ANALYTICAL APPLICATIONS OF THE HYDROLYSIS KINETICS OF SOME ORGANO-PHOSPHORUS INSECTICIDES*

By P. R. CARTER

A study of the hydrolysis kinetics of some organo-phosphorus insecticides, particularly parathion and paraoxon, can provide an approximate method of analysis for such mixtures. The mathematical treatment and experimental details are discussed.

The analysis of organic phosphorus insecticides is often closely associated with the hydrolysis of these compounds and it is proposed to discuss the application of results on the kinetics of hydrolysis of organo-phosphorus esters to the determination of the purity of phosphorus-containing insecticides, particularly parathion. The two usual impurities in this material are the *S*-ethyl isomer, $EtO{\cdot}SEt{\cdot}PO{\cdot}O{\cdot}C_6H_4{\cdot}NO_2$ (*p*), and paraoxon $(EtO)_2PO{\cdot}O{\cdot}C_6H_4{\cdot}NO_2$ (*p*). The *S*-ethyl isomer results from the action of heat on parathion[1–3] and paraoxon may be formed by reaction of small amounts of phosphoryl chloride present in the thiophosphoryl chloride used for the manufacture of parathion.

An application of hydrolysis kinetics to analysis is shown in the method of Hartley & co-workers[4, 4a] for the analysis of commercial schradan, octamethylpyrophosphoramide. Kinetic studies of the hydrolysis of related compounds have been made by Heath & Casapieri,[5] and the method of analysis is based on the different rates of alkaline hydrolysis and partition ratios of the components. Hydrolyses are conducted under different conditions and the proportions of the various components calculated. The actual determination made is that of dimethylamine, isolated by steam distillation and titrated, or that of phosphorus, measured colorimetrically by formation of molybdenum blue.

There are many examples where a determination of the actual rate of hydrolysis of a pure compound has been used as a means of identification—indeed Hartley has referred to the half-life of plant metabolites of schradan—and the question of the application of this principle to mixtures is now discussed.

In dealing with the hydrolysis of parathion and its impurities, only alkaline conditions, in which *p*-nitrophenoxide ion is produced, will be considered; resulting dialkyl phosphate or thiophosphate is hydrolysed very slowly and does not interfere with the colorimetric determination of the liberated *p*-nitrophenol. The nitrophenol group is hydrolysed off almost exclusively and the reaction can be followed colorimetrically to at least 90% of complete hydrolysis. This hydrolysis with alkali is probably bimolecular,[6] but in the presence of a large excess of alkali the kinetics are first-order, and the velocity constant, k, is given by

$$k = \frac{1}{t} \log \frac{a}{a - x}$$

where a is the initial concentration of parathion and x is the amount hydrolysed in time t.

A known weight of the parathion is hydrolysed in solution in standard sodium hydroxide solution (the solubility is about 20 p.p.m.) at constant temperature, and the intensity of the yellow colour due to the sodium *p*-nitrophenoxide formed is compared, at timed intervals, with that from a known concentration of *p*-nitrophenol. A simple colorimetric measurement with Nessler cylinders is accurate to within $\pm$ 4%, and the method could readily be adapted for use with a colorimeter if greater accuracy were required.

Hydrolysis measurements have been made by Mr. D. R. Peck, and some typical results

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Table I

Hydrolysis of parathion

Time, min.	k/[OH], l. min.$^{-1}$ mol.$^{-1}$
10	0·050
30	0·051
60	0·052
90	0·052
120	0·049
150	0·049
180	0·050
240	0·048
300	0·052
360	0·052
420	0·053
480	0·052
540	0·051
1500	0·050

are shown in Table I for pure parathion, hydrolysed at 25·1° with 0·025N-sodium hydroxide solution.

The velocity constant k applies to the whole range of hydrolysis from 0 to 84%, as would be expected for a pure sample. (The marked drift in the value of k with time, shown in Table IV, demonstrates the effect of 5% of paraoxon in the parathion, and similar effects may readily be observed with the *S*-ethyl isomer as impurity.)

Table II shows the constancy of k/[OH] over a wide range of [OH], the values recorded being mean values from Table I and other results (not given in this paper).

Table II

Hydrolysis of parathion

[OH]	k/[OH], l. min.$^{-1}$ mol.$^{-1}$	Per cent. hydrolysed
0·0250	0·0508	84
0·0590	0·0497	75
0·1085	0·0502	88
0·2650	0·0490	84
0·5655	0·0493	81

The results obtained agree well with those of other workers, as shown in Table III.

Table III

Hydrolysis of parathion and its dimethyl analogue

	Temp., ° c	k/[OH], l. min.$^{-1}$ mol.$^{-1}$	
		Ketelaar[6]	Peck
Parathion	25·1	0·060	0·050
,,	35·1	0·148	0·154
,,	37·5	0·186	0·186
Dimethyl analogue of parathion	25·1	0·246	0·248

Ketelaar's[6] velocity constants for parathion in Table III are consistent with:

$$\log (k/[\mathrm{OH}]) = 10{\cdot}95 - 16600/2{\cdot}303RT$$

and Peck's with:

$$\log (k/[\mathrm{OH}]) = 13{\cdot}72 - 20520/2{\cdot}303RT$$

Although the actual values for the rate constants in Table III agree well, the discrepancy between these equations is probably due to the small temperature range (25·1–37·5°) used by Peck, compared with that used by Ketelaar (10–75°).

Lee & Kolthoff[7] have described a method for the analysis of mixtures of compounds based on the difference in rates of reaction of the components of the mixture. Application of this method to the hydrolysis of some of these phosphorus esters enables the drift in k to be used as a measure of the impurity present. Consider two compounds A and B that react with a substance R at different rates, and suppose that the reaction of A with R is faster than that of B with R at a given temperature. Mixtures of A and B can be analysed by the following procedure:

A solution is prepared in which the total molar concentration of A and B, and the concentration of R, are known. The reaction is allowed to proceed for a specified time at a given temperature, and then the amount of (A + B) that has reacted is determined. Finally, refer-

ence is made to a graph, and from this value the initial concentrations of A and B can be read off directly. First-order reactions will be considered here, though the method is not limited to these.

The concentration of A remaining at any time t is given by the expression:

$$a = a_0 \exp(-k't)$$

where a is the concentration of A at any time t,
a_0 is the initial concentration of A, and
k' is the specific rate constant for the reaction of A.

A similar expression gives the concentration of B at any time t. The sum of the concentrations of A and B is, therefore, given by the expression:

$$a + b = a_0 \exp(-k't) + b_0 \exp(-k''t) \quad . \quad . \quad . \quad . \quad (1)$$

The calibration curve for the analysis of the mixture is a plot of the percentage of A + B that has reacted after a given period against the initial composition of the mixture. The slope of the calibration curve, and hence the accuracy of the analysis, are dependent on the choice of the reaction period. For every pair of competitive reactions there exists an optimum reaction period. It can be shown that, for first-order reactions,

$$t_{\text{opt.}} = \frac{\log_e (k'/k'')}{k' - k''}$$

and for a pseudo-unimolecular reaction,

$$t_{\text{opt.}} = \frac{\log_e (k'/k'')}{r_0(k' - k'')} \quad . \quad . \quad . \quad . \quad . \quad . \quad (2)$$

where r_0 is the concentration of R.

Calibration curves can be found empirically by analysing mixtures of known composition, or by plotting values of $(a + b)/(a_0 + b_0)$, calculated from equation (1), against values of $a_0/(a_0 + b_0)$. Since k', k'', t and $a_0 + b_0$ are constant in the analysis of mixtures, the calibration curve for first-order reactions is a straight line.

If the optimum reaction period is employed, a simplified method of calculating the calibration curve can be used. Combination of equations (1) and (2) results in the following equations:

$$a/a_0 = [\%\text{ of (A + B) remaining}]_A = \alpha^{1/(1-\alpha)} \quad . \quad . \quad . \quad . \quad (3)$$

$$b/b_0 = [\%\text{ of (A + B) remaining}]_B = \alpha^{\alpha/(1-\alpha)} \quad . \quad . \quad . \quad . \quad (4)$$

in which the subscripts A and B refer to solutions containing 100% of A and B respectively, and α is the ratio of the specific rate constants k''/k' $(k' > k'')$. The calibration curve for first-order reactions can be found by joining with a straight line the points calculated for 100% A [equation (3)] and for 100% B [equation (4)], and is determined solely by the value of α, the ratio of the rate constants.

As an example of this method, a mixture of parathion containing 5% of paraoxon will be considered.

At 21·5° for parathion, $k/[OH] = 0{\cdot}05$ l. min.$^{-1}$ mol.$^{-1}$
,, ,, ,, paraoxon, $k/[OH] = 0{\cdot}526$ l. min.$^{-1}$ mol.$^{-1}$

The hydrolysis is performed at this temperature with excess of 0·1015N-sodium hydroxide solution.

In Table IV, x_1 and x_2 are the quantities of parathion and paraoxon respectively hydrolysed in time t, and k is a composite figure calculated from

$$k = \frac{1}{t} \log \frac{a}{(a - x)}$$

where $x = x_1 + x_2$, and a is the total initial concentration of these esters.

