). The results of these analyses are presented in Table 2 and all figures are calculated on a total dry weight basis.

TABLE 2.—*Methoxyl value of pectic substances*

SAMPLE	$-OCH_3$	(mEq.) ALKALI/G USED IN SAPONIFICATION	$-OCH_3$ BY SAPONIFICATION PROCEDURE
	per cent		*per cent*
Pectin	9.22		
Wichmann Pectic Acid	0.4*	0.041	0.14
Ehrlich Acid C	0.4*	0.042	0.14
Ehrlich Acid B	0.7*		
Carré-Haynes Calcium Pectate	0.4*		

* N/100 thiosulphate used.

From the analytical results it appears that the pectic acids were not completely de-esterified. Using the Zeisel method, Link *et al.* (5b) obtained methoxyl values of 0.4 per cent to 0.6 per cent for citrus polygalacturonide after treatment with alkali, and the results of Olsen *et al.* (17) were in agreement with this. However, at the time this work was carried out, the inaccuracy of the Zeisel determinations due to retained

alcohol was not realized. Jansen *et al.* (16a) showed that their modification of Olsen's (17) saponification procedure gave methoxyl values which were equal to or greater than those obtained by the Zeisel method on a humidified sample. Hills, Ogg, and Speiser (16b) developed a modification of the saponification procedure which gave results in better agreement with those obtained by the Zeisel method on a humidified sample. Their figures show that, in general, this modification gives slightly lower results than the Zeisel method. The figures presented in Table 2 also show this trend, although the difference in these values is slightly greater than the average difference reported by Hills *et al.* (16b).

Uronic Anhydride Content.—The uronic anhydride content of the prepared pectic acids and pectin was determined by the method of McCready *et al.* (18) with the results shown in Table 3. By assuming that uronic acid is the only material present which will liberate carbon dioxide under the conditions of the determination, and that carbon dioxide is liberated

TABLE 3.—*Uronic anhydride content of pectic substances*

SAMPLE	MILLIEQUIV. CO_2/G DRY WEIGHT	URONIC ANHYDRIDE		
		PER CENT ON TOTAL DRY WEIGHT BASIS	PER CENT ON Ca-FREE DRY WEIGHT BASIS	PER CENT ON ASH-FREE DRY WEIGHT BASIS
Pectin	8.12	71.5 (74.6)[1]	71.8 (75.0)	73.3 (76.6)
Wichmann Pectic Acid	10.38	91.3[2]		91.3
Ehrlich Acid C	10.56	92.9[2]		92.9
Ehrlich Acid B	9.62	84.7	85.6	91.9
Carré-Haynes Calcium Pectate	8.56	75.3	82.2	86.5
Pectic Acid prepared from Carré-Haynes Calcium Pectate	9.10	80.1	81.3	82.1

[1] Values in parenthesis are correct for CH_3—content: (methoxyl value/31) ×14 = per cent CH_3).
[2] Corrected for insoluble ash.

by all the free carboxyl groups and also by methyl ester groups, it is permissible to calculate the uronic anhydride content on the basis that two equivalents of carbon dioxide will be evolved for each 176 g of uronic anhydride. The uronic anhydride contents determined in this work are calculated on three different bases—total dry weight basis, calcium-free dry weight basis, and ash-free dry weight basis. These calculations are meant only to indicate the approximate range of variation in reported values as they depend on the basis used, so corrections for the low and almost constant methoxyl values of the pectic acids are neglected, and the uronic anhydride content is considered a close enough approximation to the uronide content. The correction for ester content in the case of the original pectin, however, is large, and values corrected for ester content are given in parenthesis in Table 3.

Although total dry weight is the least satisfactory basis for expressing the results, the ash-free, dry weight is not a consistent basis, as the value of the correction depends not only on the nature of the non-organic contaminants but also on the duration and temperature of ashing. Expression of results on a calcium-free, dry weight basis depends on the reasonable assumption that all the calcium is combined with uronic acid residues, but is accurate only when the ash consists entirely of calcium salts. The figures presented in Table 1 showed that, for the Carré-Haynes calcium pectate and the pectic acid prepared from it, calcium was the only titratable contaminant. Calculation of the uronic anhydride content of these two substances on a calcium-free weight basis is therefore more accurate than calculation on either of the other bases, but must still be corrected for insoluble ash. Strictly speaking, a correction for the hydrogen displaced by the calcium should be made. This correction, however, is not large enough to affect the arguments presented in this work, and is neglected.

The uronic anhydride values calculated for the Wichmann pectic acid and the Ehrlich Acid C can be considered reasonably accurate, since the ash of these two acids was previously shown to consist almost entirely of insoluble ash. On the other hand, the uronic anhydride contents of the original pectin and the Ehrlich Acid B cannot be corrected for ash with any degree of certainty, since the ash contains metals other than calcium. The true value for these substances obviously lies between those obtained on the calcium-free dry weight basis and on the ash-free dry weight basis.

Neutralization Equivalent and Average Residue Weight.—Hinton (7) showed, from the electrometric titrations of a number of purified pectins, that a *p*H of 7.5 could be taken as the neutralization point of the free acidity without any risk of error from incipient action on the ester groups. Later workers have accepted *p*H 7.5 as the point of equivalence, since complete titration curves have shown this to be the point of inflection, but Speiser, Hills, and Eddy (19) have stated that theoretical considerations show the point of true equivalence to be at a *p*H somewhat higher than that of the point of inflection. Since the true point of equivalence appears to be a complicated function of the total concentration of carboxyl groups and of the dissociation constants, they accepted *p*H 7.5 as the point of equivalence and pointed out that the error in using this value was not large.

There is some confusion in the literature regarding terminology. Schultz *et al.* (20), for example, refer to the number obtained from the titration value (corrected for alkalinity of the ash) as equivalent weight. This value obviously depends on the degree of esterification of the material under investigation and also on the non-uronide content. Säverborn (21) subjected the pectic material to a preliminary saponification and expressed the titration results as the equivalent weight of the corre-

sponding pectic acid, making no allowance for alkalinity of the ash. Olsen *et al.* (17) referred to equivalent or combining weights of esterified pectic substances and of the corresponding pectic acids, and made no allowance for alkalinity of the ash. Since alkali has a secondary action on pectic substances apart from de-esterification, it is very possible that the so-called equivalent weight of the saponified samples are low, whereas failure to correct for the alkalinity of the ash would result in high values.

The nomenclature of Hills and Speiser (22) is considered more precise and is used throughout this work. If N is the number of equivalents of alkali (corrected for the alkalinity of ash) required to titrate an aqueous solution of 1 g of pectic substance to *p*H 7.5, 1/N is the *neutralization equivalent.* The additional amount of alkali which would be required by the methyl-esterified carboxyl group, if they were free, is given by: (per cent CH_3O)/3100 = Z. Then 1/(N+Z) is the *average residue weight.* It is obvious that the neutralization equivalent and average residue weight are identical only for completely de-esterified pectic acids.

It will be shown that the average residue weights as calculated from alkali titration data and from methoxyl values do not agree with the average residue weights calculated from CO_2 determinations. The neutralization equivalents reported by Schultz *et al.* (20), and the neutralization equivalents and average residue weights reported by Hills and Speiser (22) are, therefore, open to doubt.

The disagreement found here between the two methods is contrary to the findings of Schultz, *et al.* (20), who reported that uronic anhydride contents, calculated from neutralization equivalents and methoxyl values, checked with uronic anhydride contents determined by CO_2 liberation to within 1 per cent. The figures given by McCready *et al.*, (18), comparing their CO_2 method with the titration method, show the same trend as is noted here, although to a lesser extent.

The water-soluble pectic substances (e.g. pectin, Ehrlich Acid B, pectic acid prepared from Carré-Haynes Calcium Pectate, and pectic acid prepared from the calcium salt of Wichmann pectic acid) were dissolved in CO_2-free water and titrated with 0.1 *N* alkali to *p*H 7.5 using a Leeds and Northrop Research Model *p*H meter and glass electrode. The end of the burette was drawn out finely and was immersed in the solution being titrated. Alkali was added slowly to prevent localized high concentrations, and CO_2-free nitrogen was bubbled through the solution for the two-fold purpose of stirring and of excluding CO_2. (The titration vessel was sealed except for a small nitrogen outlet, and the alkali in the micro-burette was protected from CO_2 by a soda-lime tube. Since movement of the solution creates a stirring potential, the nitrogen was shut off and the solution allowed to come to rest before *p*H readings were made.)

The water-insoluble acids (e.g. Wichman pectic acid and Ehrlich Acid

C) were suspended in CO_2-free water and dissolved under nitrogen by the slow addition of 0.1 *N* alkali. These acids dissolved completely before the *p*H had risen above 4. The solutions were then made to a known volume with CO_2-free water and aliquots were further titrated by the method described above. The total amount of alkali required to adjust the pectic acid solution to *p*H 7.5 was, in this case, the sum of the alkali originally present in the aliquot and the alkali required in the final titration.

The Carré-Haynes calcium pectate was not sufficiently soluble in either water or alkali to enable a determination of its neutralization equivalent.

Table 4 gives the values of the neutralization equivalents obtained by the alkali titration method.

TABLE 4.—*Neutralization equivalent of pectin and pectic acids*

SAMPLE	NUMBER OF DETERMINATIONS	NEUTRALIZATION EQUIVALENT*		
		AVERAGE	MAXIMUM	MINIMUM
Pectin	8	1009	1021	1006
Wichmann Pectic Acid	30	187.4	187.9	186.9
Ehrlich Acid C	36	182.4	183.1	181.9
Ehrlich Acid B	3	198.5	198.9	198.3
Pectic Acid prepared from Carré-Haynes Calcium Pectate	6	362	364	359

* Corrected for alkalinity of ash on ash free dry weight basis.

There was some evidence that the rate of addition of alkali influenced the amount required to neutralize the solution. If the alkali was added rapidly, slightly more was required to adjust the solution *p*H to 7.5 than if it was added slowly. In addition, adding the alkali in drops rather than slowly and continuously from an immersed tip increased the amount of alkali required. Since this effect was observed with pectic acids of low methoxyl value, it cannot be due to localized concentrations of alkali saponifying methyl ester groups. It may, however, be connected in some way with the colloidal nature of the pectic solutions. All titrations were therefore made slowly as described. The variation found between individual results was within experimental error and, since the rate of addition was not standardized, it was concluded that the rate was sufficiently slow in all cases to minimize this secondary effect.

The values obtained for the neutralization equivalent of the pectic acid prepared from Carré-Haynes calcium pectate (362) and the pectic acid prepared from the calcium salt of Wichmann pectic acid (303) are very high, and the most probable reason for this is that ammonia was not removed completely in the preparation of these acids.

Hills and Speiser (22) point out that, in view of the variable non-galacturonide content, neither the ester content (computed as methoxyl and expressed as per cent by weight of the whole sample) nor the neutralization equivalent, is adequate for the characterization of a pectic

substance, and that the galacturonide content and the degree of esterification of the galacturonide residues better describe the properties of pectin.

Table 5 gives the results calculated by the method of Hills and Speiser (22). The average values (to the nearest unit) of the neutralization equivalents presented in Table 4, and the methoxyl values in Table 2 are used.

TABLE 5.—*Characterization of pectic substances from titration data and methoxyl values*

(method of Hills and Speiser)

SAMPLE	NEUT. EQUIV.	PER CENT GALACT-URONIDE	PER CENT NON-GALACT-URONIDE	PER CENT ESTERIFI-CATION	AVERAGE RESIDUE WEIGHT
Pectin	1009	73.9	26.1	75.1	253
Wichmann Pectic Acid	187*	96.5*	3.5*	2.4*	182*
Pectic Acid from Calcium Salt of Wichmann Pectic Acid	362†				
Ehrlich Acid C	182*	99.2*	0.8*	2.3*	178*
Ehrlich Acid B	198	93.2	6.8	4.3	190
Pectic Acid from Carré-Haynes Calcium Pectate	303†				

* Corrected for insoluble ash.
† Since the ammonia in these samples was not determined these values have little meaning.

In the determination of uronic anhydride content by CO_2 liberation, it is assumed that all galacturonide residues give CO_2 quantitatively, whether the carboxyl group is free or esterified. Thus, from CO_2 analyses and methoxyl values it is possible to calculate the same quantities as were calculated by Hills and Speiser from titration data and methoxyl values. Table 6 gives the results calculated from the CO_2 determinations given in Table 3. For comparative purposes, the values of neutralization

TABLE 6.—*Characterization of pectic substances from CO_2 analysis and methoxyl values*

SAMPLE	NEUT. EQUIV.	PER CENT GALACT-URONIDE	PER CENT NON-GALACT-URONIDE	PER CENT ESTERIFI-CATION	AVERAGE RESIDUE WEIGHT
Pectin	917	75.6	24.4	73.3	246
Wichmann Pectic Acid	198	91.5*	8.5*	2.5*	193*
Ehrlich Acid C	194	93.1*	6.9*	2.4*	189*
Ehrlich Acid B	218	85.0	15.0	4.7	208
Carré-Haynes Calcium Pectate	241	75.5	24.5	3.0	234
Pectic Acid prepared from Carré-Haynes Calcium Pectate	226†	80.3†	19.7†	2.8†	220

* Corrected for insoluble ash.
† Assuming methoxyl value the same as for Carré-Haynes Calcium Pectate.

equivalents obtained by titration and corrected for alkalinity of the ash, and the values calculated from CO_2 analyses are given in Table 7, along with the neutralization equivalents and average residue weights obtained by the two methods.

The figures presented in Tables 5, 6, and 7 are based on total dry weight of the samples (with the exceptions noted) and allow accurate comparison of the values obtained by the two methods for the same material. It is

TABLE 7.—*Comparison of average residue weights and neutralization equivalents derived by two different methods*

A. From titration data and methoxyl value
B. From CO_2 determination and methoxyl value

SAMPLE	AVERAGE RESIDUE WEIGHT		NEUTRALIZATION EQUIVALENT		$N \times 10^{-3}$	
	A	B	A	B	A	B
Pectin	253	246	1009	917	0.99	1.09
Wichmann Pectic Acid	183*	193*	187*	198*	5.35*	5.06*
Ehrlich Acid C	178*	189*	182*	194*	5.49*	5.15*
Ehrlich Acid B	190	208	198	218	5.05	4.58

* Corrected for insoluble ash.
N is the number of equivalents of alkali (corrected for alkalinity of the ash) required to titrate an aqueous solution of 1 g pectic substance to *p*H 7.5.

unsatisfactory, however, to use these values to compare different pectic substances, because of variation in the content of inorganic constituents.

NON-URONIDE CONTENT

The conclusions of Hirst and Jones (23) and Schneider and Bock (24), namely that organic non-uronide material ("ballast") is associated with pectic substances only by loose secondary valences and is not chemically combined with the polygalacturonide chains, have recently been disputed and the opinion is now held by several investigators (25) that non-galacturonic acid residues are chemically bound in the pectin molecule. McCready *et al.* (25) have demonstrated the occurrence of rhamnose as well as galactose and arabinose in purified pectic acids.

In the present work hydrolysates of the pectin substances were prepared according to the recommendations of McCready (25). Approximately 1 g of pectic substance was boiled under reflux for 6 hours with 25 ml of 1 N H_2SO_4. After cooling, the unhydrolyzed residue was filtered off and the filtrate was neutralized with barium hydroxide in aqueous solution. The material which precipitated was removed by filtration and the filtrate was concentrated under vacuum to about 5 ml. Two samples of galacturonic acid, one supplied by the Western Regional Research Laboratory and the other obtained from the Eastman Kodak Company, were treated in exactly the same way as the pectic substances.

One per cent solutions of arabinose, rhamnose, galactose and Eastman Kodak galacturonic acid in 50% ethanol were used as standards. Between 10 and 40 microliters of the hydrolysates and standards were then spotted on $18\frac{1}{4}\times24\frac{1}{2}$ No. 1 Whatman filter papers and subjected to descending solvent partition, using water as stationary phase and n-butanol or phenol as the moving phase. Phenol gave more distinct separation of sugars than did n-butanol. With phenol the galacturonic acid moved, but to a lesser extent than the other constituents, while with n-butanol the galacturonic acid remained stationary.

Brown areas on a yellow background were obtained when sodium dinitrosalicylate was used as the indicator, while *m*-phenylenediamine dihydrochloride gave a pink color with galacturonic acid and a tan color with the reducing sugars. Although the background in the latter case is initially colorless, it soon darkens. The colored spots, however, do not fade. Areas where no spot could be detected visually but which fluoresced in u.v. light occurred only when galacturonic acid was one of the components of the system and only in samples which had been heated with acid and precipitated with barium hydroxide. Highly fluorescent areas occurred only with the hydrolysates of pectin and Carré-Haynes calcium pectate. The component responsible for this phenomenon is apparently removed in the preparation of the other two pectic materials.