Table IV

Hydrolysis of parathion containing 5% of paraoxon

t, min.	x_1	x_2	k/[OH], l. min.$^{-1}$ mol.$^{-1}$	Per cent. hydrolysis
5	0·02408	0·01172	0·07126	3·58
10	0·04696	0·02069	0·06898	6·77
15	0·06956	0·02755	0·06685	9·71
20	0·09183	0·03281	0·06546	12·46
30	0·13380	0·03993	0·06263	17·37
40	0·17450	0·04409	0·06076	21·86
50	0·21300	0·04654	0·05918	25·95
100	0·3780	0·04976	0·05499	42·78
200	0·60580	0·04999	0·05253	65·58
500	0·87490	0·05000	0·05103	92·49
48·73	0·20810	0·04630		25·44

Substituting in equations (2), (3) and (4),

$$a/a_0 = 7{\cdot}424\%$$
$$b/b_0 = 78{\cdot}10\%$$
$$t_{opt.} = \frac{2{\cdot}303 \log (0{\cdot}526/0{\cdot}05)}{0{\cdot}1015 \times 0{\cdot}476} = 48{\cdot}7 \text{ min.}$$

On calculating the percentage hydrolysed after 48·7 minutes, as in Table IV, we obtain a value of 25·44%. Hence $a + b = 100 - 25{\cdot}44 = 74{\cdot}56\%$.

From the straight-line graph, by using $a/a_0 = 7{\cdot}424$ and $b/b_0 = 78{\cdot}10$, we find that for $a + b = 74{\cdot}56$, the percentage of paraoxon is 5·0%.

Thus, in the experimental analysis of a mixture of parathion and paraoxon, a known weight of the mixture is hydrolysed in a large excess of standard dilute sodium hydroxide solution at a suitable constant temperature for the calculated optimum reaction period, and the amount of *p*-nitrophenol liberated is measured. Hydrolysis of the mixture of parathion and paraoxon is then allowed to proceed to completion and the total amount $a_0 + b_0$, of *p*-nitrophenol liberated is measured. These two determinations of *p*-nitrophenol enable the percentage of *p*-nitrophenoxide esters hydrolysed during the optimum reaction period to be calculated, and hence $a + b$. Reference to the appropriate calibration curve gives the composition of the mixture.

The same method can be used for the determination of the dimethyl analogue of parathion in parathion.

An alternative application of the drift in the hydrolysis constant of impure parathion, is to give an indication of the nature of any contaminating *p*-nitrophenyl esters. If a plot of k against time is examined it is seen that towards the end of the hydrolysis k becomes substantially constant. This value of k can be taken as that of the hydrolysis of the more stable compound, in this case presumably parathion. By using this value of k, the amount of *p*-nitrophenol contributed at any time by the parathion can be found, and hence a simple calculation will give the rate of hydrolysis of the impurity. Brown & Fletcher[8, 9] describe an example of this method, which may be extended to the detection, and sometimes determination, of more than one impurity.

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References

[1] Bright, N. F. H., Cuthill, J. C., & Woodbury, N. H., *J. Sci. Fd Agric.*, 1950, **1**, 344
[2] Schrader, G., BIOS Report, No. 1808, p. 10
[3] Topley, B., *Chem. & Ind.*, 1950, p. S859
[4] Hartley, G. S., Heath, D. F., Hulme, J. M., Pound, D. W., & Whittaker, Mary, *J. Sci. Fd Agric.*, 1951, **2**, 303
[4a] David, A., Hartley, G. S., Heath, D. F., & Pound, D. W., *J. Sci. Fd Agric.*, 1951, **2**, 310
[5] Heath, D. F., & Casapieri, P., *Trans. Faraday Soc.*, 1951, **47**, 1093
[6] Ketelaar, J. A. A., *Rec. Trav. chim. Pays-bas*, 1950, **69**, 649
[7] Lee, T. S., & Kolthoff, I. M., *Ann. N.Y. Acad. Sci.*, 1951, **53**, 1093
[8] Brown, H. C., & Fletcher, R. S., *J. Amer. chem. Soc.*, 1949, **71**, 1851
[9] Brown, H. C., & Fletcher, R. S., cited by Frost, A. A., & Pearson, R. G., 'Kinetics & Mechanism', 1953, p. 149 (New York: Wiley & Sons Inc.)

THE COMPONENT ACIDS AND GLYCERIDES OF ARECA-NUT (*ARECA CATECHU*) FAT

By S. P. PATHAK and S. S. MATHUR

The fatty acid composition of areca-nut fat was determined by the usual ester-fractionation method, using an electrically heated and packed column for fractional distillation under high vacuum (0·2 mm.). The glyceride structure was studied by crystallization of the neutral fat from acetone and ether; the composition of each of these glyceride fractions was studied by the fractionation method and the final possible glyceride composition computed therefrom. The chief component acids are lauric (19·5%), myristic (46·2%) and palmitic (12·7%), and in the unsaturated portion oleic (6·2%), linoleic (5·4%) and hexadecenoic acid (7·2%). Minor proportions of stearic, decanoic and of unsaturated monoethylenic C_{12} and C_{14} acids are also present. The chief component glycerides are (i) 56% of fully saturated (trimyristin, dimyristins and lauromyristopalmitin); (ii) 30% of mono-unsaturated-disaturated (mainly hexadecenolauromyristin, with some oleo-(linoleo)myristopalmitins and dimyristins); (iii) 14% of diunsaturated-monosaturated (oleolinoleoglycerides, mostly oleolinoleopalmitin). The glyceride composition follows closely Hilditch's rule of widest distribution of acyl radicals in the glyceride molecules. The fully saturated glyceride content of the fat, determined separately by the method of Hilditch & Lea,[1] is 53·7%. The fully saturated components are found to contain (by the fractionation method): 19·4% of lauric, 54·6% of myristic, 19·2% of palmitic and 6·8% of stearic acids. The proportions of various acids in the fully saturated components are similar to the corresponding ones in the saturated acid portion of the whole fat.

Introduction

Rathje[2] studied a sample of fat from areca palm nuts of the Pacific Islands. At the time of his investigations, the modern methods were not well developed and Longenecker's fractionating column[3] was unknown. Recently a sample of areca-nut fat was extracted and examined by the authors in this Laboratory. The nuts were of areca palm (*Areca catechu*, a species of the Palmae family) grown in Bengal (India). The fatty acid composition of the fat and the probable nature of its component glycerides were studied and are recorded here.

Results

The crushed nuts (2010 g.), on extraction several times with hot acetone, yielded the fat, which was separated from aqueous extract by light petroleum. Finally 356 g. of the fat was recovered. The fat was rendered neutral by washing its ethereal solution with dilute aqueous alkali.

A solid, pale fat was thus obtained. It had the following characteristics: acid value 0·3, saponification equiv. 246·0, iodine value (I.V.) (Hanus) 27·0, unsaponifiable matter 0·6%.

Experimental

Determination of component acids

The mixed acids (I.V. 27·8), obtained by hydrolysis in the usual way of a portion of the neutral fat (97·0 g.), were separated into two groups by the lead salt–alcohol method (Table I).

Table I

Fractional crystallization of the mixed acids of areca-nut fat

Fraction	Description	Weight		I.V.
		g.	%	
A	Lead salts insoluble in alcohol	61·3	66·1	2·5
B	Lead salts soluble in alcohol	31·4	33·9	75·6

Each of these two groups of acids was separately converted into methyl esters, which were fractionally distilled through an electrically heated and packed column. The ester-fractionation results are summarized in Table II.

From the results in Tables I and II the composition of the mixed acids present in the fat was calculated[4] and is given in Table III.

Table II

Fractionation results for methyl esters of the acids of areca-nut fat

No.	Wt., g.	Saponification equiv.	I.V.
	Methyl esters of A acids		
A1	3·00	219·1	0·9
A2	3·55	231·9	0·8
A3	3·46	239·2	0·2
A4	3·82	242·5	0·2
A5	3·36	243·8	0·3
A6	3·88	244·1	0·3
A7	3·63	245·9	0·4
A8	4·21	248·4	0·6
A9	4·09	259·1	1·7
A10	4·20	279·4*	15·9
	Methyl esters of B acids		
B1	3·25	211·9	2·8
B2	3·36	215·4	1·3
B3	3·47	223·1	4·1
B4	3·37	264·3	64·6
B5	3·64	283·7	109·9
B6	3·26	284·2	112·8
B7	3·20	284·7	117·3
B8	2·25	289·0	122·0
B9	2·61	323·4*	106·1

* Equivalents of esters (freed from unsaponifiable matter): A10, 278·4; B9, 296·1

Determination of the glyceride structure

The neutral fat (187·7 g.) was crystallized from acetone (10 c.c./g.) at 15°, when 86·3 g. of glycerides (I.V. 5·1) was deposited. These were crystallized again from ether (10 c.c./g.) at 0°, when 56·9 g. of glycerides (I.V. 0·8) separated and formed the fraction A, while 29·4 g. of the glycerides (I.V. 12·8) remained in solution. The soluble portion from the first crystallization (101·4 g., I.V. 46·3) was next crystallized at 0° from acetone at the same dilution, when 32·6 g. of glycerides (I.V. 4·5) crystallized out and the portion still left in solution (68·8 g., I.V. 66·7) formed fraction C of the triglycerides.

Table III

Component acids of the various fractions and of the whole fat

Acids	A (66·1%)	B (33·9%)	Total	Excluding unsaponifiable matter	
				% (wt.)	% (mol.)
Decanoic	—	0·2	0·2	0·2	0·3
Lauric	6·8	9·7	16·5	16·6	19·5
Myristic	42·9	1·9	44·8	44·9	46·2
Palmitic	12·7	1·1	13·8	13·8	12·7
Stearic	1·9	—	1·9	2·0	1·6
Dodecenoic	0·1	0·2	0·3	0·3	0·3
Tetradecenoic	0·2	0·4	0·6	0·6	0·6
Hexadecenoic	0·3	7·5	7·8	7·8	7·2
Oleic	1·2	6·2	7·4	7·4	6·2
Linoleic	—	6·4	6·4	6·4	5·4
Unsaponifiable	Trace	0·3	0·3	—	—

Further resolution of the insoluble portion from the last crystallization (32·6 g., I.V. 4·5) and of the soluble portion of the ether crystallization (29·4 g., I.V. 12·8) was attempted at lower temperatures (— 10°), but no marked separation was obtained. The two fractions were combined and this portion of glycerides (62·0 g., I.V. 8·3) is termed fraction B. The fat was thus resolved into three simpler mixtures of glycerides, as shown by the summary in Table IV.

Component acids of the glyceride groups A, B and C

The fractions were hydrolysed and the respective mixed acids were recovered. The acids from fraction B were submitted to lead salt–alcohol separation and fractions BA and BB were obtained:

		Wt., g.	%	I.V.
BA	Lead salts, alcohol-insoluble	29·9	60·8	1·6
BB	,, ,, , alcohol-soluble	19·3	39·2	19·7

Mixed acids from glyceride fraction C were similarly resolved into two simpler fractions CA and CB:

		Wt., g.	%	I.V.
CA	Lead salts, alcohol-insoluble	16·9	31·1	4·5
CB	,, ,, , alcohol-soluble	37·5	68·9	78·8

The several mixed acid fractions (A, BA, BB, CA and CB) were separately converted into methyl esters, which were distilled through the fractionating column as already mentioned. Ester-fractionation results are not recorded, but the component acids of each glyceride fraction

Table IV

Glyceride fractions of areca-nut fat from acetone and ether

Fraction	Description	Wt., g.	%	I.V.
A	Insoluble at 0° (10 × E)	56·9	30·3	0·8
B	Soluble at 0° (10 × E) and insoluble at 0° (10 × A)	62·0	33·0	8·3
C	Soluble at 0° (10 × A)	68·8	36·7	66·7

Note: 10 × E indicates 10 c.c. of ether/g.
10 × A ,, 10 c.c. of acetone/g.

are summarized in Table V, and it is noteworthy that the totals from the fatty acid compositions of these glyceride fractions (Table V) show good agreement with those obtained by analysis of the mixed acids from the whole fat (Table III).

After grouping the acids, as in Table VI, the possible combinations of these groups of acids, and thus the possible component glycerides of the fat, were calculated on the theory of computation given by Hilditch.[4]

Determination of the fully saturated glyceride content and its component acids

The neutral fat (39·0 g.) was submitted to the acetone–permanganate oxidation method of Hilditch & Lea[1] and 21·6 g. of the fully saturated glycerides finally recovered (55·4%). Characteristics of these fully saturated glycerides were: I.V. 0·3, acid value 2·6, per cent. of neutral fully saturated glycerides 53·7.

The glycerides were hydrolysed and the mixed acids esterified and fractionated by the usual method. The fractionation results are not given. The final composition is recorded in Table VII.

Discussion

Component acids.—The major component saturated acids of areca-nut fat are lauric (19·5%), myristic (46·2%) and palmitic (12·7%), and minor amounts of stearic (1·6%) and of decanoic

Table V

Areca-nut fat fractions from acetone and ether and their component acids, % (mol.)

Component acids	A (30·9%)	B (34·1%)	C (35·0%)	Total
Decanoic	—	0·2	0·2	0·4
Lauric	3·8	9·1	6·2	19·1
Myristic	22·2	16·4	7·8	46·4
Palmitic	4·3	5·5	3·9	13·7
Stearic	0·3	0·3	0·7	1·3
Dodecenoic	Trace	0·1	0·1	0·2
Tetradecenoic	0·1	0·2	0·3	0·6
Hexadecenoic	0·2	1·8	5·4	7·4
Oleic	Trace	0·5	5·2	5·7
Linoleic	—	—	5·2	5·2

Table VI

	Component glycerides A	B	C	Whole fat
Component acid groups, % (mol.)				
Lauric (and decanoic)	3·8	9·3	6·4	19·5
Myristic	22·2	16·4	7·8	46·4
Palmitic (and stearic)	4·6	5·8	4·6	15·0
Hexadecenoic (and dodecenoic and tetradecenoic)	0·3	2·1	5·8	8·2
Oleic	Trace	0·5	5·2	5·7
Linoleic	—	—	5·2	5·2
Component glyceride groups, % (mol.)				
(*a*) Fully saturated	29·9	26·4	—	56·3
Mono-unsaturated-disaturated	1·0	7·7	21·3	30·0
Diunsaturated-monosaturated	—	—	13·7	13·7
(*b*) Trimyristin	4·8	—	—	4·8
Dimyristomono-(others)	26·1	15·2	—	41·3
Myristodi-(others)	—	18·9	23·2	42·1
Tri-(others)	—	—	11·8	11·8
(*c*) Oleodi-(others)	0·1	1·5	15·7	17·3
Tri-(others)	30·8	32·6	19·3	82·7
(*d*) Linoleodi-(others)	—	—	15·7	15·7
Tri-(others)	30·9	34·1	19·3	84·3
Possible component glycerides, % (mol.)				
Fully saturated (56·3%)				
Trimyristin	4·8	—	—	4·8
Dimyristolaurin	11·3	10·5	—	21·8
Dimyristopalmitin	13·8	4·7	—	18·5
Lauromyristopalmitin	—	11·2	—	11·2
Mono-unsaturated-disaturated (30·0%)				
Hexadecenolauromyristin	—	6·2	17·3	23·5
Hexadecenodimyristin	0·9	—	—	0·9
Oleodimyristin	0·1	—	—	0·1
Oleomyristopalmitin	—	1·5	2·0	3·5
Linoleomyristopalmitin	—	—	2·0	2·0
Diunsaturated-monosaturated (13·7%)				
Oleolinoleolaurin	—	—	1·9	1·9
Oleolinoleomyristin	—	—	1·9	1·9
Oleolinoleopalmitin	—	—	9·9	9·9

acid (0·3%) also occur. Among the unsaturated acids, oleic and linoleic acids are present (6·2% and 5·4% respectively) with traces of dodecenoic and tetradecenoic acids (0·3% and 0·6%) and significant proportions of hexadecenoic acid (7·2%). Myristic acid is thus predominant in the fat. Unsaturated C_{10}, C_{12} and C_{14} monoethenoid acids, though not of common occurrence in seed fats in general are, however, known to occur in small quantities in seed fats of certain sub-tropical plants, for instance those belonging to the Lauraceae family.

The only other study of this fat seems to be that of Rathje,[2] in which he has determined the fatty acid composition of the fat from areca palm nuts of the Pacific Islands. He found it to contain 71·0% of saturated acids and 29·0% of unsaturated (oleic) acid; lauric acid predominated (43·6% of the total) in the saturated portion. The composition of fat reported by Rathje thus differs from that of the fat obtained from the Indian variety of areca nuts

Table VII

Component acids of the fully saturated glycerides of areca-nut fat

	Wt.-%	Moles-%
Lauric	16·8	19·4
Myristic	53·7	54·6
Palmitic	21·2	19·2
Stearic	8·3	6·8

(*Areca catechu*) studied by the authors. It may be pointed out that, at the time Rathje carried out his investigations, the present method of fractional distillation of the esters of mixed fatty acids under high vacuum was not well developed and the modern electrically heated and packed fractionating column was unknown. He probably had to base his conclusions of the nature and the proportions of various saturated acids by melting-point determination of the fatty acid fractions obtained by repeated crystallizations from different solvents, or by some similar method, which will lead to erroneous results.

Component glycerides.—Areca-nut fat contains 56% of fully saturated, 30% of mono-unsaturated-disaturated and 14% of diunsaturated-monosaturated glycerides. The fully saturated components include trimyristin (4·8%), dimyristo-laurin and -palmitin (21·8% and 18·5% respectively) and 11·2% of lauromyristopalmitin. The mono-unsaturated glycerides are mainly hexadecenolauromyristin (23·5%) with some oleo- and linoleo-myristopalmitins (3·5% and 2·0%), and traces of hexadeceno- and oleo-dimyristins (0·9% and 0·1% respectively). The diunsaturated group is comprised of oleolinoleoglycerides (oleolinoleo-laurin 1·9%, -myristin 1·9% and -palmitin 9·9%).