The presence of galactose, arabinose, and rhamnose was demonstrated in all the pectic substances examined. The areas due to these sugars were more marked in the chromatograms of the original pectin and the Carré-Haynes calcium pectate than in those of the Wichmann pectic acid. No trace of arabinose or rhamnose could be detected when 40 microliters of the hydrolysate of Ehrlich Acid C were examined, and the presence of galactose was not definitely indicated. However, by using 100–200 microliters of hydrolysate, the presence of all three sugars was demonstrated. It is conceivable that the apparently low concentration of these sugars in the Ehrlich Acid C was due to slower hydrolysis of this acid. (Strong hydrolytic conditions had prevailed during its preparation, and the resulting product may have been comparatively resistant to further hydrolysis.)

A suitable basis for computation of the analytical data is critical when comparisons between the uronide and non-uronide contents of different pectic substances are to be made, and Table 8 demonstrates this. The non-galacturonide contents are the values obtained from the CO_2 determination (Table 6). The first column shows that on the total dry weight basis the non-galacturonide content of Carré-Haynes calcium pectate is almost identical with that of the original pectin. But the ash content of calcium pectate is much greater than that of pectin, so there is less "ballast" or organic non-uronide material in the former than in the latter. The second and third columns of Table 8 give the amounts of organic non-uronide material in 100 g samples, allowing in one case for

TABLE 8.—*Non-galacturonide content of pectic substances*

SAMPLE	IN 100 GRAMS OF SAMPLE			PER CENT OF Ca-FREE DRY WEIGHT	PER CENT OF ASH-FREE DRY WEIGHT
	TOTAL	TOTAL, MINUS CALCIUM	TOTAL, MINUS TOTAL ASH		
Pectin	24.4	23.9	21.9	24.0	22.5
Wichmann Pectic Acid	8.5*		8.5		8.5
Ehrlich Acid C	6.9*		6.9		6.9
Ehrlich Acid B	15.0	13.9	7.2	14.1	7.8
Carré-Haynes Calcium Pectate	24.5	16.1	11.6	17.6	13.3

* Corrected for insoluble ash.

calcium alone and in the other case for total ash. This, however, does not allow accurate comparison between the different samples, although it does amply describe the non-uronide contents of the individual substances. The last two columns of Table 8 give the "ballast" content of the various pectic substances in 100 g of calcium-free material and in 100 g of ash-free material. (It is obviously more accurate to compare non-uronide contents than non-uronic-anhydride contents, although the trends indicated in both cases are the same.)

GRAVIMETRIC INTERRELATIONS BETWEEN PECTIC ACIDS AND CALCIUM PECTATES

Wichmann (2a) showed that the same yield of pectic acid was obtained when the pectin was first precipitated by the Carré-Haynes procedure and then boiled with 1 per cent HCl, as when the pectin was converted directly to pectic acid by the Wichmann procedure. He observed that a part of the calcium precipitate was soluble in HCl. This soluble material could be precipitated from the filtrate by neutralization.

Weighed amounts of pectin, Wichmann pectic acid, Ehrlich Acid C and Ehrlich Acid B (approximating 80 mg dry weight) were treated by the Carré-Haynes procedure, using double the volumes recommended. All samples except the Ehrlich Acid B were run in duplicate and two blank determinations were also made, starting with distilled water. In addition to washing on the filter with hot water, the precipitates were boiled four times with water in the original beakers. After the second boiling the filtrate gave a negative test for chloride. Finally, the precipitates were washed from the filters with hot water, and the aqueous suspensions were evaporated to dryness in platinum dishes as described above. All samples were dried to constant weight in a vacuum oven at 70°C. and were then further dried for 12–24 hours at 105°C. in an air oven. In no case did this additional drying bring about any significant change in weight. The dried samples were then ashed at 550°C. for 24

hours and weighed, and the amount of calcium present in the ash was determined by the A.O.A.C. volumetric method.

Table 9 gives the total dry weight yields, the ash contents, and the calcium contents of the calcium salts prepared from the various pectic substances. The duplicate values are reported to demonstrate the lack of precision in the determination of total ash. The dependence of this

TABLE 9.—*Calcium precipitates of pectin substances*

	YIELD	ASH CONTENT OF CALCIUM PECTATE	CALCIUM CONTENT OF CALCIUM PECTATE
	Per cent total dry weight taken	*per cent*	*per cent*
Pectin*	96.1	15.7	8.42 } 8.36
	96.7	16.1	8.31 }
Wichmann Pectic Acid	111.8	15.6	9.25 } 9.29
	111.3	16.0	9.32 }
Ehrlich Acid C	114.7	16.4	9.57 } 9.55
	115.5	16.7	9.53 }
Ehrlich Acid B	28.9	20.1	10.72

* The calcium precipitate of pectin is the Carré-Haynes Calcium Pectate.

quality on time and temperature of ashing has already been pointed out. It is also seen that the duplicate calcium analyses are in good agreement and that the total ash is not proportional to the calcium content of the ash. (It is shown earlier in this work that the ash of the calcium precipitate of pectin (Carré-Haynes calcium pectate) consisted entirely of calcium salts and insoluble ash, and it is reasonable to assume that this applies also to the ash of the calcium salts of the other pectic materials). The high value for the total ash of the calcium precipitate of Ehrlich Acid B is probably partly due to the presence of a large amount of insoluble ash.

DISCUSSION

The pectic acids, prepared in different ways (and in spite of the hydrolysis by alkali and possible hydrolysis by hot acid, e.g., in the Ehrlich and Wichmann procedures), still contained residual methyl ester groups which were found higher by the Zeisel method than by saponification. Identical methoxyl values, however, do not necessarily indicate the same degree of esterification, in view of the variable non-galacturonic anhydride content. The galacturonic anhydride content was highest in the Ehrlich Acid C preparation and least in the Carré-Haynes calcium pectate, but the actual values varied with method of expression. The average residue weights calculated from neutralization equivalent for pectic acid reported in the literature are all about 200, whereas the theoretical value for a

polygalacturonic acid is 176. The values obtained by us varied from 362 for Carré-Haynes pectic acid to 187 for Wichmann pectic acid and 182 for the Ehrlich Acid C. This wide discrepancy cannot be explained from errors inherent in the determination. The same wide variation occurred in galacturonide content of the preparations. It was established that the non-uronide content was highest in the starting material, pectin, and least in Ehrlich Acid C preparation. Qualitatively, the chemical constituents of all the pectic substances examined were similar, since all contained galactose, arabinose, and rhamnose. The relation between the various pectic substances, however, can best be expressed in terms of yield of calcium pectate.

The theoretical yield of calcium precipitate from pure galacturonic anhydride is 110.6 per cent of the weight taken, and the theoretical yield of the calcium precipitate of a pectic substance containing 90 per cent galacturonic anhydride, assuming that all the uronide is precipitated and that the non-uronide content is included quantitatively in the calcium precipitate, is 109.7 per cent of the weight taken. The yields of the calcium precipitates prepared from the Wichmann pectic acid and the Ehrlich Acid C (Table 9) are therefore greater than theoretical.

Yields higher than theoretical have been reported before. Hinton (7) obtained a 112 per cent yield of calcium pectate from a pectic acid prepared by saponifying a solution of apple pectin with cold sodium hydroxide, precipitating with hydrochloric acid at *p*H 2, washing with acid at this *p*H, and finally washing with 80 per cent alcohol. He showed that the yield of pectic acid by this procedure was 94 per cent of the original weight of pectin, while precipitation with sufficient HCl to make the solution 1 *N* in acid resulted in a lower yield (78 per cent). Essentially, however, this treatment compares with that used in the preparation of the Wichmann pectic acid. The yield of 112 per cent reported by Hinton compares with the 111.5 per cent found here for the calcium precipitate of the Wichmann pectic acid.

Hinton found no difficulty in raising the yield of calcium precipitate to about 100 per cent of the dry weight taken by washing the pectin with 50 per cent alcohol. The variation in reported yields and the ability to increase the yield by preliminary alcohol treatment point to differences in the proportion of non-uronide to uronide material in the original pectin. If the original pectinic acid is pictured as: $(Uronide+X)+Y$ where X, loosely or tightly bound non-uronide material, is quantitatively precipitated with the calcium pectate, and Y is physically admixed non-uronide material, prior removal of the Y fraction or a part of it will result in an increased yield of calcium precipitate. It is suggested that the calcium precipitate of pectinic acid includes the uronide and X fractions but not the Y fraction. On this hypothesis, it should be possible to remove all the Y fraction by careful washing, with the consequent production of

a pectin, containing about 82 per cent uronic anhydride, which precipitates quantitatively as calcium pectate. In this case the theoretical yield of the calcium precipitate would be about 109 per cent of the dry weight of the pectin.

It is conceivable that crude pectins vary only in the proportion of the *Y*, or physically admixed, fraction. As has already been suggested, it seems likely that the *Y* fraction is completely removed during the calcium precipitation procedure, while the *X*, or chemically bound fraction, remains intact. If this were so, crude pectins having different total galacturonic anhydride contents (as a result of having different *Y* contents) would give rise to calcium precipitates containing the same percentage of calcium. The *X* fraction can be reduced under certain conditions, as in the preparation of Wichmann pectic acid and Ehrlich Acid C, and the calcium content of the calcium precipitate therefore increased. In addition, the yields would seem to indicate that treatment of Ehrlich Acid C and Wichmann pectic acid with alkali does not result in loss of *X*. The fact that variation in the alkali treatment of the original pectin did not affect the calcium content of the calcium precipitate indicates that this may also be true in the case of crude pectin. Further evidence for the quantitative precipitation of (Uronide+*X*) by the calcium procedure is derived from the figures given in Table 10. In Table 10, the calcium precipitate of pectin is in reality Carré-Haynes calcium pectate, and the uronic anhydride content calculated from calcium analysis is for Carré-Haynes calcium pectate and not for the original pectin.

TABLE 10.—*Relationship between calcium content and uronic anhydride content of calcium pectates*

A. Free galacturonic anhydride content of starting material
B. Galacturonic anhydride calculated from CO_2 analysis

CALCIUM PRECIPITATE OF	CALCIUM CONTENT OF CALCIUM PRECIPITATE	A	B
	per cent		
Pectin*	8.36	81.3	81.3
Wichmann Pectic Acid	9.29	90.4	90.6
Ehrlich Acid C	9.55	92.9	92.7

* The calcium precipitate of pectin is the Carré-Haynes Calcium pectate.

SUMMARY

Pectic acids were prepared from citrus pectin in accordance with the original procedure of Ehrlich, Wichmann, and Carré and Haynes. The pectic acids so prepared were shown still to contain arabinose, galactose, and rhamnose, as well as galacturonic acid. These non-uronides, however, were greatest in the Carré-Haynes preparation.

The working hypothesis which has been found useful in explaining the

differences in some of the observed properties is: $(Uronide+X)+Y$ where X represents organic constitutents, other than free or methyl esterified anhydrogalacturonic acid residues (which are chemically combined in the pectin molecule), and Y consists of physically admixed non-uronide, organic material.

The Carré-Haynes procedure is considered to be better than the Wichmann procedure for the estimation of the pectic content of a crude pectin. It is suggested that calcium precipitation eliminates the Y fraction completely and quantitatively determines the (Uronide$+X$) fraction, while the Wichmann procedure results in the cleavage of some of the X material from the pectin molecule and in the loss of uronide.

It has been shown that the galacturonic anhydride content and the neutralization equivalent calculated from titration data differ from the respective values obtained from measurement of carbon dioxide evolution. The uronic anhydride contents of pectic substances which, in terms of the hypothetical structure suggested above, contained no Y material and varied in X content, have been computed from the calcium content of their calcium precipitates, and the values obtained in this way were found to be in good agreement with those calculated from carbon dioxide analyses. For this and other reasons, it is considered that the carbon dioxide method is more accurate than the titration method.

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CHEMISTRY OF ANALYTICALLY IMPORTANT PECTIC ACIDS

II. ACIDIC PROPERTIES, OPTICAL ACTIVITY, SPECIFIC VISCOSITY, AND OSMOTIC PRESSURE OF EHRLICH, WICHMANN, AND CARRÉ-HAYNES PECTIC ACIDS*

By ROBERT P. NEWBOLD† and M. A. JOSLYN (Department of Food Technology, University of California, Berkeley 4, California)

The structure, size, and shape of the molecules of the pectic acids have been investigated by determinations of their acidic properties, their behavior towards acids and bases, viscosity, optical properties (double refraction, streaming double refraction, refractive index and optical rotation), sedimentation velocity, osmotic pressure, and X-ray scattering. Their natural heterogeneity, and the fact that most of the studies of the physical properties of pectic acids have been carried out with ill-defined mixtures of various molecular size, has led to some confusion. The available literature has been reviewed by Kertesz (1) and in the recent theses of Deuel (2) and Sävenborn (3).

To establish the nature of the pectic acids prepared from citrus pectin by the methods of Ehrlich, Wichmann, and Carré-Haynes, the acidic properties, optical activity, specific viscosity, and osmotic pressure of these pectic acids were determined, and some of the factors influencing these physical properties were established. The results are reported here; data on composition and chemical properties of these pectic acids have been reported in the previous paper (4).

EXPERIMENTAL METHODS

The preparation of the pectic acids used has been described (4). The titration curves were obtained both by the constant titration method of Briggs (6), and Speiser *et al.* (6), and by titrating solutions of pectic substances directly with acid and alkali. The data by the first procedure are referred to throughout this work as "Constant Concentration," while those determined by the second procedure are referred to as "Direct Titration." In addition, owing to the insolubility of some of the pectic acids in water, two variations of each of the above procedures were employed, and these variations are referred to as "Method 1" and "Method 2."

Method 1 consisted of titrating an aqueous solution or suspension of the substance under investigation with acid or alkali.

Method 2 consisted of titrating a solution, which had been prepared by the addition of a known amount of alkali to give a *p*H of about 11, with standard acid.

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* Based on data presented in Ph.D. Thesis, University of California, 1950.
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Blank titrations were run by all of the above procedures, using CO_2-free water in place of pectic solution. To avoid inaccuracies brought about by the production of a "stirring potential" (7), all *p*H readings were made after the solution under investigation had come to rest. The figures reported as averages (arithmetic) of several determinations are the averages of readings which differed by less than 0.03 *p*H units. (The accuracy of the *p*H readings is considered to be within ± 0.02 *p*H units.)

For optical rotation measurements, solutions containing known concentrations of pectic substances were prepared, and sufficient alkali was added to aliquots of these solutions to give *p*H values of about 4.5, 6.0, and 7.5 when the total volume was made up to 50 ml. The optical rotations of these solutions were measured on a Schmidt and Haensch Polarimeter reading to 0.01°. A 1 dm. tube and a sodium lamp were used. Duplicate solutions were examined and at least 10 readings were taken on each solution.

For viscosity measurements, standard solutions of the pectic substances were prepared by direct solution in water in the case of the soluble materials, and by slow addition of sodium hydroxide to an aqueous suspension in the case of the water insoluble acids (Ehrlich Acid C and Wichmann pectic acid). In the latter case the *p*H was not increased at any time beyond 4. In all cases the pectic substance was not dried before solution, and correction for moisture was made from a separate moisture analysis. The standard solutions were used only on the day they were made.

A sufficient quantity of a standard solution to give the required concentration when diluted to 50 ml was measured out and made up to about 35 ml with water. The amount of sodium hydroxide (0.1 *N*) necessary to adjust the *p*H of the final solution to the required value was added slowly and with vigorous agitation, and the volume was made up to 50 ml with water (after the addition of 10 ml 1 *M* NaCl or 1 *M* sodium phosphate buffer in the cases where the viscosity was to be measured in the presence of excess electrolyte). The alkali requirement was determined by a preliminary titration. The final *p*H of the buffered solutions was 5.87. The viscosities were measured in 5 ml Ostwald capillary viscometers having delivery time for water of between 80 and 100 seconds. The viscometers were held in a constant temperature water at $27.3°C \pm 0.05°C$. and the solution under investigation was equilibrated at this temperature before pipetting out 5 ml for analysis. Three successive time measurements were recorded on each solution and these rarely differed by more than 0.1 second.

The relative viscosity of a pectic solution was calculated from the relation: $\eta_r = d_2 \cdot t_2 / d_1 \cdot t_1$, where d_1 is the density of the solvent at 27.3°C. (the solvents were water, 0.2 *M* NaCl and 0.2 *M* phosphate buffer), d_2 is the density of a pectic solution at 27.3°C., t_1 is the time of efflux of the solvent, and t_2 is the time of efflux of the solution.

Osmotic pressures were measured at 27.3°C. by the method of Bull (8) using Visking cellulose sausage casing (8/32 "NoJax") as membranes. The pectic solutions were adjusted to *p*H 6 with 0.1 *N* NaOH prior to the addition of sufficient sodium phosphate buffer to give a final concentration of 0.2 *M*. The *p*H of the final solution was 5.87. In addition to using toluene as the manometric fluid, a trace of toluene was added initially to all the pectic solutions to protect them against bacterial action.