Areca-nut fat thus follows closely the rule of even distribution. Only myristic acid, which is the predominant fatty acid in this fat, occurs twice in an appreciable number of glyceride molecules and three times in a few. The rest of the acyl groups occur only once in some of the glyceride molecules, and the number of such molecules depends on the actual proportion of the particular acid in the total mixed acids.

In the other fats of this family (Palmae) that have been examined for their glyceride structure (e.g. coconut, palm kernel, babassu, gru-gru, tucuma and turluru kernels) and have been found to follow the rule of widest distribution, it was observed that the content of fully saturated glycerides is in each case of an order that leaves the ratio of saturated to unsaturated acids in the remaining part of the fat (i.e. in the mixed saturated-unsaturated glycerides) at approximately 1·4 : 1 (Hilditch[4]). For the same 'association ratio' of 1·4 : 1 for areca-nut fat, the proportions of glyceride groups are very similar to those actually observed.

The actual fully saturated glyceride content of areca-nut fat, determined by the method of Hilditch & Lea,[1] was found to be 53·7%, and that obtained in the glyceride structure determination (Table VI) is 56·3%. The two values thus compare well. Fully saturated glycerides contain 19·4% of lauric, 54·6% of myristic, 19·2% of palmitic and 6·8% of stearic acids. Proportions of the various acids in the saturated acid portion of the whole fat are decanoic 0·4, lauric 24·3, myristic 57·5, palmitic 15·8 and stearic acid 2·0%. This similarity in the proportions of saturated acids in the fats as a whole and in their fully saturated components has been observed in a number of fats (e.g. coconut, palm kernel, nutmeg butter, dika fats), in which unsaturated acids are a very minor component, and in which, consequently, the fully saturated glycerides form a very large proportion of the fat. This is confirmed by the present study.

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References

[1] Hilditch, T. P., & Lea, C. H., *J. chem. Soc.*, 1927, p. 3106

[2] Rathje, A., *Arch. Pharm., Berl.*, 1908, **246**, 702; cf. Hilditch, T. P., 'The Chemical Constitution of Natural Fats', 1947, 2nd edn., p. 202 (London: Chapman & Hall Ltd.)

[3] Longenecker, H. E., *J. Soc. chem. Ind., Lond.*, 1937, **56**, 119T

[4] Hilditch, T. P., 'The Chemical Constitution of Natural Fats', 1947, pp. 235, 505

THE SMELL PROBLEM IN HERRING-MEAL FACTORIES

By J. A. LOVERN and JUNE OLLEY

Herring press-cake was dried under both laboratory and commercial conditions and effluent air from the drier passed through acid or permanganate solutions. Production of volatile base per unit of water evaporated varied with the drying rate, but production of total volatile reducing substances, probably a truer index of smell production, was apparently independent of rate and method of drying.

The manufacture of fish meal is subject to the Offensive Trades Act. For white fish meal (non-oily raw material), the type of drier in general use[1] permits effective deodorization of the effluent gases, but the driers normally used in herring-meal production discharge such large volumes of air that effective deodorization would be difficult and is seldom practised. Lack of information on the nature and amount of offensive material produced in the manufacture of herring meal hampers the administration of the Offensive Trades Act in herring-meal and -oil factories.

Virtually the only published information on the effluent gases from fish-meal driers is that of Ronald,[2] and this relates only to white fish meal. Ronald found that trimethylamine and similar bases were quantitatively the most important constituents, but that minor amounts of some water-insoluble gas were responsible for a very unpleasant fraction of the smell. This latter material could be destroyed by oxidation, e.g. chlorination or incineration.

Experiments have now been made with herring press-cake, dried both in the laboratory and in a commercial pneumatic (air-lift) drier. In the laboratory tests air was drawn over the press-cake (500 g.), heated in a metal container placed in an oven. Effluent air was passed through a chain of wash bottles and then through a gas meter calibrated in litres. In the factory tests effluent air was withdrawn through a muslin screen, a chain of wash bottles and a gas meter. The performance of the drier was measured and it was calculated that approximately 22,000 cu. ft./min. of effluent air was discharged and 68 lb./min. of water evaporated.

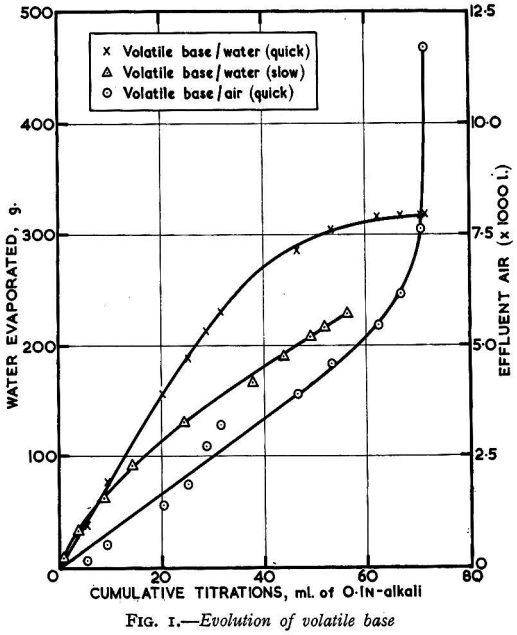

FIG. 1.—*Evolution of volatile base*

Despite Ronald's emphasis on trimethylamine, he noted that the odour was virtually unchanged after absorption in acid. It was, however, largely water-soluble. The same has been found to hold for herring-meal-drier effluent. The smell is almost completely destroyed by alkaline permanganate, which leaves a very faint smell resembling that of acetaldehyde. Alkaline permanganate has been employed for measuring the odour of staling fish.[3, 4] We used both acid (0·1N) and permanganate (0·1N or 0·5N in N-alkali) in various experiments. The temperature of the oven was maintained at 135° during the evaporation of the first two-thirds of the water in the press-cake, and was then reduced to 95° to avoid the possibility of scorching. Rate of drying, observed by loss of weight of the container and contents, was dependent largely on rate of air flow under our laboratory conditions, and in a 'slow'-drying series the rate of drying was about half that in the 'quick' series. The slow experiments were not continued to complete dryness of the meal, nor were air volumes plotted. The results obtained in the laboratory tests are given in Fig. 1 for volatile base, and in Fig. 2 for volatile reducing substances (V.R.S.), in which titration values of the material in the wash bottles are plotted both against water evaporated and against volume of effluent air. Alkaline permanganate was acidified and potassium iodide added for titration with thiosulphate.

It can be seen from Fig. 1 that volatile base is evolved over the whole drying period, and to a less extent per unit of water evaporated at a higher drying rate. A certain amount of base is evolved after drying is complete, but the process terminates soon after this. It is unlikely that the volatile base is produced by decomposition during drying, since steam distillation of the press-cake yields far more volatile base (titration > 145 ml.) than is evolved in the drying tests.

The results on V.R.S. are probably more relevant to smell production than those on volatile base. Fig. 2 shows that, in contrast with the volatile base, V.R.S. production is independent of drying rate over the range investigated. Moreover it continues long after drying is complete, and had not ceased when the experiments were discontinued. This accords with the fact that dry herring meal has an odour. Again, steam distillation of press-cake gave a value for V.R.S. (> 250 ml.) far in excess of that obtained in the drying experiments, and there is no reason to suspect production by thermal decomposition. It may also be noted that in neither Fig. 1 nor Fig. 2 is there any abrupt change in the curves when the temperature was lowered from 135° to 95° (at approx. 200 g. of water evaporated).

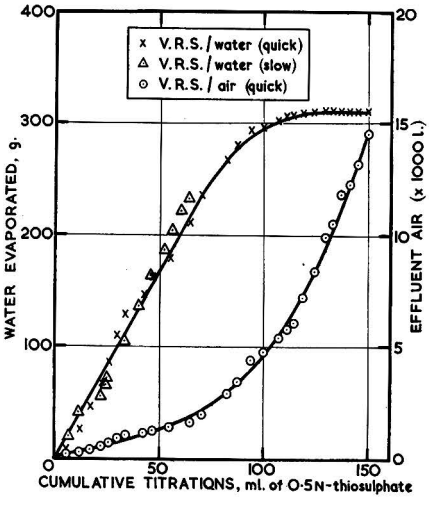

FIG. 2.—*Evolution of volatile reducing substances*

In Table I the results obtained on the air-lift drier are compared with those in Fig. 2. For this purpose the water evaporated during each sampling of effluent gas was calculated for the volume of gas withdrawn, and the titration value for such evaporation calculated from Fig. 2, using the total titration for evaporation of 300 g. of water as a basis, since this corresponds roughly to the degree of drying in commercial operations (10% of moisture in the meal). This gives the calculated value in Table I. The actual titrations obtained at the factory show general agreement, and any disagreement shows the air-lift drier as producing more V.R.S. per unit of water evaporated than the laboratory drier, a result possibly associated with some scorching. The quality of the press-cake was similar in all experiments.