Measurements were taken over a period of several days. The differences between the level of the column of buffer and the levels of the other two liquid columns were measured directly. Only slight variation, throughout the entire period during which readings were taken, was noted in the difference between the pectic solution and buffer heads. However, the actual heights of these two columns, determined by comparing the liquid levels with a fixed mark on the capillary tube, decreased slowly and regularly, probably due to evaporation. With the Wichmann pectic acid, the Ehrlich Acid C, and the Ehrlich Acid B the stopcock was opened after several days, reclosed, and a second set of readings was taken. The

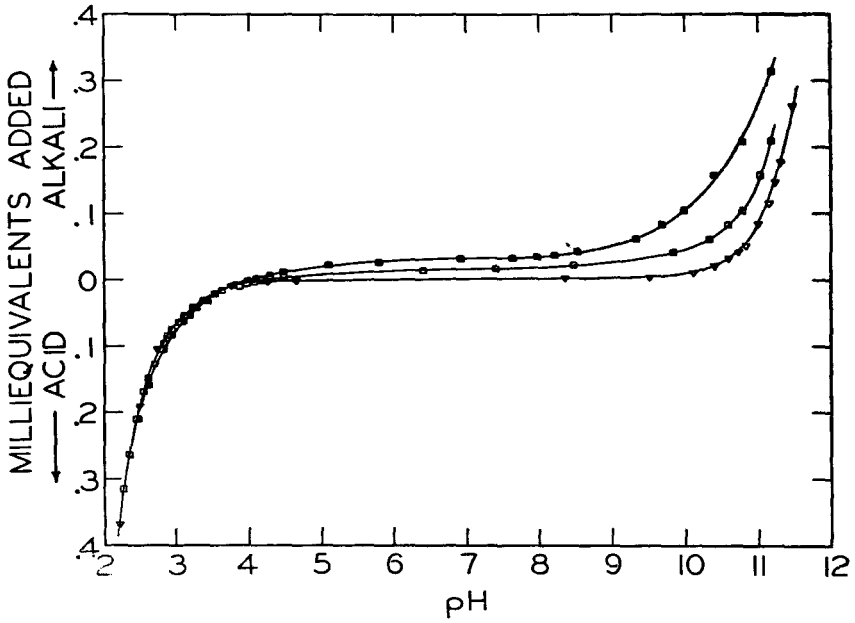

FIG. 1.—Titration curves. Pectin—constant concentration—Method 1.
Open squares —0.500 g/liter
Solid squares —1.000 g/liter
Inverted triangles—Blank

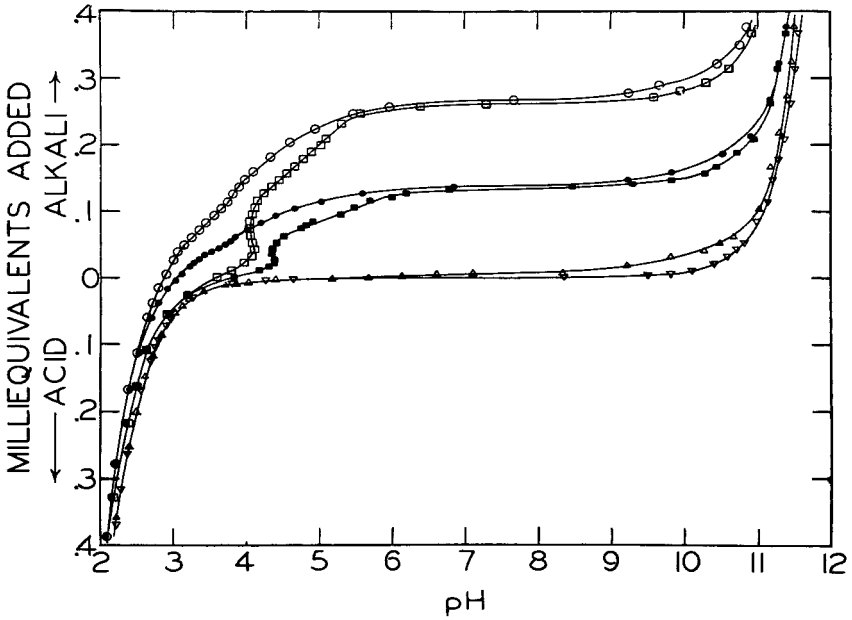

FIG. 2.—Titration curves. Wichmann Pectic Acid—constant concentration.
Small solid circles —Method 2, 0.496 g/liter
Large open circles —Method 2, 0.992 g/liter
Small solid squares—Method 1, 0.496 g/liter
Large open squares—Method 1, 0.992 g/liter
Normal triangles —Method 2, Blank
Inverted triangles —Method 1, Blank

individual readings were calculated to cm of H_2O and are given in the Tables.

RESULTS

Titration Curves.—Typical titration curves obtained with the various preparations are shown in Figures 1 to 4.

The blank titration curve obtained at constant concentration by Method 2 is seen to coincide with that obtained by Method 1 at *p*H values below 6. At higher *p*H values, however, the curves differ markedly. In Figures 2, 3, and 4 a corresponding dissimilarity between the curves determined for pectic substances by Methods 1 and 2 is apparent in the alkali range.

It is seen that the titration curves plotted from data obtained by any of the described procedures have the same characteristics. The pectic substances which are soluble in water (e.g. pectin and Ehrlich Acid B) have smooth titration curves with a single buffer range. The titration

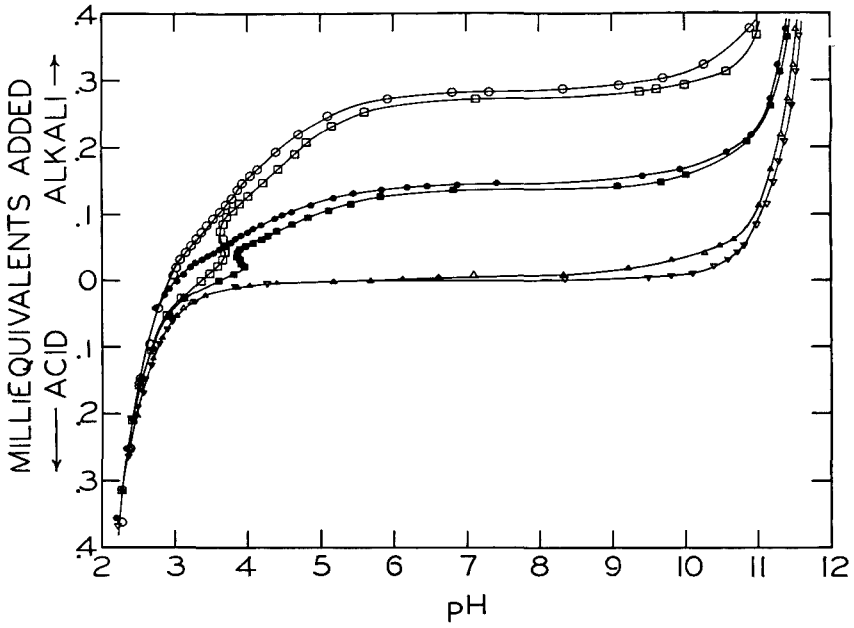

FIG. 3.—Titration curves. Ehrlich Acid C—constant concentration.
Small solid circles —Method 2, 0.500 g/liter
Large open circles —Method 2, 0.998 g/liter
Small solid squares—Method 1, 0.498 g/liter
Large open squares—Method 1, 0.998 g/liter
Normal triangles —Method 2, Blank
Inverted triangles —Method 1, Blank

curves of pectic substances which are insoluble in water (e.g. Wichmann pectic acid and Ehrlich Acid C) show a distinct irregularity at about *p*H 3.75–3.90 when examined by Method 2. This irregularity seems more pronounced in the case of the Wichmann pectic acid but is also readily apparent in the case of the Ehrlich Acid C.

Wichmann pectic acid and Ehrlich Acid C show some solubility in water, as is evidenced by the *p*H value of their suspensions. With the slow addition of alkali the *p*H remains essentially constant while the pectic acid dissolves. This "solution" phenomenon has been reported for pectinic acids by Jansen and MacDonnell (9) who were able to prepare 2 per cent solutions of these acids without raising the *p*H of the solution above 4. (The apparent reversal of the curve is probably due to the fact that the *p*H readings were made before sufficient time had elapsed for the attainment of equilibrium between the suspended acid and the solution.)

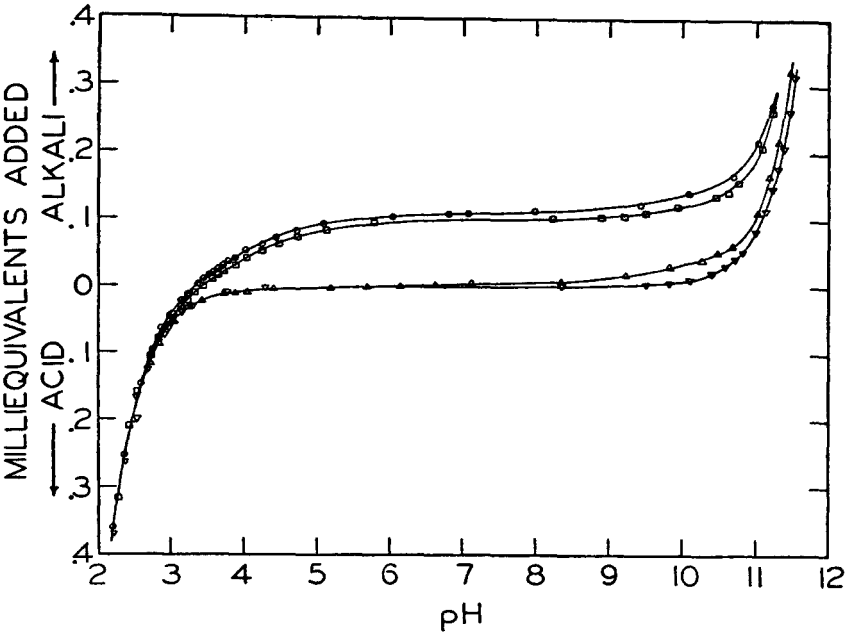

FIG. 4.—Titration curves. Ehrlich Acid B—Constant concentration.
Open circles —Method 2, 0.500 g/liter
Open squares —Method 1, 0.500 g/liter
Normal triangles —Method 2, Blank
Inverted triangles—Method 1, Blank

It has been noted by Myers and Baker (10) and by Lotzkar *et al.* (11) that the addition of a neutral salt to a pectinic acid solution causes a change in *p*H. The effect of added salt on the *p*H of pectic acid solutions can be seen best in Figure 4 where equal concentrations of Ehrlich Acid B are titrated by both Method 1 and Method 2.

Neutralization equivalents calculated from the titration curves are given in Table 1. A method for determining the neutralization equivalent of a pectic substance has been described previously (4). The procedures by which titration curves were obtained show certain significant variations when the neutralization equivalents calculated from these curves are compared with those found previously.

Buffer Capacity.—Buffer capacity curves were plotted according to the treatment of titration data developed by Van Slyke (12). Typical of the data obtained is that for Wichmann pectic acid (Fig. 5).

The buffer capacity of pectin was much lower than the buffer capacity of pectic acid. This is natural since the acidity, and consequently the

TABLE 1.—*Neutralization equivalents of pectic substances*
(from titration curves)

PECTIC SUBSTANCE	SAMPLE	PROCEDURE	mEq. ALK REQUIRED*	NEUT. EQUIV.
	(*grams*)			
Pectin	0.0250	Const. Conc.—Method 1	0.026	962
(1009)	0.0500	Const. Conc.—Method 1	0.053	952
	0.0500	Direct —Method 1	0.049	1020
	0.1000	Direct —Method 1	0.098	1020
	0.2000	Direct —Method 1	0.197	1015
Wichmann Pectic	0.0248	Const. Conc.—Method 1	0.132	187.9
Acid	0.0496	Const. Conc.—Method 1	0.262	190.0
(187.4)	0.0248	Const. Conc.—Method 2	0.132	187.9
	0.0496	Const. Conc.—Method 2	0.261	190.0
	0.0893	Direct —Method 1	0.472	189.2
	0.0898	Direct —Method 2	0.484	185.5
Ehrlich Acid C	0.0249	Const. Conc.—Method 1	0.136	183.1
(182.4)	0.0499	Const. Conc.—Method 1	0.273	182.8
	0.0250	Const. Conc.—Method 2	0.139	179.5
	0.0499	Const. Conc.—Method 2	0.278	180.0
	0.0928	Direct —Method 1	0.500	185.6
	0.0878	Direct —Method 2	0.488	180.0
Ehrlich Acid B	0.0250	Const. Conc.—Method 1	0.129	189.4
(198.5)	0.0250	Const. Conc.—Method 2	0.132	193.8
	0.0535	Direct —Method 1	0.266	198.9
	0.1070	Direct —Method 1	0.538	201.1
	0.0535	Direct —Method 2	0.272	196.7
	0.1070	Direct —Method 2	0.565	189.5

* Corrected for blank titration and alkalinity of the ash.
Figures in parenthesis are neutralization equivalents determined by a method described earlier in this work.

buffering power, of pectic substances depends on the number of free carboxyl groups.

Wichmann pectic acid and Ehrlich Acid C show a maximum in the buffer capacity curves at about *p*H 3.8 (the *p*H at which there is a distinct irregularity in the titration curves). There may also be another maximum at about *p*H 3.4 but from the results presented here the presence of this second maximum could not be definitely established. It is interesting to note in this connection that the *p*K value of galacturonic acid is about 3.4.

The curves for suspensions of the insoluble pectic acids show, as would be expected, an infinite buffering capacity at the *p*H at which solution occurs. The *p*H at which solution occurs varies with the nature of the pectic substance and its concentration. Comparison of the data obtained indicates the curves cross at a given concentration, the *p*H at which

solution of the Wichmann pectic acid occurs, and that, with both these acids, an increase in concentration decreases the *p*H at which they dissolve.

The buffer capacities of Ehrlich Acid C and Wichmann pectic acid were compared. It was found that these two acids have slightly different neutralization equivalents and very different molecular weights, yet their buffer capacity curves are identical from *p*H 7.5 to the *p*H 3.8 maximum.

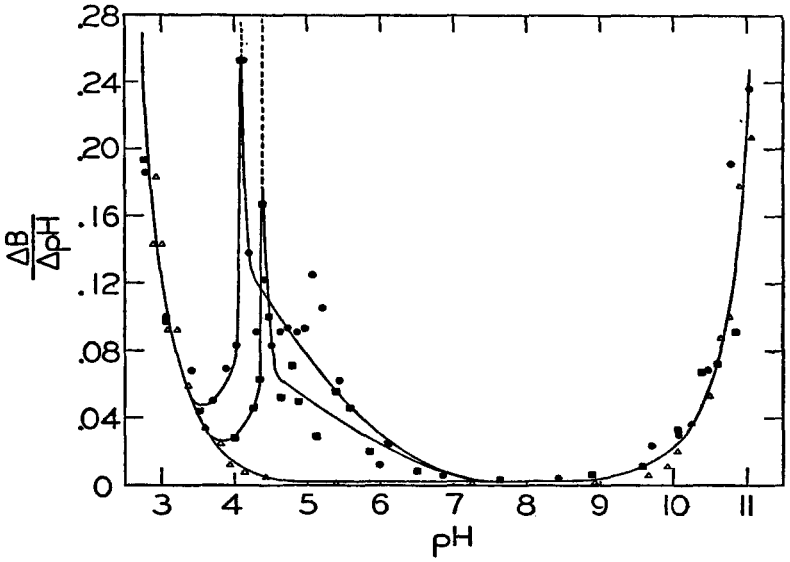

FIG. 5.—Buffer capacity curves—Wichmann Pectic Acid.
Constant concentration—Method 1.
Solid circles —0.992 g/liter
Solid squares—0.496 g/liter
Triangles —Blank
Showing variation in "solution *p*H" with concentration.

Below *p*H 3.8 there appears to be a difference in their buffering power. The significance of the maximum at *p*H 3.8 will be discussed later.

Titration Constants.—The degree of dissociation and the titration constant of each pectic substance at several *p*H values were calculated according to the method of Speiser, Hills, and Eddy (6). These authors designate the ratio of the number of carboxyl groups in the system to the total number of galacturonide residues as T, and consider this the significant quantity in comparing and characterizing pectic substances of different ester content and neutralization equivalents, since it is independent of "ballast" materials which do not contribute directly to the electrolytic behavior.

The T values for the substances under investigation here are:

Pectin	0.27
Wichmann Pectic Acid	0.98
Ehrlich Acid C	0.98
Ehrlich Acid B	0.96

If the methoxyl values of the three pectic acids are assumed to be due entirely to retained alcohol, the T values of these acids are 1.00.

Typical of the data obtained on the degrees of dissociation and the dissociation constants is that shown in Figures 6 and 7. The curves for pectin calculated from titration data obtained by a method identical with that employed by Speiser, *et al.* (constant concentration—Method 1) show an increase in the degree of dissociation at any given *p*H value with increase in concentration, c.f. Speiser, *et al* (6).

The curves describing the behavior of Wichmann Pectic Acid and Ehrlich Acid C, when titrated at constant concentration by Method 2 (Fig. 6), do not show any concentration effect. One curve describes the

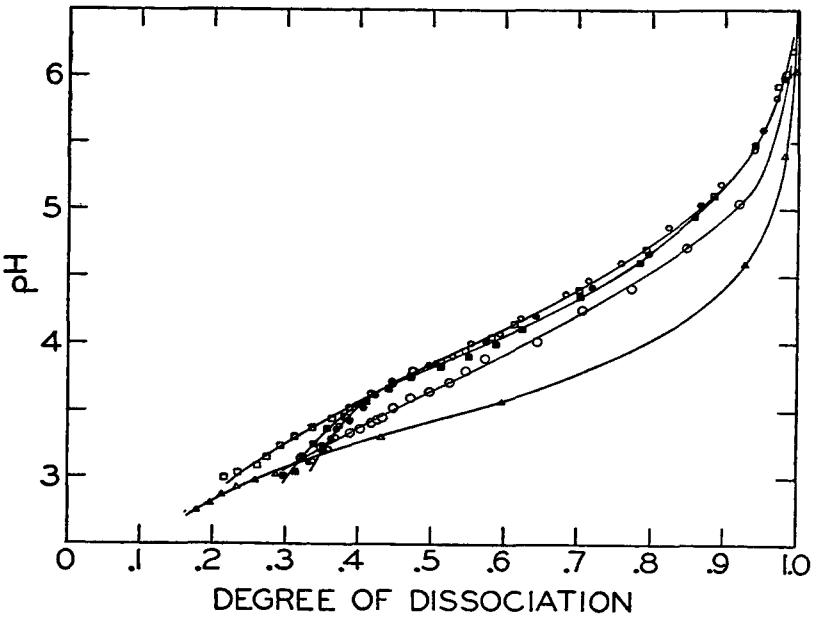

FIG. 6.—Dissociation curves. Constant concentration.
Small open circles—Ehrlich Acid C, Method 2, 0.500 g/liter.
Open squares —Ehrlich Acid C, Method 2, 0.998 g/liter.
Solid circles —Wichmann Pectic Acid, Method 2, 0.496 g/liter.
Solid squares —Wichmann Pectic Acid, Method 2, 0.992 g/liter.
Large open circles—Ehrlich Acid B, Method 2, 0.500 g/liter.
Triangles —Galacturonic Acid, Method 1, 2.13 g/liter.

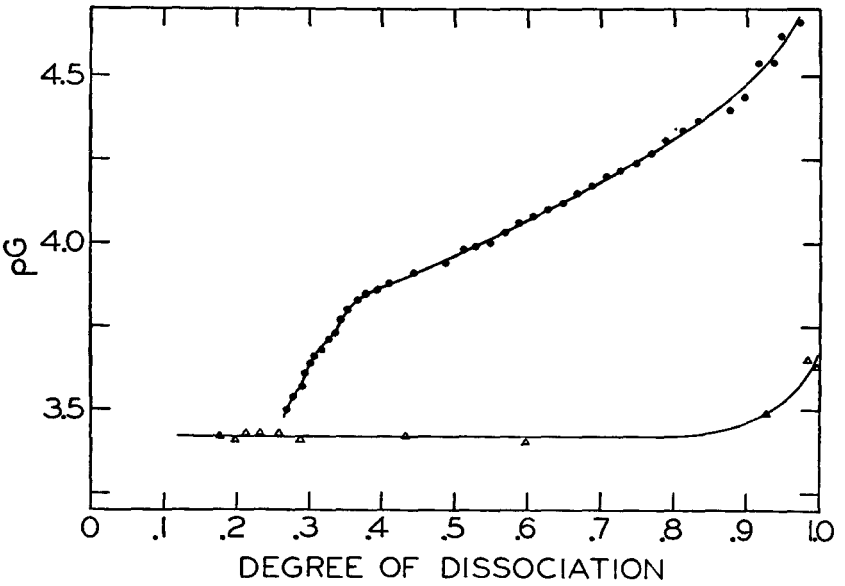

FIG. 7.—Dissociation curve—Wichmann Pectic Acid. Direct titration—Method 2.
Solid circles—Wichmann Pectic Acid, 0.599 g/liter.
Triangles —Galacturonic Acid 2.13 g/liter.

behavior of each of these acids at both the concentrations employed, except at *p*H values below about 3.7. The curves for these two acids lie very close together at *p*H values above 3.7, while the curve for Ehrlich Acid B is significantly displaced. From a comparison of the curves obtained by titrating the same concentration of Ehrlich Acid B and pectin by the same procedure (constant concentration—Method 2), it is seen that an increase in the number of carboxyls per unit length of chain causes the degree of dissociation at any given *p*H value to increase. This is contrary to the findings of Speiser, *et al.* (6), and may be due to the presence of different and considerable amounts of ash in each of the samples, inaccuracies in the alkali titration method referred to in detail earlier in this work, and/or difference in molecular weights of the two pectic substances. The molecular weight of the pectin, as determined by osmotic pressure measurements (see later), is over 20,000, while that of the Ehrlich Acid B is about 3,500. That molecular weight has no effect on acid behavior has, however, been reported by Speiser, *et al.* for pectinic acids prepared from apple pomace.

Naturally many of the trends seen in the degree of dissociation vs. *p*H. curves can also be seen in the degree of dissociation vs. *p*G curves (Fig. 7). The presence of sodium chloride decreases the *p*G value at any given

degree of dissociation. This agrees with the observation of Speiser, *et al.* regarding the effect of excess electrolyte on the *p*G values of pectinic acids. From these results, it is apparent that the *p*G values of a pectic or pectinic acid are influenced by added electrolyte and, although Speiser and co-workers have concluded that the variation in *p*G cannot be due to ionic atmosphere effects, the effect of concentration on the acid behavior of a pectic substance may be explicable on some such basis. If the small

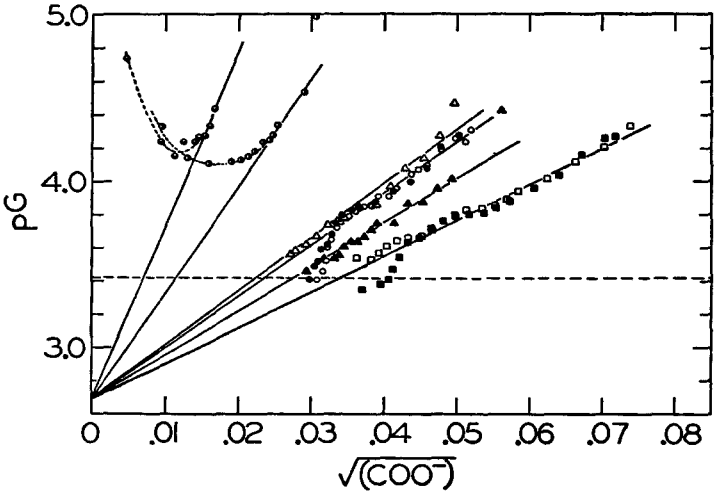

FIG. 8.—Dissociation curves. Constant concentration.

Open circles	—Ehrlich Acid C, Method 2, 0.500 g/liter.
Open Squares	—Ehrlich Acid C, Method 2, 0.998 g/liter.
Solid Circles	—Wichmann Pectic Acid, Method 2, 0.496 g/liter.
Solid squares	—Wichmann Pectic Acid, Method 2, 0.992 g/liter.
Open triangles	—Ehrlich Acid B, Method 1, 0.500 g/liter.
Closed triangles	—Ehrlich Acid B, Method 2, 0.500 g/liter.
◑	—Pectin, Method 1, 1.000 g/liter.
◒	—Pectin, Method 1, 0.500 g/liter.

Horizontal broken line—Galacturonic Acid, Method 1, 2.13 g/liter.

amounts of sodium chloride present in the solutions analysed by Method 2 were sufficient to completely neutralize a certain ionic effect, the degree of dissociation at a given *p*H and the corresponding *p*G value should be constant and independent of concentration. This would explain the observed behavior of Ehrlich Acid C and the Wichmann pectic acid.

From the curves obtained for Wichmann pectic acid and Ehrlich Acid C it was found that the variation of *p*G with degree of dissociation decreases when the latter has a value of about 0.5. The corresponding *p*G value is approximately the same in both cases and coincides with the *p*H value at which irregularities were noted in the titration curves

(3.8–3.85). In addition, erratic behavior is apparent at pG values below 3.8. In this region the behavior depends both on the acid itself and on its concentration. At both concentrations of Wichmann pectic acid there is a marked increase in the effect of degree of dissociation on pG while a similar effect is obvious only with the lower concentration of Ehrlich Acid C. These effects are not obvious in the case of the Ehrlich Acid B, although there are slight indications of such trends; the significant pG value in this case is about 3.65. It is suggested that this behavior is due to a change in state of these acids at a certain pH, such as a change in the degree of aggregation of the molecules.

Speiser, Hills, and Eddy (6) reported a possible linear relationship between pG and $\sqrt{(COO^-)}$ and found that, for all pectinic acids, the plots of this relationship passed through the same intercept for zero dissociation. They suggested that this common value (pG = 2.7) might perhaps be the fundamental dissociation constant for a single carboxyl group in a pectinic acid molecule when none of the adjacent carboxyls had dissociated. In Figure 8 the dissociation constants reported in this work are plotted against $\sqrt{(COO^-)}$, the lines all drawn to pass through pH = 2.7 at $\sqrt{(COO^-)} = C$. It is noted: that the linearity is only approximate and the plotted points show consistent non-linear trends; that pectin deviates markedly from the assumed linearity; that the curves for Ehrlich Acid C and Wichmann pectic acid are coincident at a given concentration; that concentration effects are very marked, the pG value of Ehrlich Acid B and Wichmann pectic acid at a given $\sqrt{(COO^-)}$ value decreasing with increase in concentration; and that there is a difference between the results obtained by different titration procedures. No explanation for the suggested relationship between pG and $\sqrt{(COO^-)}$ can be found.

Specific Rotation.—The specific rotation $[\alpha]_D^{24}$, of pectin in the range of 0.05 to 0.25 g dry weight per 100 ml was found to be 206; for Ehrlich Acid C in the range of 0.1 to 0.5 g ash-free dry weight per 100 ml it was 290; for Ehrlich Acid B in the range of 0.1 to 0.2 g ash-free dry weight per 100 ml it was 235; and for Wichmann pectic acid in the range of 0.1 to 0.5 g ash-free dry weight per 100 ml, it was 281. For pectic acid prepared from the calcium precipitate of Wichmann pectic acid it was 283; and for pectic acid prepared from Carré-Haynes calcium pectate it was 246, both expressed on total dry weight per 100 ml of solution in the range of 0.1 to 0.3 g/100 ml. Optical rotations above 1 per cent were difficult to read accurately because of turbidity. All the pectic substances were dextrorotatory.

The specific rotations determined here for the Wichmann pectic acid, the Ehrlich Acid C, and the Ehrlich Acid B are similar to the values reported for these materials by Nelson (13) and Ehrlich (14). It was found that, at any one of the concentrations used, there was no detectable difference in rotation at pH between 4.5 and 8.0, both in aqueous solution and in a solution 0.2 N in NaCl.

Table 2 presents values for specific rotations calculated to 100 per cent galacturonic anhydride content. Because of the lack of agreement between results obtained by CO_2 analysis and those calculated from titration data, and the indecision as to which is more correct, the galacturonic anhydride contents determined by both methods are included. Nothing definite can be concluded from an examination of these few results, but an interesting trend is noted, and it is considered likely that further investigation of

TABLE 2.—*Relationship between specific rotation and galacturonic anhydride content*

SAMPLE	$[\alpha]_D^{24}$	GALACTURONIC ANHYDRIDE CONTENT†		$[\alpha]_D^{24}$ CALCULATED TO 100 PER CENT GALACTURONIC ANHYDRIDE CONTENT†	
		A	B	A	B
Pectin	206	69.7	71.5	296	288
Wichmann Pectic Acid	281*	96.4*	91.3*	291	308
Ehrlich Acid C	290*	98.9*	92.9*	293	312
Ehrlich Acid B	235	92.9	84.7	253	277
Pectic Acid from Carré-Haynes Calcium Pectate	246	—	80.1	—	307

* Corrected for insoluble ash.
† A. From titration data.
B. From CO_2 determination.

the relationship between uronic anhydride content and specific rotation might lead to the conclusion that the uronic anhydride content of a pectic acid can be deduced from its specific rotation. The numerical values of (specific rotation$\times$100)/(uronic anhydride content), listed in the last two columns of Table 2, are independent of the basis of calculation; the same basis is used for both numerator and denominator and both are liable to the same corrections. The specific rotations and anhydride contents can therefore be expressed on a total dry weight basis for this purpose.

Viscosity in Aqueous Solution.—At all concentrations of Wichmann pectic acid, the viscosity increases between *p*H 4.5 and *p*H 6. Thereafter it appears to remain constant to about *p*H 8, when it begins to fall off again. The increase in viscosity, as pectic acid is converted to its salt, is similar to the behavior observed with pectinic acid (9, 10), and with solutions of other negatively charged hydrophilic colloids, such as gum arabic and carboxymethyl cellulose. The occurrence of a maximum at *p*H 6 has been established by several investigators.

Figure 9, drawn from the average viscosity values, indicates a linear relationship, over the concentration range examined between the relative viscosity and concentration of solutions of Wichmann pectic acid. The position of the curve is seen to depend upon the *p*H at which the viscosity

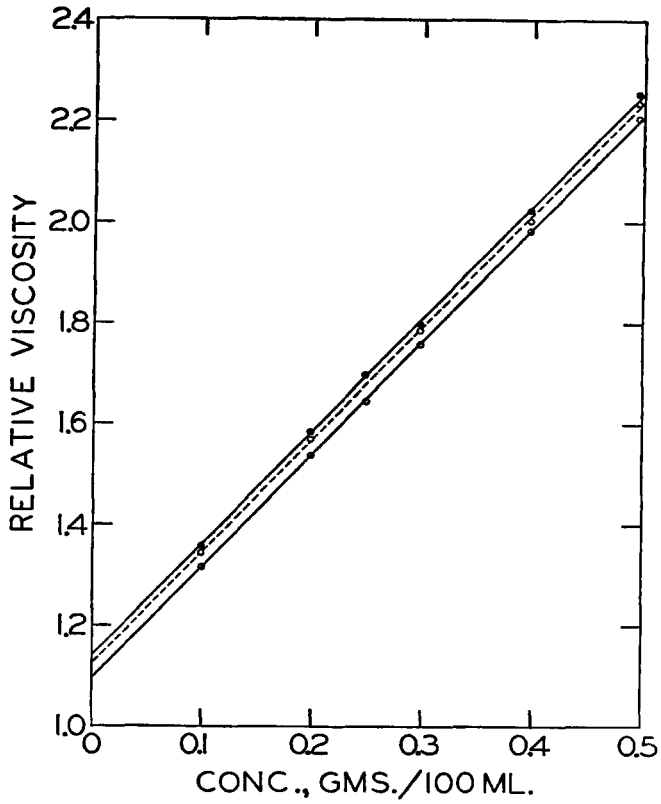

FIG. 9.—Viscosity of Wichmann Pectic Acid in water.
Open circles, solid line —*p*H 4.5
Solid circles, solid line —*p*H 6–8
Open circles, broken line—*p*H 9–9.5
Showing relationship between η_r and concentration and the effect of *p*H on this relationship.

is measured, although the slope is independent of *p*H. From this it would appear that for a pectic acid the overall viscosity, including that due to the electroviscous effect, can be expressed by the straight-line relation: $\eta_r = kc + x$, where k is a constant determined by the slope, and x is the intercept indicated by extrapolation of the straight line obtained experimentally to zero concentration. The intercept depends on the *p*H at which the viscosity measurements are made.

Figure 10 includes the curves obtained, at *p*H values of about 6, for pectin, Wichmann pectic acid, Ehrlich Acid C, and Ehrlich Acid B. The pectin curve differs from the other three in that it is only approximately linear. It is seen that the actual values of k and x in the above equation

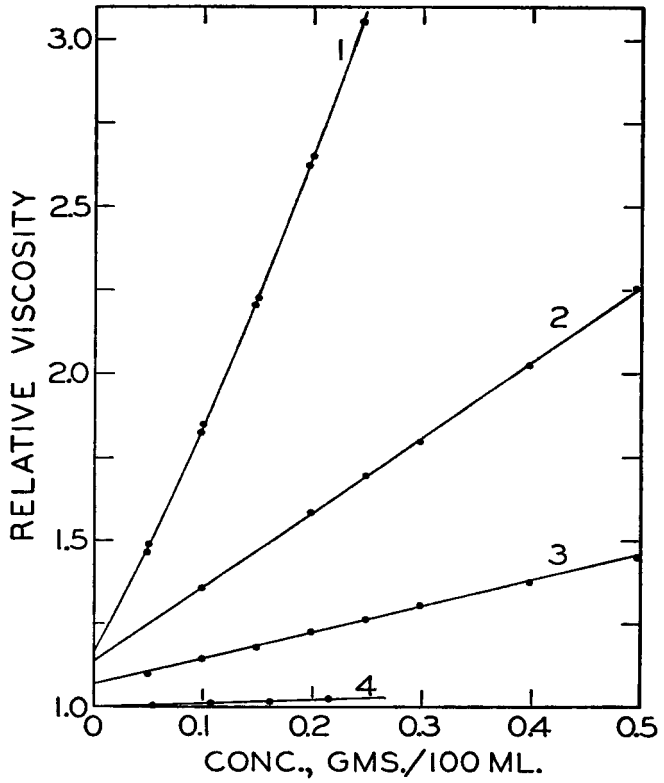

FIG. 10.—Viscosities in water (*p*H 6).
Curve 1, Pectin.
Curve 2, Wichmann Pectic Acid.
Curve 3, Ehrlich Acid C.
Curve 4, Ehrlich Acid B.
Showing relationship between η_r and concentration.

are different for each of the pectic substances. Approximate values of k and x derived from the curves are given below, together with the intrinsic viscosity determined by a method which will be discussed later. (As an approximation, it was assumed that the slope of the pectin curve was constant from $c=0$ to $c=0.066$.)