The general conclusion is that odour production per unit of water evaporated from herring press-cake is probably largely independent of rate and method of drying, assuming that no scorching occurs.

Table I

Comparison of laboratory and commercial driers in production of volatile reducing substances per unit of water evaporated

Expt. No.	Vol. of air, l.	Water evapd., g.	Titration, ml. of 0·5N-thiosulphate	
			Observed	Calculated from Fig. 2
1	325	15·9	5·5	5·6
2	84	4·1	3·5	1·4
3	78	3·8	2·4	1·3
4	103	5·0	1·7	1·7
5	83	4·1	1·6	1·4

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References

[1] Lovern, J. A., *Fish. News, Lond.*, 1951, No. 1970, 10
[2] Ronald, D., ' Handbook on Offensive Trades ', 1935 (Edinburgh: Hodge and Co. Ltd.)
[3] Lang, O. W., Farber, L., Yerman, F., & Beck, C., *Industr. Engng Chem.* (*Anal.*), 1945, **16**, 490
[4] Farber, L., & Cederquist, A., *Food Tech., Champaign*, 1953, **7**, 478

THE SWELLING EFFECT OF POLYPHOSPHATES ON LEAN MEAT

By J. R. BENDALL

(1) Orthophosphate, pyrophosphate, Calgon and Phosphate Glass all increase the ' uncooked volume ' of minced rabbit muscle by 10–20%, when added at an over-all concentration of 0·5% to a mixture of equal parts of meat and water. On cooking, the volume falls to between 65 and 75% of the volume of fresh meat. These effects are about the same as that of sodium chloride at an over-all concentration of 1%.

(2) When sodium chloride is added to a final concentration of 1%, the uncooked volume of the mince is increased by about 30% in the presence of 0·5% of orthophosphate, Calgon, Phosphate Glass or metaphosphate, but by at least 55% in the presence of 0·5% of disodium dihydrogen pyrophosphate (anhydrous). The ' cooked volume ' in 1% sodium chloride solution only is about 75% of the original volume of fresh meat. This is increased to about 80% in the presence of the phosphate compounds, except pyrophosphate, when it is increased to at least 95%.

(3) Detailed study of the effect of 0·25% and 0·5% of pyrophosphate in 1% sodium chloride solution (over-all concentrations) gives the following average results for the increase in uncooked volume: 1% sodium chloride solution 20 ± 6·0%; 1% sodium chloride solution/0·25% pyrophosphate, 51 ± 14%; 1% sodium chloride solution/0·5% pyrophosphate, 64 ± 14%. The cooked volumes, as a percentage of the volume of fresh meat, were: 1% sodium chloride solution, 71 ± 4·0%; 1% sodium-chloride solution/0·25% pyrophosphate, 89 ± 8·0%; 1% sodium chloride solution/0·5% pyrophosphate, 99 ± 6·0%.

(4) Pyrophosphate exerts an effect in 1% sodium chloride solution at least twice as great as that of sodium chloride of corresponding ionic strength. The effect is therefore specific, and it is suggested that it consists in the splitting of the contractile protein, actomyosin, into its components and the partial conversion of these from the gel into the sol form.

(5) Addition of Ca or Mg ions at the concentrations found in tap-water does not significantly affect the action of pyrophosphate.

The use of polyphosphates as a means of increasing the water-holding power of sausage-meat has been extensively investigated in Germany,[1] where the use of water-retaining fillers,

such as rusk, is forbidden. It has been shown that potassium ' metaphosphate ', in particular, brings about marked swelling when extra water is added to the meat, although the effect decreases as the amount of added water increases. Some of the extra water is retained when the meat is cooked, and this, it is claimed, improves the texture. Claims are also made that metaphosphate improves the emulsification and retention of fat.

The exact nature of the ' metaphosphate ' used by the German workers is not specified, and the term could be taken to cover almost any of the known polyphosphates. In the present work, therefore, the effects of the following series of compounds were investigated: orthophosphate; pyrophosphate ($Na_2H_2P_2O_7$); Calgon, the registered trade name adopted by Albright & Wilson Ltd. for a glassy polymer containing 12–14 phosphate residues per molecule; Phosphate Glass No. 696, a ' glass ' containing 20–30 phosphate residues; and potassium metaphosphate, a crystalline powder, of ill-defined composition but probably containing at least 10,000 phosphate residues per molecule. It is this last substance that the German workers are likely to have used.

In order to avoid complications arising from variable fat-content, rabbit muscle was chosen as the most suitable type of meat, since the fat content is very low and fairly constant. The results are therefore virtually free from errors such as those arising from pockets of fluid within partly emulsified fat, and can be taken to relate entirely to the proteins of the meat.

In the preliminary work, the compounds mentioned above were dissolved in water and added in the proportion of 1 vol. of solution to 1 vol. of meat. The over-all concentration of solutes was 0·5% in each case. Under these conditions, none of the compounds had a marked effect on the volume of uncooked meat or on the cooked volume. However, on addition also of 1% of sodium chloride the swelling was greatly increased, both before and after cooking, particularly in the presence of pyrophosphate, which was found to be more effective at a concentration of 0·25% than were 0·5% solutions of any of the other compounds. All these gave very similar results, orthophosphate being as effective as metaphosphate.

As no significant difference could be found between sodium chloride and potassium chloride, it is clear that the increase in swelling on adding salt is due to the increase in ionic strength of the medium. This is, of course, already considerably increased by the phosphate compounds themselves, but although the effect of orthophosphate and the other polyphosphates can be explained satisfactorily in terms of this increase, that of pyrophosphate is almost double that of sodium chloride of corresponding ionic strength and must, therefore, be a specific effect.

In view of these results, it is seen that addition of the more complex polyphosphates to meat is not likely to lead to greater retention of water in commercial practice. On the other hand, addition of pyrophosphate in 1% sodium chloride solution might result in a firmer and more spongy texture in cooked sausages in the presence of rusk, and would certainly improve the texture of sausages such as Frankfurters, which contain no rusk. It is also possible that addition of metaphosphate together with pyrophosphate, a procedure sometimes adopted in Germany, might be effective, the metaphosphate contributing the increase in ionic strength necessary for the pyrophosphate to act.

Experimental methods

Preparation of the mince.—The musculature of the back and hind limbs was removed after killing the rabbits by stunning and decapitation. The meat was left 24 h. at 0° to pass fully into *rigor mortis* and then minced once through a plate with perforations of $\frac{1}{8}$ in. in diameter. Samples (50 g.) were weighed out and treated as below.

Measurement of ' free-fluid ' losses and cooking losses.—To 50-g. samples of minced meat in 200-ml. centrifuge cups was added 50 ml. of the solution to be tested, and the mixture was well stirred to ensure that all clumps of muscle were broken up. The mixtures were left for $\frac{1}{2}$ h. at room temperature and then stored for 20–24 h. at 0°. At the end of this time they were centrifuged at a relative centrifugal force of 1200 for $\frac{1}{2}$ h. The supernatant fluid, referred to as ' free fluid ', was then removed and its volume measured. This value was subtracted from the total volume, assumed to be 100 ml., to give the volume of the meat after centrifuging; this is called the ' uncooked volume '.

The remaining muscle-mass was carefully transferred to boiling-tubes (8 in. × $1\frac{1}{2}$ in.) and

packed down tightly to avoid air pockets. The tubes were fitted with air condensers and placed in a boiling-water bath for 20 min. On removal, the fluid that had cooked out was decanted carefully into funnels fitted with small cotton-wool plugs and draining into 100-ml. measuring cylinders. This introduces a small but constant error. When most of the fluid had filtered through, the cooked meat was tipped out carefully into the funnel and allowed to drain for 10 min. The total volume of the filtrate was noted. This is referred to as ' cooking-out loss '. Subtraction of this volume from the ' uncooked volume ' gives the volume of the cooked meat, called ' cooked volume '. The operation was carried out at an elevated temperature to avoid setting of any fat that might have cooked out. As a result some losses occur, owing to evaporation. The total losses, however, are low and fairly constant (about 3% of the total fluid), as shown in Table I. This Table shows the results of an experiment in which four 50-g. samples of the same mince were mixed with 50 g. of 2% sodium chloride solution, and treated as outlined above, except that the free-fluid and cooking-out losses were weighed instead of being measured as volumes. The cooked meat was also weighed. It is seen that the over-all errors in the weights of the uncooked and cooked meat do not exceed $\pm$ 2% of these weights, and that the observed ' cooked weights ' and those estimated by difference do not differ by more than 1·5 g. in 33 g.