PECTIC SUBSTANCE	k	x	η	k/η	$(x-1)/\eta$
Pectin	6.4	1.17	3.72	1.7	.05
Wichmann Pectic Acid	2.2	1.14	1.15	1.9	.12
Ehrlich Acid C	0.8	1.07	0.50	1.6	.14
Ehrlich Acid B	0.1	1.00	0.04	2.5	—

The viscosities of solutions of Ehrlich Acid B are extremely small, and hence the magnitude of possible errors is great. Consequently, little account will be taken of the viscosity data obtained for this acid.

Viscosity in the Presence of Added Salt.—In determining viscosities the *p*H was adjusted to approximately 6, because of the tendency of pectinic acids of low ester content to aggregate at low *p*H values. The work of Owens *et al.* (16) and Lotzkar *et al.* (11) indicates that, in the presence of excess sodium chloride, the viscosity of a pectin solution shows little if any variation with *p*H above 4.5. Hence the actual *p*H values of the solutions used, although they vary slightly, are not considered to be critical. The electroviscous effect is reduced to a minimum by the addition of electrolyte, and Figure 11 shows that under these conditions there is a linear

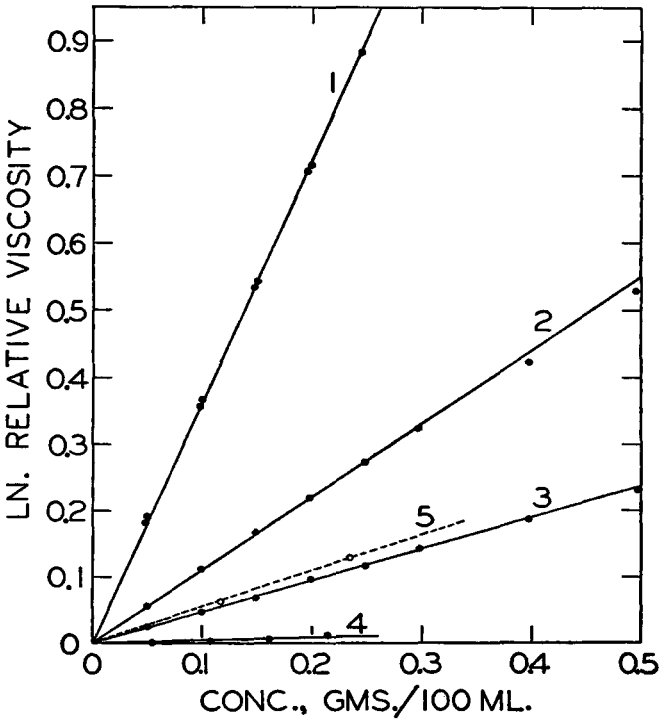

FIG. 11.—Viscosities in 0.2 *M* electrolyte.
Curve 1, Pectin, in NaCl.
Curve 2, Wichmann Pectic Acid in NaCl.
Curve 3, Ehrlich Acid C, in NaCl.
Curve 4, Ehrlich Acid B, in phosphate.
Curve 5, Pectic Acid prepared from calcium precipitate of Wichmann Pectic Acid, in phosphate.
Showing relationship between $\ln\eta_r$ and concentration.

relationship between the natural logarithm of the relative viscosity and the concentration of all the pectic solutions examined, and that all these lines pass through the origin. This relationship can be written: $\ln\eta_r = Kc$ where K is a constant, and is determined by the slope of the line. Thus when the electorviscous effect is minimized, the viscosity of a pectic solution conforms to the Arrhenius viscosity equation for electrolytes. A difference in behavior between Wichmann pectic acid and the pectic acid prepared from its calcium salt is again noted.

An exception to this rule was found in the case of the pectic acid prepared from Carré-Haynes Calcium Pectate. Although the viscosity of this substance in water could be readily determined it was found that the addition of sodium chloride or sodium phosphate increased the relative viscosity at all concentrations (except at the lowest concentration with 0.2 *M* phosphate). This is contrary to the behavior of the other pectic substances, where addition of sodium chloride or sodium phosphate was found to decrease the relative viscosity. In addition, with the pectic substances discussed above, the addition of chloride or phosphate had the same effect on the relative viscosity, while with the pectic acid prepared from Carré-Haynes calcium pectate the two salts have different effects. Since the pectic acid prepared from the calcium precipitate of Wichmann pectic acid behaved normally, it seems unlikely that the presence of ammonia and other possible contaminants can entirely explain the behavior of the pectic acid prepared from Carré-Haynes calcium pectate. Because the efflux times showed no constancy, except at lower concentration, the values of η_r and $\ln\eta_r$ were calculated from the initial measurement of the efflux time. The viscosity was found to increase with time of standing but, owing to the difficulty of making accurate viscosity measurements (due to the effect on the viscosity of shaking—see below), it is not certain whether a constant value was reached or whether there was a decrease after a maximum value had been attained.

Intrinsic Viscosity.—Since, in the presence of electrolyte, the pectic solutions (with the exception noted above) conform to the Arrhenius viscosity equation for electrolytes, the intrinsic viscosity, which has been defined as

$$(\ln\eta_r/c)_c = c \quad \text{or} \quad (\eta_{sp}/c)c = 0$$

is readily determinable for these substances. The slope of the $\ln\eta_r$-c curve is constant and equal to $\ln\eta_r/c$, and therefore the intrinsic viscosity can be measured by the slope of the curves in Figure 11.

Various methods are employed for the graphical determination of intrinsic viscosity:

(1) Extrapolation of the $(\ln\eta_r/c)$-c curve to $c = 0$
(2) Extrapolation of the (η_{sp}/c)-c curve to $c = 0$
(3) Extrapolation of the $\ln(\eta_{sp}/c)$-c curve to $c = 0$

All three methods were found to be equally satisfactory for accurate extrapolation of the data obtained for the pectic acids. Methods 1 and 3 were preferable in the case of pectin, since they gave straight line plots, while the plot by Method 2 was not strictly linear. The intrinsic viscosities found for the pectic substances examined here were:

Pectin	3.72
Wichmann Pectic Acid	1.15
Ehrlich Acid C	0.50
Ehrlich Acid B	0.04
Pectic Acid prepared from the calcium precipitate of Wichmann Pectic Acid	0.55

Weight Average Molecular Weight.—Since $[\eta]$ has been shown by Owens, *et al.* (16) to vary with temperature, the equation developed by these investigators to relate intrinsic viscosity and "weight average molecular weight" is strictly applicable at only one temperature. However, it was assumed to be reasonably accurate at 27.3°C., the temperature used in this work. Weight average molecular weights calculated from this equation are:

Pectin	62,000
Wichmann Pectic Acid	26,000
Ehrlich Acid C	14,000
Ehrlich Acid B	2,200
Pectic Acid prepared from the calcium precipitate of Wichmann Pectic Acid	15,000

Relationship between viscosities determined in the presence of added salt and those determined in the absence of added salt.—According to Kern (17) the viscosity behavior of polybasic acids can be interpreted by assuming the cooperation of two factors—an "ionic factor" and a "macromolecular factor." These allow for the influence on viscosity of the charges and the degrees of polymerization respectively; thus: $\eta_{sp}/c = I$ times $(\eta_{sp}/c)_m$ where I is the ionic factor, and $(\eta_{sp}/c)_m$ is the macromolecular factor. Since both factors influence the viscosities in water, and the influence of the ionic factor is completely repressed or minimized in solutions containing added salt, the ratios of the η_{sp}/c values obtained at *p*H 6 in the absence and in the presence of salt were investigated. From an analysis of viscosity data in this way, it was found that I, or $\eta_{sp}H_2O/\eta_{sp}$ salt increased with decreasing concentration and that I times $c^{1/3}$ was constant.

The value of I times $c^{1/3}$ is about 0.9 for pectin and varies a little. This is to be expected, since in the absence of added salt the η_r—c curve was not strictly linear. For Wichmann pectic acid the value of $I \cdot c^{1/3}$ is strictly constant (1.39), and for the Ehrlich Acid C, where the measurements of η_{sp} are smaller and therefore liable to greater error, the product is constant within experimental error and has a value about 1.34. The η_{sp} values for Ehrlich Acid B were so small and the possible error so great

that the values calculated for I are of little significance. The two $I \cdot c^{1/3}$ values obtained for the pectic acid prepared from the calcium precipitate of Wichmann pectic acid have an average close to the value obtained for Wichmann pectic acid. As a general empirical rule it may be stated that the product $I \cdot c^{1/3}$ is a constant, the value of which is the same or nearly the same for all pectic acids, and that $I \cdot c^{1/3}$ does not appear to be the same for pectinic and pectic acids.

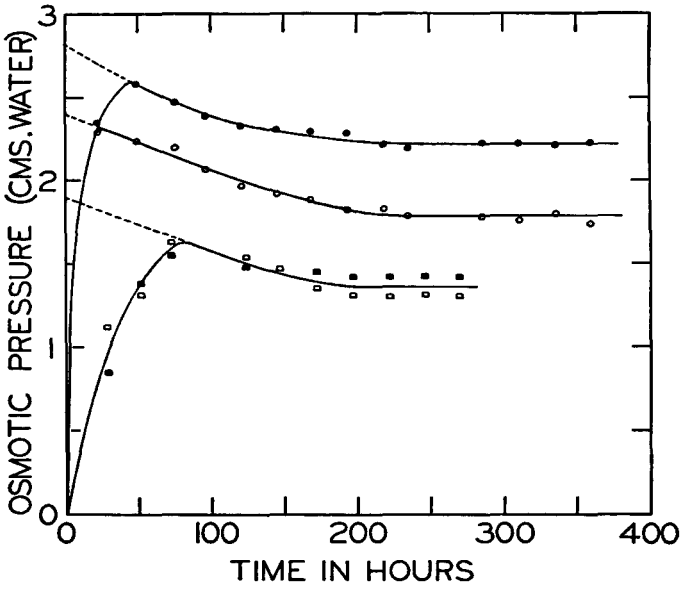

FIG. 12.—Osmotic pressures in 0.2 *M* phosphate buffer (*p*H 5.87)
Open circles —Pectin, 0.200 g/100 ml
Solid circles —Pectin, 0.250 g/100 ml
Open squares—Pectic Acid prepared from Carré-Haynes calcium pectate, 0.0948 g/100 ml
Solid squares—Pectic Acid prepared from Carré-Haynes calcium pectate, 0.0948 g/100 ml

Osmotic Pressure.—The osmotic pressure measurements obtained are shown in Figures 12–16. Phosphate buffer was used as solvent to minimize possibility of aggregation and to reduce osmotic contribution due to diffusible ion distribution. Measurements in 0.2 *M* NaCl solutions were found to give results identical with those obtained with buffer.

It is seen from the graphs that there is a maximum in all the osmotic pressure-time curves (except that given in Figure 15) beyond which the osmotic pressure decreased, sometimes to a constant value and sometimes continuously. A decrease in osmotic pressure with time was observed by

Jansen, *et al.* (9) for a pectic substance having a molecular weight of about 6,000. They considered that part of the material dialysed and that it appeared justifiable to accept the pressure indicated by extrapolation to zero time as the true osmotic pressure. Jansen *et al.* reported that the decrease in osmotic pressure with time was almost linear and this is borne out in the present work. In all the determinations reported here (with the exception already noted) there is an initial linear decrease in osmotic pres-

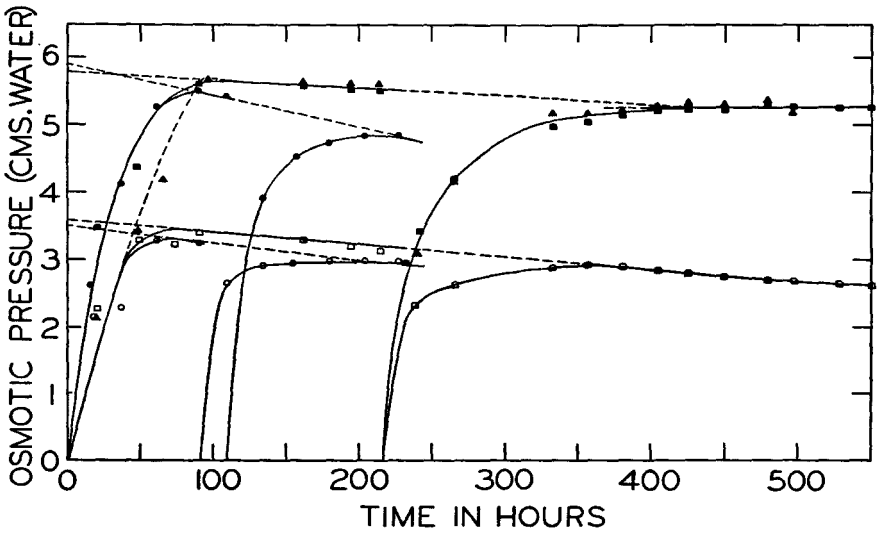

FIG. 13.—Osmotic pressure of Wichmann Pectic Acid.
Open circles —In 0.2 *M* Phosphate Buffer (*p*H 5.87) 0.149 g/100 ml
Solid circles —In 0.2 *M* Phosphate Buffer (*p*H 5.87) 0.248 g/100 ml
Open squares —In 0.2 *M* Phosphate Buffer (*p*H 5.87) 0.149 g/100 ml
Solid squares —In 0.2 *M* Phosphate Buffer (*p*H 5.87) 0.248 g/100 ml
Solid triangles—In 0.2 *M* NaCl (*p*H 6.50). 0.248 g/100 ml

sure with time after the maximum has been reached. With some of the solutions this linear decrease continues for as long as readings were taken, while with other the slope of the descending curve decreased until the osmotic pressure no longer varied with time. This latter behavior is to be expected if there is dialysis of smaller molecular weight constituents.

When a second set of pressure readings was made on any solution the resulting osmotic pressure-time curve, after reaching a maximum, was found to be a continuation of the curve drawn from the first set of readings. In the accompanying graphs the solid lines denote the curves obtained, and the broken lines show the continuity between the two sets of readings and also the extrapolation of the linear decrease to zero time.

Number Average Molecular Weight.—In calculating molecular weights

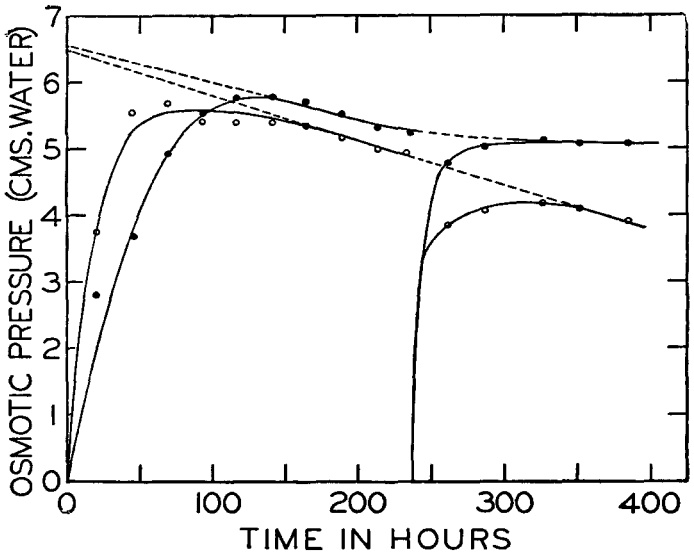

FIG. 14.—Osmotic pressure in 0.2 *M* phosphate buffer (*p*H 5.87)
Ehrlich Acid C.
Open circles—0.150 g/100 ml
Solid circles—0.150 g/100 ml

from osmotic pressure determinations, Bull (8) used the equation of Burk and Greenberg (19) which requires the concentration to be expressed in terms of grams of solute per 100 grams of solvent. The concentrations used in the determination of the osmotic pressures reported in this work are known in terms of grams of solute per 100 ml of solution and hence another equation was used, viz: $M = W \cdot RT/P$, where M is the molecular weight, R is the gas constant, 84.8 liter-cm of H_2O per degree, T is the absolute temperature (300.5°), P is the observed osmotic pressure in cms of H_2O and W is the weight concentration of solute (g per liter of solvent). With dilute solutions the error introduced by using g/liter of solution instead of g/liter of solvent in expressing concentration is very small, so that, if c represents the concentration in grams of solute per 100 ml solution, the equation becomes: $M = 10 \cdot cRT/P$. Table 3 gives two series of molecular weights calculated by this method, those calculated from the osmotic pressure determined by extrapolation to zero time, and those calculated from the value of the equilibrium osmotic pressure in the cases where an equilibrium was attained.

Regardless of which of the calculated molecular weights is considered to be accurate, certain general observations can be made, as follows:

1. The molecular weights determined here show no marked variation with concentration of the solutions employed. Owens, *et al.* (16) have

demonstrated this for pectin over a wider range of concentration. It would therefore seem that, with pectic acid under the conditions used, aggregation is not important.

2. The "number average molecular weight" as calculated from osmotic pressure measurements is smaller than the "weight average molecular weight" as determined from viscometric measurements in all cases (except that of Ehrlich Acid B). It has already been pointed out that heterogeneousness will cause the two types of molecular weight to vary in this way. The reason for the anomalous behavior of the Ehrlich Acid B is not clear.

3. The number average molecular weights of the Wichmann pectic acid and the pectic acid prepared from its calcium salt are comparable, although the weight average molecular weights are very different.

4. The number average molecular weight calculated for Ehrlich Acid C from the zero time pressure is close to the value of 4,700 determined by a similar extrapolation by Jansen, *et al.* (18) for the methylglycoside of polygalacturonic acid methyl ester.

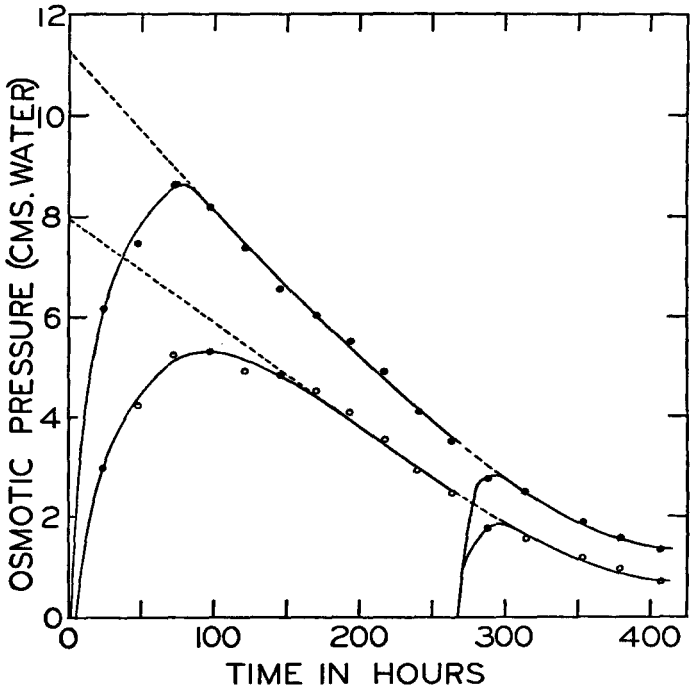

FIG. 15.—Osmotic pressure in 0.2 *M* phosphate buffer (*p*H 5.87)
Ehrlich Acid B.
Open circles—0.107 g/100 ml
Solid circles—0.161 g/100 ml

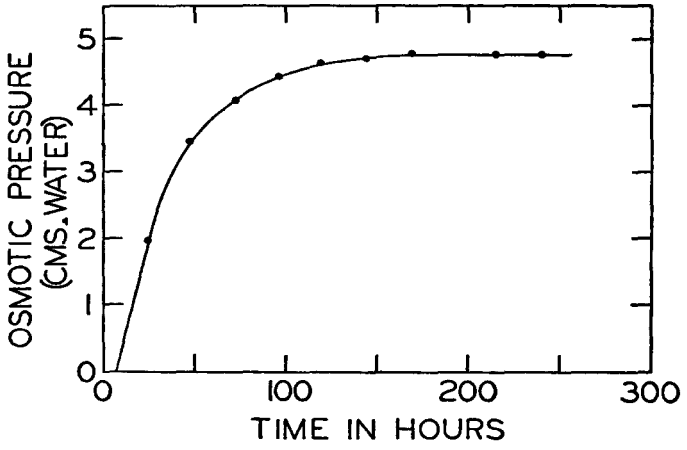

FIG. 16.—Osmotic pressure in 0.2 *M* phosphate buffer (*p*H 5.87). Pectic Acid prepared from calcium precipitate of Wichmann Pectic Acid.

5. The number average molecular weight of the original pectin is of the same general magnitude as the molecular weights reported by Owens, *et al.* (16).

6. The number average molecular weight of the pectic acid prepared

TABLE 3.—*Number average molecular weights of pectic substances*

PECTIC SUBSTANCE	CONC. GRAMS/100 ml	AT ZERO TIME		AT EQUILIBRIUM	
		OSMOTIC PRESSURE	MOLECULAR WEIGHT	OSMOTIC PRESSURE	MOLECULAR WEIGHT
Pectin	.250	2.82	22,600	2.22	28,700
	.200	2.40	21,200	1.78	28,600
Wichmann Pectic Acid	.248	5.85 (3)	10,800	5.26 (2)	12,000
	.149	3.55 (2)	10,700	—	—
Pectic Acid prepared from calcium precipitate of Wichmann-Chernoff Pectic Acid	.234	4.76	12,500	4.76	12,500
Ehrlich Acid C	1.50	6.50 (2)	5,900	5.08	7,500
Ehrlich Acid B	.161	11.26	3,600	—	—
	.107	7.93	3,400	—	—
Pectic Acid from Carré-Haynes Calcium Pectate	.095	1.89 (2)	12,800	1.36 (2)	17,800

(Figures in parenthesis refer to number or readings averaged.)

from Carre-Haynes Calcium Pectate does not differ greatly from that of the Wichmann pectic acid.

7. As would be expected from their methods of preparation, the molecular weights of the pectic acids decreased in the order Wichmann pectic acid, Ehrlich Acid C, Ehrlich Acid B.

The number average molecular weights of the pectic substances investigated in this work appear to form the following series in which each substance has a molecular weight approximately twice that of the substance below it: 1. Pectin; 2. Wichmann Pectic Acid; 3. Ehrlich Acid C; 4. Ehrlich Acid B. Owing to the heterogeneity of all these pectic substances and to the fact that the molecular weights are average values, this is considered to be entirely fortuitous.

SUMMARY

1. The acidic properties, specific optical rotation, specific and intrinsic viscosity, and osmotic pressure of preparations of Ehrlich, Wichmann, and Carré-Haynes pectic acids were measured.

2. The neutralization equivalent of Wichmann pectic acid was found to be 187.4, that for Ehrlich Acid C 182.4 and for Ehrlich Acid B 198.5. The parent citrus pectin had a neutralization equivalent of 1009. The equivalent for galacturonic acid is 194.1, that for digalactronic acid is 185, that for polygalacturonic acid containing 50 anhydro-galacturonic acid residues is 176. The buffering capacity of Wichmann pectic acid and Ehrlich Acid C showed a maximum at *p*H 3.8, close to the *p*K value of 3.4 for galacturonic acid. These two pectic acids have slightly different neutralization equivalents, very different molecular weights and yet have identical buffer capacity in the region of *p*H 7.56 and *p*H 3.8. The pectic acid prepared from Carré-Haynes calcium pectate is somewhat similar in behavior. The calculated ratio of the number of carboxyl groups to the number of galacturonide residues, T, was 0.98 for both Wichmann and Ehrlich pectic acids. The pectic acids investigated were similar in their behavior when analyzed either in terms of variation of degree of dissociation with *p*H or variation in degree of dissociation with *p*G. Unlike galacturonic acid, however, their *p*G values were not constant with increase in $\sqrt{(COO^-)}$.

3. The specific rotation of pectic acids was considerably higher than that for pectin and varied with the method of preparation. The nonuronide material present apparently reduced the specific optical rotation of pectic acid preparations as the specific rotation was proportional to per cent uronide matter.

4. The viscosity of aqueous solutions of Wichmann pectic acid increases between *p*H 4.5 and *p*H 6 with *p*H, similarly to the pectinic acids. A linear relation was found between relative viscosity and concentration of all the pectic acids examined; the slope of the straight line was greatest for the Wichmann pectic acid and least for Ehrlich Acid B.

5. The addition of salt minimized the electro-viscous effect and resulted in a linear relationship between the logarithm of relative viscosity, and concentration. From the relationship, the intrinsic viscosity and the corresponding weight average molecular weights were calculated, as follows:

	Intrinsic viscosity	*Weight average M.W.*
Pectin	3.72	62,000
Wichmann Pectic Acid	1.15	26,000
Ehrlich Acid C	0.50	14,000
Ehrlich Acid B	0.04	2,200

6. The product of the ratio of the specific viscosities in water to those in salt solution times the cube root of concentration was found to be constant for all pectic acids and appreciably higher than that for pectin.

7. The osmotic pressure behavior of the pectin and pectic acids investigated indicated a considerable heterogeneity in all preparations. The number average molecular weights calculated from osmotic pressures observed were:

Pectin	22,000
Wichmann Pectic Acid	11,000
Ehrlich Acid C	6,000
Ehrlich Acid B	3,500
Carré-Haynes pectic acid	13,000

Wichmann pectic acid and the Carré-Haynes pectic acid appear to be structurally analogous and to consist of polymers containing approximately 60–70 anhydro-galacturonic acid residues. They differ essentially in the lower content of non-uronide material present in the former. Ehrlich Acid C is made up of polymers having on an average between 30–40 anhydro-galacturonic acid residues while Ehrlich Acid B has, on an average, about 15–20 units. Our preparation of Ehrlich Acid C is similar in degree of polymerization to the structural unit described by Jansen *et al.* which corresponded to a polymer of 32 anhydro-galacturonic acid residues but differed from it in being less homogeneous.

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PROCEDURE FOR THE ANALYSIS OF ZINC PHOSPHIDE RODENTICIDES

By ERVIN BELLACK and JAMES B. DEWITT (Fish and Wildlife Service, U. S. Department of the Interior, Laurel, Maryland)

Zinc phosphide is widely used as a rodenticide, and there is a need for suitable methods for the analysis of this toxicant. The procedures of Elmore and Roth (1), Hunold (2), and Boirie (3) involve the determination of phosphorus as $Mg_2P_2O_7$ and appear too complicated for routine use. A modification of the Elmore method has been suggested (4, 5), but experience in this laboratory has indicated that further modifications are necessary before satisfactory accuracy can be attained. Many zinc phosphide rodenticides contain antimony potassium tartrate, and require preliminary purification before analysis. Antimony potassium tartrate interferes with quantitative evolution of phosphine, and leads to formation of an insoluble black residue containing much of the phosphide phosphorus. None of the above methods provide for separation of components of common zinc phosphide rodenticides.

The following procedure, based upon a modification of the method of Elmore and Roth, has been found satisfactory for the rapid quantitative assay of commercial rodenticides.

EXPERIMENTAL

Apparatus—(Apparatus for hydrolysis of zinc phosphide and the collection of the evolved PH_3 is shown in Figure 1. It consists of a water bath maintained at $50 \pm 1°$ C.; a 200 ml reaction flask fitted with a rubber stopper carrying a 125 ml separatory funnel; and three 200 ml absorption flasks. The separatory funnel is fitted with an inlet tube for the introduction of nitrogen gas, and the stem of the funnel is extended to the bottom of the reaction flask).

Reagents.—Nitrogen gas and the following solutions: *potassium permanganate* (0.5 *N*); *sulfuric acid* (1:9); *sulfuric acid* (1:1); *oxalic acid* (0.5 *N*); *sulfuric-oxalic acids* (1 part 1:1 sulfuric acid solution plus 3 parts 0.5 *N* oxalic acid solution); *sodium bicarbonate* (saturated); 0.1 *N iodine; starch indicator*, 0.5 per cent.

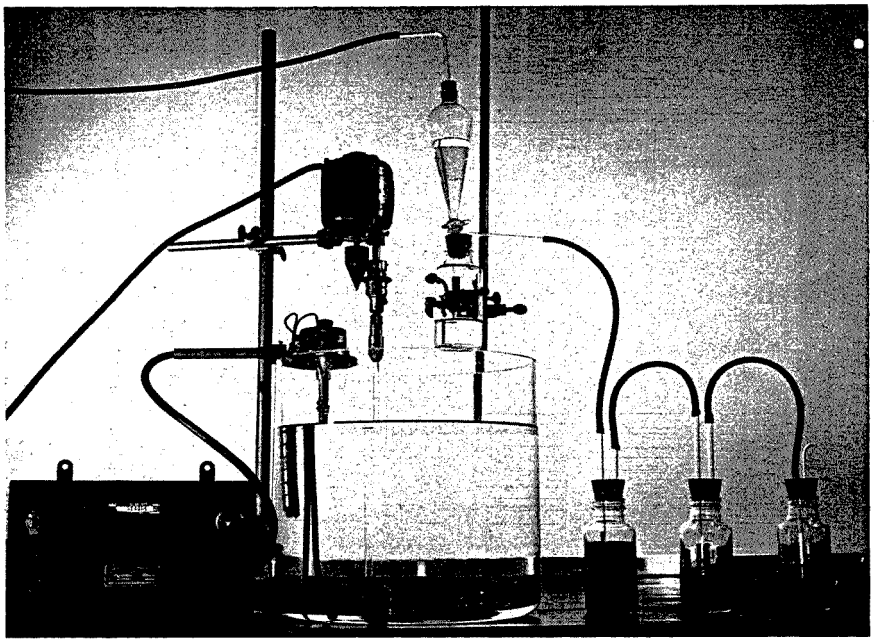

FIG. 1.—Apparatus for the hydrolysis of zinc phosphide.

PROCEDURE

Accurately weigh approximately 0.6 g of the rodenticide powder and place in a 250 ml beaker. Add 50–75 ml of freshly boiled and cooled (15°C.) distilled water, stir, and filter with light suction through ashless, double-acid washed filter paper. (Use minimum suction from water aspirator to avoid tearing of filter paper.) Wash residue on paper 5 times with 15 ml portions of freshly boiled and cooled distilled water. Combine all filtrates and reserve for antimony potassium tartrate determination. Transfer filter paper and residue to the reaction flask. Place 100.0 ml of standard potassium permanganate in the first absorption flask, and 50.0 ml in each of the other two absorbers. Pour 100 ml of the 1:9 sulfuric acid solution in the separatory funnel, and connect the apparatus as shown in Figure 1. Add the acid to the reaction flask slowly, regulating addition rate so that a steady stream of bubbles appear in the absorbers. After all acid has been added, adjust nitrogen gas pressure so that a steady flow of bubbles is maintained, and place reaction flask in water bath. Allow reaction to continue for at least one hour. At the end of reaction period, quantitatively transfer potassium permanganate solution from absorbers to an 800 ml beaker. Rinse absorbers and tubes with 225 ml of sulfuric-oxalic acid solution, taking care to dissolve all manganese dioxide, and add rinsings to the potassium permanganate solution in the beaker. Rinse absorbers and tubes with distilled water and add the rinsings to the beaker. Warm oxalic-manganous solution to approximately 50°, and titrate the excess oxalic acid with 0.5 N potassium permanganate solution. Calculate the Zn_3P_2 content of the sample by the formula:

$$\text{Per cent } Zn_3P_2 = \frac{\text{net ml } KMnO_4 \times 0.5 \times 1.614}{\text{weight sample (grams)}}$$

To the filtrate from the separation of components, add 10 ml of a cold, saturated solution of sodium bicarbonate, and a few drops of starch indicator solution. Titrate immediately with 0.1 *N* iodine solution until a persistent blue color is obtained. Calculate the percentage of antimony potassium of tartrate in the sample by the formula:

$$\text{Per cent antimony potassium tartrate} = \frac{\text{ml } I_2 \text{ soln} \times 1.67}{\text{wt. sample (grams)}}$$

DISCUSSION

The described method for the analysis of zinc phosphide rodenticides differs from earlier procedures in that it provides for the separation of components, and for the direct determination of phosphine by its reduction of potassium permanganate rather than by determination of phosphoric acid as magnesium pyrophosphate. The change in procedure necessitates modifications of apparatus to insure quantitative absorption of phosphine. The principal modification is the introduction of nitrogen through the mouth of the separatory funnel to provide a neutral atmosphere and to prevent escape of phosphine during the addition of the acid. Dilute sulfuric acid is substituted for hydrochloric acid in the hydrolysis in order to avoid error from the reaction of HCl with potassium permanganate.

This procedure has been evaluated in comparative assays involving a number of samples of known and unknown composition. Typical results are shown in the following tabulation:

TABLE 1.—*Results of assays of zinc phosphide rodenticides by different methods*

SAMPLE	PER CENT Zn_3P_2 FOUND BY METHODS:			PER CENT $K(SbO)C_4H_4O_6$
	X[1]	Y[2]	Z[3]	
A[4]	11.1	73.0	80.3	Not determined
B[5]	86.6	91.7	93.9	—
C[6]	—	91.7	94.4	—
D[7]	—	—	—	24.6
E[8]	—	—	—	24.8

[1] Method X—procedure specified in U. S. Army Specification 4-115.
[2] Method Y—procedure X preceded by separation of components.
[3] Method Z—procedure described in this paper.
[4] Sample A—composition unknown.
[5] Sample B—commercial zinc phosphide—approximately 95% Zn_3P_2.
[6] Sample C—sample B mixed with antimony potassium tartrate—values expressed in terms of proportion of zinc phosphide used.
[7] Sample D—three parts sample B mixed with 1 part antimony potassium tartrate.
[8] Sample E—three parts sample B mixed with 1 part antimony potassium tartrate.