Table I

' Free-fluid ' losses and cooking-out losses of four samples of the same rabbit-mince treated with an equal weight of 2% sodium chloride solution; total initial wt. = 100 g.; pH of all samples 5·74 $\pm$ 0·005

	' Free fluid ', g.	Wt. of treated muscle (by diff.), g.	Cooking-out loss, g.	Total loss, g.	Wt. of cooked muscle, g.	
					By diff.	Observed
1	43·2	56·8	22·6	65·8	34·2	33·0
2	43·2	56·8	23·7	66·9	33·1	32·0
3	43·0	57·0	24·2	67·2	32·8	32·5
4	42·7	57·3	23·7	66·4	33·6	32·1
Average	—	57·0	—	66·6	33·4	32·4
S.E.	—	$\pm$ 0·2	—	$\pm$ 0·6	$\pm$ 0·6	$\pm$ 0·5

Estimation of percentage protein in the ' free fluid '.—The percentage of protein in the ' free fluid ' was measured in some cases on a refractometer, 0·00183 being taken as the refractive-index increment per 1% of protein. It was found satisfactory to use the ' cooked-out ' fluid as control. Then, if the refractive index of the ' free-fluid ' is R_1 and that of the ' cooked-out ', protein-free fluid is R_2, the percentage of protein is given by $(R_1 - R_2)/0{\cdot}00183$.

Determination of pH.—pH values of all the samples of ' free fluid ' were measured by means of a glass electrode, and were found to fall within a narrow range. The average pH after addition of 1% of sodium chloride was 5·71, with a standard error of $\pm$ 0·09, and, after addition of 0·5 or 0·25% of pyrophosphate + 1% of sodium chloride, 5·76, with a standard error of $\pm$ 0·12 (25 samples in each case).

Samples of polyphosphates.—The samples of Calgon, Phosphate Glass No. 696 and potassium metaphosphate were kindly supplied by Albright & Wilson Ltd. None of these contained more than 0·5% of the total phosphate as orthophosphate. Pyrophosphate was used in the form of the tetrasodium salt, $Na_4P_2O_7{,}10H_2O$ (Hopkin & Williams Ltd.), and orthophosphate as the dry salt KH_2PO_4 (British Drug Houses Ltd.). All solutions were adjusted to pH 6·5 before use. As the final pH in the presence of the meat is about 5·75, this leads to a considerable increase in ionic strength, μ, for tetrasodium pyrophosphate, the increment per mole being approximately 3·0 for disodium pyrophosphate, but 5·0 after neutralization of the tetra-salt to pH 5·75. This is taken into account in the calculation of μ. ($\mu = \frac{1}{2} \Sigma m_i z_i^2$, where m_i is the molarity and z_i is the valency of the species of ion present.)

It should be noted, however, that the percentage of pyrophosphate is given in all cases in terms of the dry salt, $Na_2H_2P_2O_7$, which has a total phosphorus content of 28%. As Calgon and Phosphate Glass have similar total phosphorus contents (28 and 30% respectively), these three salts are directly comparable at the same percentage concentrations.

Results

The results are given in terms of the volume of the meat, obtained by difference after the various treatments, on the basis of a volume of fresh meat of 100 ml. The concentration of the various added solutes is given as the over-all percentage after addition of 100 ml. of solution to 100 ml. of meat.

Effect of polyphosphate solutions without added sodium chloride.—A few preliminary experiments were carried out with 0·5% solutions of disodium dihydrogen pyrophosphate (pyrophosphate), Calgon and Phosphate Glass (' glass '), and 0·61% solutions of potassium dihydrogen phosphate (phosphate), against distilled water as control. Potassium metaphosphate could not be tested under these conditions as it is insoluble. Such an experiment is illustrated in Fig. 1, where it is seen that water increases the uncooked volume to 104, phosphate to 112, pyrophosphate to 119, Calgon to 114 and ' glass ' to 120. The same order is observed on cooking, although in no case does the cooked volume exceed 75% of the volume of the fresh meat. Experiments of this type were discontinued when it was realized that addition of sodium chloride to an over-all concentration of 1% modified the results markedly and led to greater swelling all round.

Effect of polyphosphate solutions with added sodium chloride.—When sodium chloride is added to give an over-all concentration of 1%, the effect of the polyphosphate compounds is considerably modified, 0·5% of pyrophosphate now giving far greater swelling than 0·5% of ' glass ', either alone or with added sodium chloride (see Fig. 1). The effects of 0·61% of phosphate, i.e. total phosphorus content equivalent to 0·5% of pyrophosphate, and of 0·5% of potassium metaphosphate are also shown and are seen to be about the same as that of Calgon. These gains are maintained after cooking.

As a consequence of this experiment and three others, which gave substantially the same results, the effects of Calgon, ' glass ' and metaphosphate were not investigated further, but the effect of pyrophosphate was studied in detail at concentrations of 0·25 and 0·5%. The average results of 12 experiments (with the standard error of the means) are given in Fig. 2, where it is seen that water increases the uncooked volume to 107, 1% sodium chloride solution to 120, 0·25% pyrophosphate/1% sodium chloride solution to 151 and 0·5% pyrophosphate/1% sodium chloride solution to 164. Similarly, on cooking, the volume of the meat falls from 100 to 70, without additions; to 64 in water; to 71 in 1% sodium chloride solution; to 89 in 0·25% pyrophosphate/1% sodium chloride solution and to 99 in 0·5% pyrophosphate/1% sodium chloride solution. It will be seen that these values can vary considerably from the mean, but, as in practice the increase due to 0·25% of pyrophosphate is always at least 10 units higher than that due to sodium chloride alone, the results are highly significant in spite of the variations, a conclusion supported by the *t*-test ($P = 0{\cdot}001$).

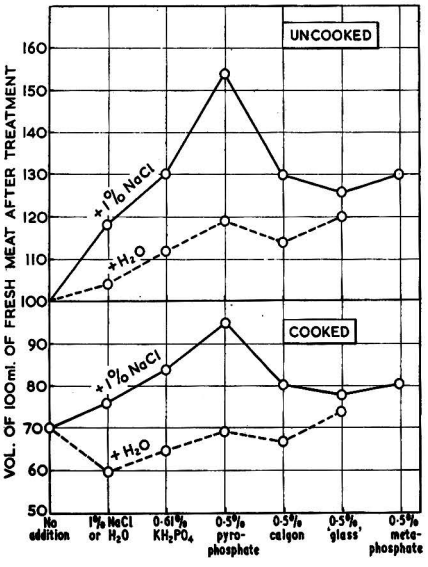


FIG. 1.—*Effect of various phosphate compounds on the uncooked and cooked volume of meat after centrifuging a mixture of 100 ml. of fresh meat + 100 ml. of solution under standard conditions*

Broken curve, in H_2O; full curve, in 1% sodium chloride solution
Concentrations given as over-all % of the various compounds

The cause of the variations in uncooked volume, particularly in the presence of pyrophosphate, is uncertain, but they are not likely to be due to the degree of fineness of the mince, or to the pH, since the fineness of the mince has only minor effects on the volume and the

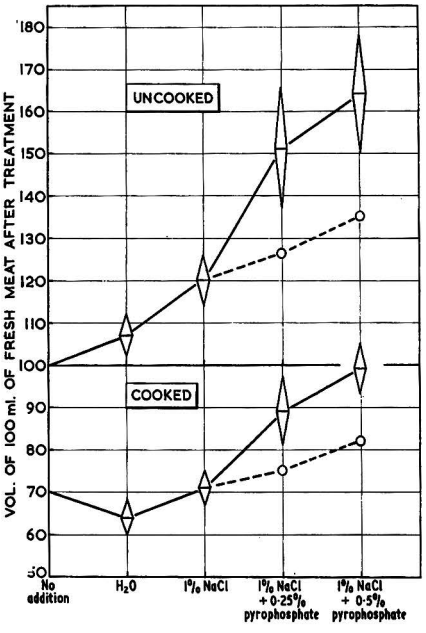


FIG. 2.—*Effect of water, 1% sodium chloride and 0·25 and 0·5% pyrophosphate in 1% sodium chloride, on the uncooked and cooked volume of meat (as Fig. 1)*

12 samples in each category; the S.E.'s of the means are indicated by the diamonds; broken lines represent effect of sodium chloride at same ionic strength as the mixtures of sodium chloride and pyrophosphate

pH varies randomly and within too narrow limits to affect the volume significantly. There are two other possible sources of the variations: (1) the stage of *rigor mortis* the muscle has reached at the time of mixing, although it would be predicted from previous work[2] that the rigor process should be complete after the 24-h. storage given to the meat in these experiments; (2) the length of time the mince is left in contact with salt solutions before centrifuging. The experiment shown in Fig. 3 indicates that the first explanation is improbable. In this case, very large increases were obtained with 0·25 and 0·5% of pyrophosphate with mince prepared 24 h. after death. These values were scarcely altered, and certainly not significantly decreased, by a further 24-h. storage of the mince at 0°, by which time rigor was undoubtedly complete. Incidentally, metaphosphate was included again in this experiment, and is seen to have a small effect compared with that of pyrophosphate.