These results indicate that the procedure described in this paper is more accurate than the modified Elmore-Roth method prescribed in the U. S. Army Specifications. They also show that for accurate chemical assay, separation of components of commercial zinc phosphide rodenticides is essential. Furthermore, they indicate that volumetric determination of phosphorus as phosphine is much simpler and more rapid than either volumetric or gravimetric determinations as magnesium pyrophosphate.

SUMMARY

A simplified and more accurate method for the analysis of zinc phosphide content of rodenticides is presented. It involves preliminary treatment of samples for the removal of interfering antimony potassium tartrate. Following this the sample is subjected to acid hydrolysis, and the liberated phosphine is determined by absorption in standard solutions of potassium permanganate.

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SEPARATION AND IDENTIFICATION OF CHLORINATED ORGANIC PESTICIDES BY PAPER CHROMATOGRAPHY

I. THE ISOMERS OF BENZENEHEXACHLORIDE

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In 1946 Ramsey and Patterson (1) separated the main stereoisomers of benzenehexachloride (BHC) on a column of silica gel, employing the immiscible solvents, nitromethane and n-hexane. Aepli, et al. (2) extended this procedure to the quantitative determination of the gamma isomer. Harris (3) improved the method by using D and C Violet No. 2 (4) to mark the position of the gamma band on the column. However, there is no convenient visual means of locating the bands of the other BHC isomers as they move down the column.

Recently, Moynihan and O'Colla (5) and O'Colla (6) have shown that the isomers can be separated and identified by paper chromatography. They used acetic anhydride as the stationary solvent and n-hexane as the mobile phase, and located the isomers on the chromatogram by spraying with ferrous sulfate in acetic acid and heating. Presumably catalytic activity of the iron salt under these conditions dehydrochlorinates the separated isomers; the released hydrochloric acid partially hydrolyzes the cellulose of the paper to reducing sugars which interfere in the local production of iron oxides and produce brown spots which mark the separations.

In the interim between appearance of the first and second publications of these authors, the writer developed the following chromatographic method for the separation and identification of the stereoisomers of BHC.

As observed by Moynihan and O'Colla (5), impregnation of the paper with the stationary solvent by dipping and pressing out the excess may

result in a non-uniform distribution. The writer was unable to obtain satisfactory results by their procedure and accordingly devised the alternate spraying technique described herein. A more direct and (in the writer's hands) more sensitive system for the detection of the BHC isomers is also described.

METHOD

APPARATUS

1. *Glass Jars*—12×25×25 cm high,[1] for 8″×8″ or 9½″×5½″ paper, 12×25×50 cm high,[2] for 18¼″×5½″ paper, 7.5×45 cm high,[3] cylindrical, for 15″×1″ paper; with flat ground top edges and flat glass covers.

The smaller rectangular jar is provided with a fitted cover and with notches in the sides of the jar to support rods across the upper end, to which the paper is attached with stainless steel clips.[4] Use flat glass plates, cut to fit, as covers for the the other jars. Bore ½″ holes in the corners of the cover for the larger jar, about 1¼″ from its inner edges, and insert, through stoppers, hook-shaped rods to hold lateral rods. Bore a single ½″ hole in the cover for the cylindrical jar to accommodate a rod, bent at a 90° angle and mounted through a stopper.

The paper is supported so that it dips into an approximately half-inch layer of mobile solvent and its lower edge hangs just free of the bottom of the jar. With the larger rectangular jar and the cylindrical jar, proper clearance is obtained by vertical adjustment of the position of the hanger rods in their stopper mountings. The smaller rectangular jar accommodates 9½″×5½″ paper. To adapt it to 8″×8″ paper, which this writer prefers, employ auxiliary glass troughs[4] to hold the mobile solvent, supported at proper height by a glass grid.

Covers are conveniently sealed with 1″ cellophane tape.

2. *Drying Rack.*—Metal or wood, to support paper hung from rods in oven at 130–133°C.

3. *Filter Paper.*—Whatman No. 1, in 18¼″×22½″ sheets which are cut into 9⅛″×5½″ and 18¼″×5½″ sizes; also in 8″×8″ sheets and 1 inch rolls.

4. *Sprayer.*—For application of stationary solvent and indicator solutions. A suitable spraying device is supplied by University Apparatus Co.[4]

5. *Gloves.*—The paper should not be touched by hand until the chromatogram is fully developed. Cloth gloves may be worn in handling the paper except that, when spraying, rubber gloves are best for personal protection.

6. *Hood.*—Protect the inner surfaces of the hood from excessive spray contamination by lining it with heavy wrapping paper.

REAGENTS

(a) *Stationary solvent.*—Acetic anhydride diluted with ethyl ether (1+9). Prepare daily.

(b) *Mobile solvent.*—Commercial grade mixed octanes[5] (not 2,2,4-trimethylpentane) nearly saturated with acetic anhydride (100+6, v/v). Prepare daily.

(c) *Development reagents.*—(1) Hydrolysis reagent: normal potassium hydroxide in methanol. (2) Indicator reagent: 0.05*N* silver nitrate in dilute nitric acid (1+3).

(d) *Standards.*—Alpha, beta, gamma, delta isomers of BHC. Prepare 0.01 and 0.1 molar solutions of each isomer and mixtures of the four isomers in ethyl acetate. Keep in glass-stoppered containers.

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[1] Fisher Scientific Co. cat. no. 11-780, size #11.
[2] Standard Scientific Supply Co. cat. no. 7060, size #23.
[3] Kimble Glass Co. cat. no. 20070.
[4] University Apparatus Co., Berkeley, California.
[5] Phillips Petroleum Co., Bartlesville, Okla.

PROCEDURE

To identify the BHC isomers and for most separations, use the 8×8 inch paper (or 9⅛×5½). Rule the paper with a hard pencil one inch from bottom of sheet, placing dots at 1½ inch intervals along the line, beginning 1 inch from either side. By means of capillary pipets spot the various solutions (preferably the 0.01 molar solutions on one sheet and the 0.1 molar solutions on a second sheet) at the marked intervals along the line. The spots should not be more than 7 mm in diameter. Add ca ½ inch layer of mobile solvent to trough (or jar). Clip top edge of paper to the rod which is to be used to suspend paper in jar, invert and clip bottom edge of paper to an auxillary glass rod supported in a well-ventilated hood. Wearing rubber gloves, impregnate the filter paper with freshly prepared stationary solvent (Reagent **a**) by spraying rapidly and uniformly in horizontal strips, beginning at the marked base line and continuing to the opposite edge or "top" of paper. The entire sheet from the base line must be covered; any unsprayed spots will interfere with the proper development of the chromatogram. Immediately invert, unclip the auxiliary glass rod, transfer the paper to the jar so that the lower edge dips into the mobile solvent (Reagent **b**), and seal with tape. Allow to stand until the mobile solvent front approaches (but does not reach) the top of paper (ca 1½ hours). Remove paper from jar and hang from rod in hood until dry (ca 1 hour). Wearing rubber gloves, spray paper with hydrolysis reagent **c1**. Transfer paper on rod to drying rack and, as soon as excess methanol has evaporated, heat in oven at 130–133° for ½ hour. Remove from oven and allow to stand overnight. Wearing rubber gloves, spray in hood with indicator reagent **c2**. After most of the nitric acid has left the paper develop the silver chloride deposits by exposing to the sun.

For a greater spread among the various isomers, use 18¼×5½ inch paper strips. Develop in the large glass jar (ca 12 hours). In making the longer run, use 0.1 molar solutions, since the farther the isomers travel on the paper the more diffused and less distinct the separations become.

EXPERIMENTAL

Whatman No. 1 filter paper was spotted with the various isomers and sprayed with methanolic potassium hydroxide, heated, and then sprayed with the 0.05 *N* silver nitrate in dilute nitric acid. Gradually the amounts of potassium hydroxide and nitric acid were increased and the paper was exposed to increasingly higher temperatures for longer periods. When sprayed with the silver nitrate solution shortly after the hydrolysis with the alkali, white spots on a brown-yellowish background were produced. The background changed fairly rapidly to a deep brown, almost black color and masked the spots. On the other hand when the paper stood overnight after hydrolysis with alkali, the silver nitrate spray caused no discoloration of the paper, and produced no visible spots even after standing for hours in the dark hood. However, when the paper, after most of the nitric acid fumes had disappeared, was exposed to the sun, the deposits of silver chloride developed on a background which slowly turned to an ash-grey. The silver chloride spots on papers which had become so dark on prolonged exposure to light (three months) that they were no longer visible were revived by spraying the papers with concentrated nitric acid followed by exposure to light.

Five lambda (0.005 ml) portions of an ethyl acetate solution, which

was 0.01 molar in respect to the four isomers of BHC, were spotted at the center of a line drawn 1½ inches from the bottom of 15×1 inch strips of No. 1 Whatman filter paper. Duplicate strips were sprayed with varying concentrations of acetic anhydride in ethyl ether as the stationary solvent, and each strip was run in a different glass cylinder which contained a half-inch layer of a mixture of isooctanes and acetic anhydride, 100+6 (v/v), as the mobile solvent. As the mobile phase ascended, its demarcation on the strip was easily visible on the strips sprayed with 10% acetic anhydride, but was less and less visible with increased proportions of the anhydride, and became practically invisible with 14 to 16 per cent. (However, the upper limit of the mobile phase could be momentarily noted shortly after removal of the strip from the cylinder.)

One set (odd numbers, Table 1) of the duplicate strips were treated with the hydrolysis reagent, heated, and sprayed with the indicator reagent immediately after their removal from the oven. These strips darkened to such a degree that it was difficult to locate the silver chloride

TABLE 1.—*Chromatographic data and R_F values*

PAPER STRIP NUMBER	TIME OF RUN	DISTANCE TRAVELED BY MOBILE SOLVENT	STATIONARY PHASES (VOLUME OF ACETIC ANHYDRIDE IN ETHYL ETHER)	R_F VALUES OF ISOMERS OF BENZENE HEXACHLORIDE			
				BETA	DELTA	GAMMA	ALPHA
	hours	*inches*	*per cent*				
1		11¼		.51	.65	.77	.84
2	5	11¼	10	.50	.64	.76	.83
3		10¾		.44	.56	.68	.77
4	6	11	12	.42	.55	.66	.74
5		11		.41	.55	.70	.79
6	7	11¼	14	.39	.53	.66	.74
7		11¼		.35	.48	.63	.71
8	8	11¼	16	.32	.45	.57	.66
9		10½		.32	.44	.57	.67
10	8	10½	18	.30	.42	.55	.64

deposits. The darkness of the paper was removed by respraying the dry strips with dilute nitric acid (1+3). The second set of duplicates (even numbers, Table 1) was sprayed with the hydrolysis reagent, heated, and allowed to stand 16 to 20 hours (overnight). When sprayed with the indicator the strips did not darken, perhaps because the potassium hydroxide in the paper had been converted to carbonate by the action of atmospheric carbon dioxide.

TABLE 2.—*Chromatographic data and R_F values*

PAPER SHEET NUMBER	TIME OF RUN	DISTANCE TRAVELED BY MOBILE SOLVENT	STATIONARY PHASE (VOLUME OF ACETIC ANHYDRIDE IN ETHYL ETHER)	R_F VALUES OF ISOMERS OF BENZENE HEXACHLORIDE							
				BETA		DELTA		GAMMA		ALPHA	
				AV. OF 7	RANGE	AV. OF 7	RANGE	AV. OF 7	RANGE	AV. OF 7	RANGE
	hours	*inches*	*per cent*								
1		5¾		.37	.04	.52	.03	.65	.05	.73	.05
2	1½	5⅞	10	.40	.05	.56	.05	.70	.03	.78	.04
3		5¾		.36	.06	.50	.09	.65	.07	.72	.09
4	1¾	5⅞	12	.34	.02	.50	.03	.63	.06	.71	.05
5		6		.30	.06	.45	.06	.58	.07	.67	.04
6	2	5⅞	14	.31	.03	.45	.04	.58	.04	.66	.06
7		5¾		.31	.02	.43	.04	.56	.02	.63	.01
8	2½	5¾	16	.29	.01	.41	.03	.53	.02	.61	.01
9		5¾		.32	.04	.47	.05	.61	.05	.70	.06
10	2¾	5¾	18	.31	.04	.44	.04	.56	.07	.63	.06
11		4½		.32	.09	.47	.02	.59	.07	.67	.09
12	3	4⅞	20	.35	.03	.48	.02	.62	.04	.70	.03
13		5⅞		(One spot at solvent front)							
14	1	5⅞	2								
15		6		.66	.05	.81	.05	.92	.06	.96	.05
16	1	5⅞	4	.71	.06	.85	.05	.94	.03	.97	.01
17		6¼		.56	.02	.70	.04	.83	.05	.90	.07
18	1	6	6	.60	.05	.75	.08	.87	.06	.91	.06
19		5¾		.53	.06	.68	.08	.81	.07	.89	.07
20	1¼	5½	8	.53	.03	.68	.06	.81	.06	.88	.07
21		6		.44	.07	.60	.06	.74	.05	.82	.05
22	1½	5⅞	10	.51	.08	.66	.08	.80	.10	.87	.10

In a subsequent experiment the concentration of acetic anhydride in ethyl ether was varied from two to twenty per cent. The base line was drawn one inch from the bottom of 8×8 inch sheets of Whatman No. 1 filter paper. Seven 5 lambda portions of 0.01 *M* solutions of each of the four isomers of BHC were spotted at one inch intervals along the line. Duplicate sheets were run in the jar at one time and the series of experiments was completed in three days. Although the mobile solvent was prepared fresh each day, runs made during a single day employed a single preparation of mobile solvent.

The data given in Table 2 are divided into groups to identify sheets run with the same preparation of mobile solvent. Duplicate sets of sheets were run for the ten per cent acetic anhydride, one at the beginning of the series and another at the end, inasmuch as room temperatures had risen about 8 to 10°C. between the first two groups of experiments and the final one.

IDENTIFICATION OF ISOMERS

To identify the individual isomers and to locate the order in which they separate on the paper, five lambda portions of a 0.01 *M* solution of each isomer, and of a solution 0.01 *M* in all four isomers, were spotted along the base line of 8″×8″ sheets. Runs were made at various times during the course of the work (February–May 1952) at room temperature. The paper was impregnated by spraying with ten per cent acetic anhydride.

TABLE 3.—*R_F values*

MONTH OF RUN	R_F VALUES OF ISOMERS OF BENZENE HEXACHLORIDE			
	BETA	DELTA	GAMMA	ALPHA
February	0.31 to 0.32	0.52 to 0.56	0.64 to 0.66	0.74 to 0.79
March	.37 .43	.54 .59	.67 .71	.75 .81
March	.31 .37	.45 .53	.59 .70	.73 .80
March	.23 .25	.38 .43	.56 .60	.70 .76
April	.28 .32	.42 .46	.54 .60	.76 .80
May	.29 .35	.42 .50	.55 .64	.62 .74
May	.30 .34	.43 .50	.57 .63	.67 .73
Max. Range	0.23 to 0.43	0.38 to 0.59	0.54 to 0.71	.62 to .81

Table 3 shows the range of R_F values obtained for the four isomers of BHC at different times, and Table 4 gives those obtained for two commercial samples.

Table 3 clearly shows the variations to be expected in runs conducted at different times, under presumably varying conditions. This points up

TABLE 4.—*R_F values of commercial samples and knowns*

ISOMER OF BENZENE HEXACHLORIDE	R_F VALUES OF THE SAMPLES		
	KNOWN	SAMPLE NO. 1	SAMPLE NO. 2
Beta	0.28 to 0.32	0.28 to 0.33	0.29 to 0.32
Delta	.42 .45	.42 .46	.42 .45
Gamma	.55 .59	.54 .60	.56 .57
Alpha	.64 .68	.63 .67	.63 .66
Unknown*	—	.78 .80	.76 .78

* Ramsey and Patterson (1) found 4% heptachlorocyclohexane and 0.6% octachlorocyclohexane in Sample No. 1.

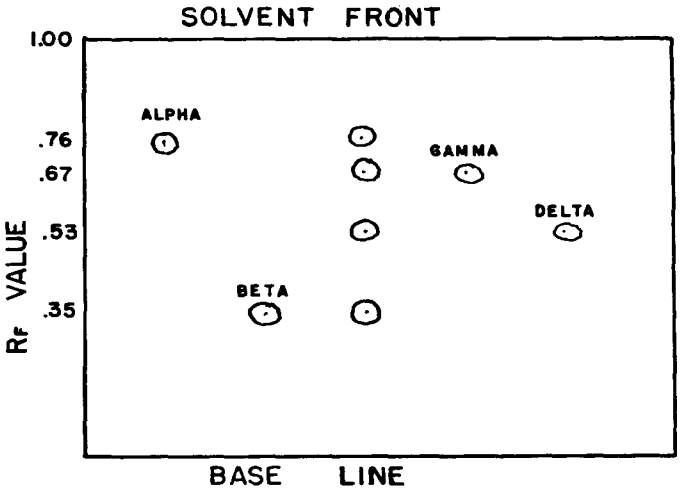

FIG. 1.—Identification of isomers of BHC.

the general observation that R_F value is useful for identifying the separated constituent only when it may be related to a reference R_F value established under comparable conditions. The most convenient means of assuring comparable conditions is to develop the unknown chromatogram simultaneously, in the same equipment, and preferably on the same sheet with the reference chromatogram. Identification of the BHC isomers, both pure, and as they occur in technical BHC, is illustrated in Figures 1 and 2.

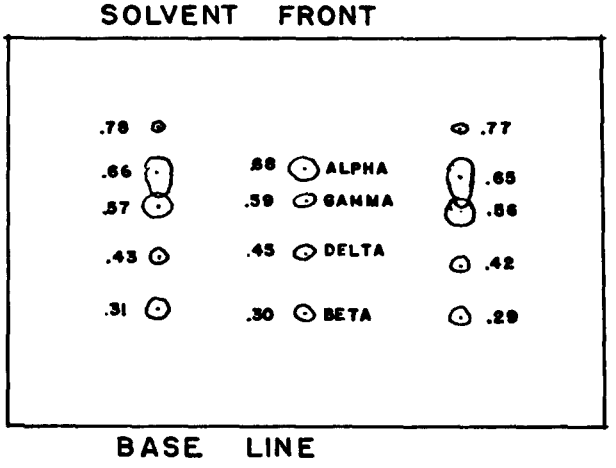

FIG. 2.—Identification of isomers in a commercial sample of BHC

SUMMARY

Mixtures of alpha, beta, gamma, and delta isomers of benzene hexachloride are separated by an application of paper chromatography in which (1) the paper is pre-impregnated by spraying with an ether solution of the stationary phase (acetic anhydride), (2) mixed octanes are employed as the mobile phase, and (3) the chromatogram is developed by dechlorination of the separated isomers with methanolic potassium hydroxide, followed by treatment with silver nitrate and sunlight to fix and reveal the released chloride ion. The writer finds the techniques described to be preferable to those employed for the same purpose by others. As little as ten micrograms of each isomer, including the relatively stable beta isomer, may be clearly separated and distinctly revealed.

ACKNOWLEDGEMENT

The writer sincerely thanks Dr. Wilbur I. Patterson, Chief, Organic Analytical Methods Branch, Division of Food, for advice and encouragement during the course of the work.

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(4) Coal-Tar Color Regulations, Federal Security Agency, S.R.A., F.D.C.3, page 45.
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(6) O'COLLA, P., *J. Sci. Food Agric.*, **3**, 130 (1952).

NOTES

A NEW INDICATOR FOR THE DETECTION OF THE CHLORINATEL PESTICIDES ON THE PAPER CHROMATOGRAM

By Lloyd C. Mitchell (Division of Food, Food and Drug Administration, Federal Security Agency, Washington, D. C.)

The indicator mechanism developed to detect the isomers of benzenehexachloride (*This Journal* **35**, 920 (1952)) in paper chromatograms was based on the conversion of part of the organic chlorine to the inorganic form. The reagents can be used for all halogen compounds which split off at least one halogen atom under the conditions of the test.

Aldrin, isodrin, dieldrin, and endrin do not react in this way, as the chlorine in these substances is very resistant to alkaline dehalogenation. However, when the paper chromatograms of these substances were treated as follows* a test was obtained with 0.01 molar solutions:

(1) Spray with 0.05*N* silver nitrate in 95% ethanol and allow to air dry† for about ½ hour;
(2) Spray with formaldehyde solution (ca 37%) and allow to air dry for ½ hour;
(3) Spray with *N* potassium hydroxide in methanol, air dry for a few minutes to remove excess methanol, and heat in an oven at 130–133° for ½ hour;
(4) Spray with a mixture of concentrated nitric acid and 30% hydrogen peroxide (1+1) and allow to air dry until nitric acid has disappeared (overnight); and
(5) Expose to the sun or bright daylight until the spots are fully developed;

This test is positive on the paper chromatogram for all of the chlorine containing pesticides so far tested; these include the BHC isomers, chlordane, DDT isomers, heptachlor, methoxychlor and toxaphene. All the latter substances were also detected on the chromatogram by the method used for BHC isomers (previous paper).

IDENTIFICATION OF CUMARIC ACID IN ROUGH RICE

By V. E. Munsey, Division of Food, and Charles Graichen, Division of Cosmetics (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.)

In a recent analysis of a sample of converted or parboiled rice for suspected artificial color, the ultraviolet absorption spectrum revealed the presence of cumaric acid. In the absence of information on cumaric acid in rice, four common varieties of rough rice, Pearl and Zenith (short type) and Rexaro and Blue Bonnet (long type) were obtained, through the courtesy of W. B. Smith, U. S. Department of Agriculture. The samples were treated according to the following procedure:

Twenty-five g of rice was extracted by decantation with three 50 ml portions of 0.1 *N* NaOH; the combined extracts were acidified and extracted with 50 ml of ether, which in turn was extracted with 50 ml 0.1 *N* NaOH. This was acidified and re-extracted with ether, which was again extracted with the NaOH. The cycle was repeated once more, and a final extract of 25 ml 0.1 *N* NaOH was used for the spectral analysis.

The extracts from the Rexaro, Blue Bonnet, and Pearl varieties showed the characteristic ultraviolet spectrum of cumaric acid, and a probably related compound

* The operator should apply the reagents to the paper in a well-ventilated hood and wear rubber gloves and goggles.
† The air temperature at the time this test was developed was 30–35°.

was found in the Zenith extract. The extracts gave a strong positive test when treated according to the procedure for coumarin given in *This Journal*, 22, 392 (coupling with diazo-*p*-nitroaniline).

The above method of sample preparation would indicate that cumaric acid appears mainly in the hulls of rough rice; however, small amounts may appear in or be transferred to the endosperm of processed converted rice.

ANNOUNCEMENTS

REFEREE ASSIGNMENTS, CHANGES, AND APPOINTMENTS

CEREAL FOODS

Edward F. Steagall, Food and Drug Administration, Los Angeles 15, Calif., has been reappointed Associate Referee on Starch in Cereal Products.

DECOMPOSITION AND FILTH IN FOODS (CHEMICAL INDICES)

R. A. Baxter, Food and Drug Administration, Los Angeles 15, Calif., has been appointed Associate Referee on Uric Acid in Nuts, to succeed H. M. Bollinger.

EXTRANEOUS MATERIALS IN FOODS AND DRUGS

O'Dean Kurtz, Food and Drug Administration, Washington 25, D. C., has been appointed Associate Referee on Methods for the Identification of Insect Contaminants.

PRESERVATIVES AND ARTIFICIAL SWEETENERS

L. L. Ramsey, Food and Drug Administration, Washington 25, D.C., has been appointed Associate Referee on Dehydroacetic Acid.

BOOK REVIEWS

The Lemon Fruit, Its Composition, Physiology and Products. By ELBERT T. BARTHOLOMEW and WALTON B. SINCLAIR, University of California Press, Berkeley 4, California, 1952, 163 pp. Price $4.50.

The authors have brought together an up-to-date summary of published and hitherto unpublished investigations on the domestic lemon fruit, with some comparisons with lemons produced in other countries and with other species of citrus fruits. The book is not intended to contain a complete discussion of the origin, distribution, varieties, and similar information concerning the lemon previously published by Webber and Batchelder (Vol. I (1943); Vol. II (1948), Univ. Calif. Press), or to take the place of books such as those by Guenther, Parry, or Gildemeister and Hoffmann. The results of the research summarized in the book should be of interest to growers, processors, shippers, and consumers, as well as food chemists, food technologists, and other research workers who may be interested in this important fruit.

The first chapter contains general information. It deals briefly (12 pages) with the origin and history, distribution, and production, variety, harvest, maturity, storage, structure, size, and the chemical changes which occur in lemons during growth and maturation. Chapter II (123 pages) deals with the composition and physiology of the lemon. The authors draw information from a number of original publications and investigations. The composition of whole lemons, the peel, albedo, and juice are discussed under the headings specific gravity, oil, juice, acids, pH, soluble solids, sugars, pectin, nitrogen, proteins, enzymes, vitamins and inorganic constituents. Sections of the chapter are devoted to a discussion of color in peel, lipids, hesperidin, limonin, and nomilin, the effect of storage on juice quantity, respiration, buffer properties, polysaccharides, pentosans, cellulose and hemicellulose, glutathione, and seeds. Chapter III deals briefly (8 pages) with products of the lemon, under the subtitles of whole fruit, peel, juice, and seeds.

A bibliography of 12 pages refers to the original literature from which the material in the text is largely drawn. It is interesting to contrast a reference to an article published in 1771 with articles published as recently as 1950. The 27 tables and 20 figures in the text are of real assistance to the reader and help to summarize the information presented.

Bartholemew and Sinclair have made an excellent contribution to the scientific literature relating to the composition and physiology of the lemon fruit.

R. A. OSBORN

Solubilities of Inorganic and Organic Compounds. Supplement to the Third Edition. By ATHERTON SEIDELL and WILLIAM F. LINKE. D. Van Nostrand Co., New York, N. Y. 1952. 1254 pp. Price $12.50.

Seidell's "Solubilities" has often supplied the basic data for a new method, or has led to the solution of problems that arise in the analysis of complex samples which often come the way of the regulatory chemist. This 1254-page supplement covers the ten year period 1939 to 1949, subsequent to the publication of the third edition. It picks up data not previously published in the main part and corrects errors. Therefore, although desired data usually may be found in the previous volumes it is well to routinely check the supplement for corrections or additions.

The book consists of 567 pages of solubilities of inorganic compounds and 251 pages of solubilities of organic compounds. Ternary systems, aqueous and non-aqueous which separate into two liquid layers, are covered in 299 pages by Alfred W. Francis of Socony-Vacuum Oil Co. The preface to this section mentions their value

in "the growing science and art of solvent extraction" but the analytical chemist will promptly use the information in his search for new solvent systems for chromatography. The final 32 pages of text is a review of the theory of solubility by Roger G. Bates of the National Bureau of Standards.

The most notable omissions are data for admittedly recent but highly important compounds in the antitoxidant, insecticide, and antibiotic fields. Data on DDT are presented, but other recent insecticidal chemicals are not mentioned, although the solubilities of the isomers of BHC were published by Slade in *Chemistry and Industry* in 1945. Similarly the field of antibiotics is covered by the single entry of "Penicillin G, Na, K, NH_4 salts in aq. acetone." On the other hand, data are given for some dyes and quaternary ammonium salts (under their chemical names). An interesting entry is "Solubility of 2-naphthylamine and other carcenogenic amines, each separately, in water and in aqueous caffeine solutions." Of the 5 other amines listed, the identical data for two of them is given under the individual entry; two others are omitted entirely from the index and in the fifth case, reference is made in the index to data under different conditions without a cross reference to this entry.

As a source of information on the solubility and collateral data of "classical" compounds this and its companion volumes stand next only to the handbooks in their usefulness in the chemical library.

WILLIAM HORWITZ

The Design and Analysis of Experiments. By OSCAR KEMPTHORNE. John Wiley and Sons, Inc., 440 Fourth Avenue, New York, 16, N. Y. xix+631 pp., $8.50.

This timely book fulfills a great need, because experimental design and the statistical analysis of experiments has become of increasing importance in many fields of science and especially in chemistry where its full impact has only recently been felt. This book, which presupposes a fundamental knowledge of statistics, is an excellent, readable, comprehensive treatise for the advanced worker and contains a thorough explanation of statistical methodology with the inclusion of many formal mathematical proofs. To the experimenter, this book will help to demonstrate that statistical methods are not a "magic black box" for the indiscriminate development of statistical designs, or from which use of statistical data can be made. Instead, it emphasizes the intelligent application and the limitations of statistics.

The author is very lucid and detailed in his explanations of statistical methods. For example, in the section "The 2-Way Classification with Unequal Numbers and No Interaction," he first treats the subject in detail from a formal viewpoint and then, "for the benefit of the less mathematically minded reader," he works through an example with full artificial data. He may use four pages to explain an example which utilizes only rudimentary mathematics. On the basis of the excellence of this book, the reviewers recommend it for both the experimenter and the more advanced student of statistics.

In writing this book the author has supplied a much needed reference book for workers in the field of experimental design and for the statistical analysis of these designs.

C. RICCIUTI
C. O. WILLITS

Field Crop Insects. By F. A. FENTON, Professor of Entomology, Oklahoma A. & M. College. 405 pp., 224 illus., tables, bibliog., Macmillan Co., 60-5th Ave., New York 11, N. Y. 1952, $5.75.

Dr. Fenton, a widely known authority on economic entomology, after long preparation has now made available a comprehensive up-to-date book that covers,

in a practical way, pretty much the entire field of staple crop insect control. It is based on years of teaching and intensive field research. Due to limitations of space, the scope of this notice is limited to consideration of only that portion of the book that would appear to be of greatest interest to readers of this periodical—the 39 pages dealing with insecticides.

It is recognized by the author at the outset that control with insecticides is an ever changing subject, and that there are, almost daily, new and unpredictable changes and trends in this field. But there are also certain elementary considerations that are not permitted to be forgotten, as, for example, that chemical control must be supplemented with other measures, that many injurious species cannot be controlled by insecticides or that such procedure is uneconomical, and that there frequently exists a very narrow margin of safety between toxicity to the given insect and toxicity to the plant, particularly when the factors of relative susceptibility, temperature, and the like enter, as they often do, into a given case.

Since there already exist a number of excellent texts covering these fields, no attempt is made by Dr. Fenton to go into the chemistry of insecticides, nor engineering as it concerns machinery for the application of chemicals. Rather his concern has been to outline the practical use of and results to be expected from these in insect control, with especial emphasis on recent development of the newer remedies—and these from the viewpoint of a dirt-farmer seeking crop protection. Considerable attention is given to various types of insecticide formulations, particularly the toxicity of some of the newer insecticides to man and to plant life, as well as to legal requirements, including the Federal Insecticide, Fungicide and Rodenticide Act of 1947 which governs their manufacture and sale.

Detailed discussion obviously would be given in a work of this kind to petroleum oils, chlorinated hydrocarbons, organic phosphates, dinitro compounds, fumigants and repellents. Considerable space is devoted to DDT, DDD or TDE, Chlordane, Aldrin, Dieldrin, Lindane, Toxaphene, Benzene Hexachloride, Parathion, Methyl Bromide, and Chloropicrin. In the section on application of insecticides, comparative data are presented on costs and results of various common methods of application, notably, aerosols, dust, dilute and concentrated sprays, also on thoroughness of application, selection of the right insecticides, formulation and dosage, auxiliary spray materials, compatibility, bait application, and on fumigation and use of gas masks. His treatment of the subject of crop sprayers and dusters includes much valuable and highly practical information relative to best types of sprayers for given purposes, notably, engine powered, row-crop, low volume, and mist and speed sprayers, and best results to be expected from the use of various types of nozzles. The discussion of different kinds of crop dusters include the sifter, plunger, bellows, crank, traction, and power operated dusters. The use of airplane dusters, spraying by plane, and use of airplanes and helicopters in scattering baits and in spraying and dusting, and the respective advantages and limitations of these are indicated.

The other sections of the book, not here discussed, appear to be fully comparable in scope and value to the one on insecticides. It is believed that the author has brought together in compact form in this little volume an array of information that will be of lasting usefulness.

J. S. WADE

Forest Entomology. By SAMUEL ALEXANDER GRAHAM, Professor of Economic Zoology, School of Natural Resources, University of Michigan. American Forestry Series. Third edition. McGraw-Hill Book Co., 330 W. 42nd St., New York, N. Y., 334 pp., 85 illus., Bibliog., Index. 1952, $6.00.

It has been an interesting and revealing experience to make a page by page com-

parative check of the insecticide sections in the second edition of this book, issued in 1939, with the present third edition of 1952. The enormous advances made in recent years in use of the newer insecticides in the control of forest insects is much more keenly realized when a revision such as this appears. The changes have been so great that in order to bring the facts to date it has become necessary for the author to rewrite entirely that portion of the work. As would be expected, much attention had to be given to present investigations of various formulations of DDT, benzenehexachloride, toxaphene, chlorinated camphene, chlordane and the like. Then too, the advent of these highly toxic chemicals has brought about marked changes in methods of application, particularly from the air. Instead of dusting, as formerly, with dry powders, the new materials are more often used in oil solutions or as emulsions. It is now possible to conduct large scale control operations that would have presented insurmountable difficulties in 1929, when the first edition of this book appeared, and scarcely less so in 1939, when the second edition was published. New chapters also have been added describing latest methods of making forest insect surveys and latest procedures in detecting and appraising damage. The remainder of the book maintains the same high standard of scholarship as the previous editions. In addition to Introduction and Historical Review, there are discussions of such topics as: Detection and Appraisal; Reproductive Potential; Environmental Resistance; Population Levels; Direct Control of Tree Insects; Direct Control by Chemical Methods; Insecticides and Their Effects; Methods of Indirect Control; Indirect Control by Parasites and Predators; Indirect Control by Silvicultural Practices; Leaf-eating Insects; Sap-sucking Insects; Meristematic Insects; Phloem Insects; Phloem-wood Insects; Wood Destroyers and the like. Principles and technique and practical and ecological aspects of the subject have been particularly emphasized, and the presentation adds definite value to the latest edition of this well known and highly useful work.

J. S. WADE

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