The second explanation is more likely to be correct. As noted earlier, the mince was usually left in contact with the salt solutions for not longer than 24 h. before centrifuging, since this was considered to be the maximum time allowable in commercial practice. However, in an experiment that gave average values, after 24 h., for the increases in uncooked volume in 1% sodium chloride and in 1% sodium chloride/0·25% pyrophosphate solutions respectively, it was found that a further 24 h. in these solutions resulted in an increase in the volume in pyrophosphate from 151 ml. to 175 ml. (per 100 ml. of fresh meat), but did not affect the volume in 1% sodium chloride solution alone. From this it is evident that the effect of pyrophosphate reaches its maximum rather slowly, and the variations are thus likely to be due to the asymptotic nature of the swelling/time curve.

The effect of increasing ionic strength.—The marked differences between the effects of pyrophosphate in water and in the presence of 1% sodium chloride solution might be due to the considerable increase in ionic strength μ brought about by the sodium chloride itself together with the increase due to pyrophosphate. The value of μ for 1% sodium chloride solution is 0·17, whereas for 0·25% pyrophosphate solution (0·011M) it is 0·055 and for 0·5% pyrophosphate solution 0·11. The total added μ is thus 0·225 at the lower and 0·28 at the higher pyrophosphate concentration, when sodium chloride is also present. The percentage of sodium chloride necessary to give $\mu = 0{\cdot}225$ is 1·32% and, to give $\mu = 0{\cdot}28$, 1·64%. Addition of these

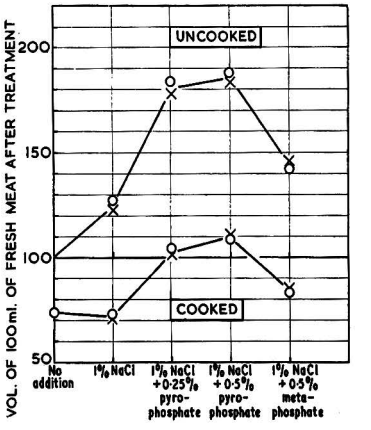


FIG. 3.—*Uncooked and cooked volume of meat treated 24 h. (○) and 48 h. (×) after death, respectively, with pyro- and meta-phosphate in 1% sodium chloride*

concentrations of sodium chloride to samples giving average values for the pyrophosphate effect increases the swelling, as shown by the broken lines in Fig. 2; these increases are seen to be well below the level of the pyrophosphate values. Another way of demonstrating the same effect is shown in Fig. 4, where the value of μ, due to additions, is held constant at 0·225, but where the concentrations of sodium chloride and pyrophosphate are varied. It is seen that pyrophosphate again has an effect much greater than that of sodium chloride alone.

In this connexion it is worth considering the total value of μ for the system, 50 ml. of meat + 50 ml. of additions. The value of μ for the muscle without additions[3] is approximately 0·26. When 50-ml. additions are made the following over-all values for μ are obtained: water, 0·13; water/0·5% pyrophosphate, 0·24; 1% sodium chloride solution, 0·30; 1% sodium chloride solution/0·25% pyrophosphate, 0·355; 1% sodium chloride solution/0·5% pyrophosphate, 0·41. The pyrophosphate effect is low at a value of μ of 0·24 and about equal to that of 1% sodium chloride solution (total μ, 0·30). However, it is more than double that of 1% sodium chloride solution at a value of μ of 0·355. Thus, in commercial practice, it would be advisable to keep the value of μ above 0·35, whatever the proportion of water added to the meat, and to keep the pyrophosphate concentration at about 0·5%. Total values of μ greater than 0·40 should be avoided, since above this value the ionic strength is approaching that necessary to extract the main structural protein of the muscle, actomyosin (0·5–0·6), and some dissolution of this protein might therefore be expected.[4] In fact, even when 0·5% pyrophosphate/1% sodium chloride solution is used (total μ, 0·41) the 'free fluid' is very viscous and, when diluted tenfold with water, shows the flocculation characteristic of the presence of myosin or actomyosin. The protein concentration of this fluid can reach values of 5·5% as compared with an average of 3·5% at the lower ionic strengths. No such dissolution of actomyosin or myosin is found, however, when the high ionic strength (0·41) is due to sodium chloride alone.

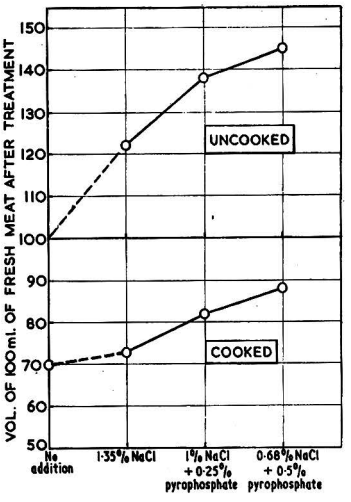


FIG. 4.—*Effect of pyrophosphate on swelling at constant ionic strength (parameters as in other Figures); added* $\mu = 0{\cdot}225$, *total* $\mu = 0{\cdot}355$

The effect of calcium and magnesium ions.—A few experiments were carried out to determine the effect of calcium and magnesium ions on swelling in the presence of pyrophosphate, since these ions occur in tap-water, and are therefore likely to be present in commercial sausage preparations. Moreover, the calcium ion might be expected to inhibit the effect and the magnesium ion to increase it, as they do in the lengthening of muscle strips brought about by addition of pyrophosphate and adenosine triphosphate.[5]

The experiments showed, however, that 0·0025M calcium ion, i.e. the concentration in Cambridge tap-water, lowered only slightly the volume of uncooked meat and had no effect on the cooked volume in the presence of 1% of sodium chloride and 0·25 or 0·5% of pyrophosphate, and that 0·0025M magnesium ion had no significant effect on either of these values. The latter result is perhaps not surprising, since under the conditions of the experiments the muscle itself is contributing magnesium ion to the extent[3] of at least 0·005M. The failure of the calcium ion to act is not so easily explained, but it may be that it can inhibit lengthening or swelling only when adenosine triphosphate is also present to provide the energy necessary for shortening of the muscle fibres.

The relation between uncooked volume and cooked volume of the meat.—When the uncooked volume of all the samples is plotted against the cooked volume (Fig. 5) it is seen that the two

are linearly related; this relation can be expressed by the equation for the regression of V_2 upon V_1:

$$V_2 = 0{\cdot}563(V_1 + 7{\cdot}6) \pm 2{\cdot}96 \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where V_1 is the volume of uncooked meat after centrifuging a mixture of 100 ml. of meat and 100 ml. of added solution, and V_2 is the volume of the cooked meat. Since the relation is linear within fairly narrow limits ($r = 0{\cdot}975$; $P < 0{\cdot}001$) for all the samples, irrespective of the treatment, it appears unlikely that the polyphosphates, pyrophosphate or phosphate have any direct effect on cooking losses, but rather that the gains in cooked volume due to these compounds depend on the retention in the more swollen uncooked meat of more of the soluble, heat-coagulable proteins which, on cooking, coagulate within the mass of meat, and thus hold a high proportion of water.

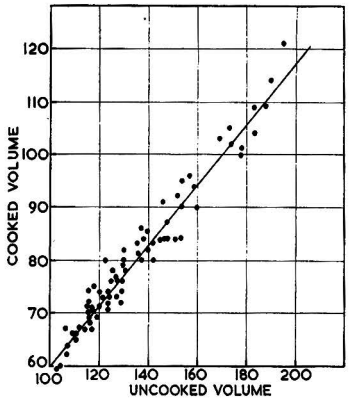


FIG. 5.—*Uncooked volume plotted against cooked volume for all samples, irrespective of treatment*

A measure of the extent of this retention of water can be obtained by rewriting equation (1). We know from Fig. 5 that when V_1 is equal to 100, V_2 is 60. The equation can thus be written:

$$V_2 = 60 + k(V_1 - 100) \quad .. \quad .. \quad (2)$$

k, from the curve, is 0·563, as in equation (1), but the term within the bracket now represents the extra fluid retained as V_1 increases. Hence k is a constant representing the water retained, after coagulation, by the soluble proteins remaining in the centrifuged material; that is, for every ml. of fluid retained 0·563 ml. remains after cooking, irrespective of the solute present. Since this value is so large, much of the water must be retained mechanically.

Effect of cooking without removing 'free fluid'.—When the mixtures of 50 ml. of meat and 50 ml. of solution are cooked without removing the 'free fluid' (see Table II) the effect of 0·25% pyrophosphate/1% sodium chloride is considerably reduced and is not much greater than that of 1% of sodium chloride alone. This would be expected from the form of equation (2) and should hold independently of the nature and concentration of added solute, since in this case none of the soluble protein has been removed and all the coagulable protein is retained by the cooked meat. However, addition of 1% sodium chloride/0·5% pyrophosphate increases the cooked weight from about 101 to about 123. This difference is well outside the range of error and must be due to some change in the nature of the meat at this concentration of pyrophosphate. As we have seen, this change is likely to be the partial extraction of myosin or actomyosin. If this is so, it is to be deduced that these proteins retain more water when coagulated in the sol form than when coagulated in the gel form.

Table II

Effect of cooking mixtures of equal volumes of meat and solution without first removing 'free fluid'; three separate samples of mince

Solute and over-all concn...	1% NaCl	1% NaCl/0·25% pyrophosphate	1% NaCl/0·5% pyrophosphate
Vol. of 100 ml. of fresh meat after cooking	101	107	128
	99	101	119
	102	106	122
Average	101	105	123

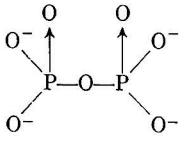
Conclusions

The results of the present study are to a certain extent in conflict with those of the German workers, Ellerkamp & Hannerland, in that they show that only pyrophosphate, of all the compounds tested, has a specific swelling effect on lean meat, and that this effect increases

with increasing ionic strength. Although it is true that orthophosphate, Calgon, Phosphate Glass and metaphosphate all have some effect on swelling, which is also increased by adding salt, yet this effect is small compared with that of pyrophosphate, and is, in fact, no greater than that of 1·65% of sodium chloride. Thus, it is possible to distinguish between these minor effects, which are probably due solely to the increase in ionic strength contributed by the compounds themselves, and the much larger effect of pyrophosphate in 1% sodium chloride solution, which is more than double that of a solution of sodium chloride of equivalent ionic strength.

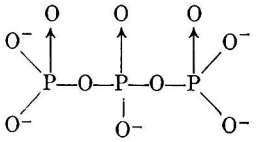
This effect of pyrophosphate is to a certain extent predictable from the work of Hasselbach,[4] who has shown it to facilitate the extraction of myosin and actin from muscle at high ionic strength (0·5–0·6), although it is not likely that the actual extraction of these proteins proceeds very far under the conditions of the present experiments. In this connexion, it is notable that pyrophosphate, alone of the compounds tested, increases the protein concentration and the viscosity of the ' free fluid ' obtained by centrifuging the wet mince.

This behaviour of pyrophosphate is probably due to its ability to split the link between the two components, actin and myosin, of the main contractile protein of muscle. To do this, the specific links (I) or (II) are required :[6]



(I)

and



(II)

Both of these are found in the compound adenosine triphosphate, which is the source of the energy for muscular contraction, and also in inorganic triphosphate (II). Thus, the triphosphate would be expected to behave in the same manner as pyrophosphate, although it has not actually been tested in these experiments. Whether compounds intermediate in chain length between triphosphate and Calgon (12–14 phosphate residues) would also behave in this way is not known, but it is rather unlikely because of the tendency of such compounds[7] to form rings which would not fit the specific groupings on the actomyosin molecule.

The applicability of these results in commercial practice will depend so much on the type of sausage mix to be used and the amount of water to be added that few general conclusions can be reached. There seems little doubt, however, that addition of pyrophosphate would give rise to a more spongy and tender texture in the meat of cooked sausage. These characteristics were very noticeable in tasting tests carried out in conjunction with the present study, and were considered to improve the palatibility above that of meat cooked in the presence of 1% of sodium chloride only, which was dry and tough.

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References

[1] Ellerkamp, W., & Hannerland, H., *Dtsch. Lebensmittlrdsch.*, 1952, **48**, 32
[2] Bendall, J. R., *J. Physiol.*, 1949, **110**, 47
[3] Dubuisson, M., Proc. Symp. Muscle, Royaumont, France, 1950, p. 8
[4] Hasselbach, W., *Z. Naturf.*, 1953, **8b**, 449
[5] Bendall, J. R., *Nature, Lond.*, 1953, **172**, 586
[6] Weber, H. H., *Proc. roy. Soc.*, 1952, [B] **139**, 512
[7] Topley, B., *Quart. Rev. chem. Soc.*, 1949, **3**, 345

DETERMINATION OF FAT PEROXIDES IN THE PRESENCE OF PHOSPHOLIPIDS

By L. HARTMAN

A colorimetric method of estimating fat peroxides in materials such as animal tissues has been developed, which is free from interference by phospholipids. The peroxides are reduced with ferrous chloride in benzene–methanol solution and the amount of ferric iron produced is determined by the addition of 2 : 6-dichlorophenolindophenol and pyrophosphoric acid. The extent of phospholipid interference with the ferric thiocyanate method of peroxide estimation has been measured by comparison with the new method. This interference has been found on the whole appreciable owing to the solubility of phospholipids in solvents used for fat extraction.

The prolonged argument as to whether the iodometric or the ferric thiocyanate method gives correct peroxide values appears now to have been settled by Lea,[1] who produced strong evidence that the ferric thiocyanate and other colorimetric methods yield impossibly high results as shown by oxygen-absorption data. Nevertheless, Lea considers that for the purpose of detecting rancidity in its earliest stages, or on a micro-scale, the ferric thiocyanate method might be more useful than the iodometric procedure. The results of the ferric thiocyanate method, especially in the form suggested by Hills & Thiel[2] with benzene–methanol as solvent, are readily reproducible, but phospholipids interfere,[3] as do phosphates and organic oxy-acids in the thiocyanate test for ferric iron in aqueous solution. To avoid this interference in peroxide estimation in dried buttermilk, Hills & Wilkinson[3] suggested the replacing of benzene–methanol as solvent by the otherwise much less satisfactory acetone, in which phospholipids are only sparingly soluble. On the other hand, Smith[4] stated that interference caused by phospholipids is eliminated in the estimation of peroxides in meat tissues if the fat is extracted with benzene only. It will be shown below that this elimination is by no means complete. Further, owing to the considerable mean unsaturation of their component fatty acids,[5] phospholipids are susceptible to autoxidation possibly even to a greater extent than the corresponding fatty acids.[6] Thus the part they play in the formation of peroxides, and in the ensuing rancidity, should not be disregarded, particularly as they form a substantial proportion of the total lipids in muscular tissues of various animals.[7]

A colorimetric method of estimating peroxides, including phospholipid peroxides, appears therefore to be of value when it is inconvenient to carry out this determination iodometrically. The dichlorophenolindophenol method of Hartmann & Glavind[8] does not suffer from interference by phospholipids, but has several weaknesses that render it unsuitable for exact work. The strictly reproducible (although not stoicheiometric) character of the reaction between organic peroxides and ferrous salts[9] makes this reaction preferable to others. The purpose of the present paper is to describe a method of peroxide estimation that uses ferrous chloride as reducing agent and yet is free from interference by phospholipids, and to present results obtained when applying this method to various fat-containing foodstuffs.

Experimental

Development of method

According to Vanossi,[10] the interference of phosphates with the thiocyanate test for ferric iron in aqueous solution can be prevented by the addition of aluminium or zirconium salts, which liberate iron from its complex with the phosphate. However, zirconium oxychloride was found completely ineffective in preventing interference of phospholipids in the estimation of peroxides, whereas aluminium chloride was effective only at low concentration of phospholipids in benzene–methanol solution.

In view of the difficulty of estimating ferric iron colorimetrically in the presence of phospholipids, the possibility of measuring the excess of ferrous iron was next considered. The *o*-phenanthroline reagent appeared suitable owing to the stability of the ferrous–*o*-phenanthroline complex. Any possible interference due to the reducing action of tocopherols and related substances was eliminated by adding oxalic acid (5 mg. of acid to 10 ml. of benzene–methanol

solution). However, even with the use of *o*-phenanthroline the interference of phospholipids was not entirely suppressed, and therefore a novel procedure, referred to below as the ferrous chloride–indophenol method, was finally developed. This consists in reducing the fat peroxides in benzene–methanol solution with a measured amount of ferrous chloride and oxidizing the excess of ferrous iron with a measured quantity of dichlorophenolindophenol. Both reactions are carried out at room temperature. Phosphoric acid and its derivatives do not interfere, and some pyrophosphoric acid is even added to facilitate a quantitative oxidation of ferrous chloride by dichlorophenolindophenol. A blank is run under similar conditions and the difference between the colour of the sample and of the blank is a measure of the ferric iron produced by the peroxides.

Reagents and apparatus

1. Benzene and methanol: A mixture of 7 vol. of benzene and 3 vol. of methanol is used as solvent. Both are purified as described by Hills & Thiel.[2]

2. Ferrous chloride stock solution: The solution is prepared according to the method of Hills & Thiel,[2] but in double concentration, i.e. by dissolving 0·8 g. of $BaCl_2,2H_2O$ and 1 g. of $FeSO_4,7H_2O$ in 100 ml. of water acidified with 2 ml. of 10N-hydrochloric acid and filtering off the precipitate. It remains usable for several weeks and is discarded when 1 ml. gives a distinct coloration with 1 drop of 30% ammonium thiocyanate (see Note 1 below).

3. Ferrous chloride solution in benzene–methanol: Aqueous ferrous chloride solution (1 ml.) is added to 20 ml. of benzene–methanol (1 : 1) (Note 2). This reagent is best prepared shortly before use, since ferrous chloride is oxidized in organic solvents much more rapidly than in aqueous solution.

4. Ammonium thiocyanate solution: Ammonium thiocyanate (30 g.) is dissolved in water and made up to 100 ml.

5. 2 : 6-Dichlorophenolindophenol solution: Approximately 0·04 g. of commercial dyestuff is dissolved in 10 ml. of methanol and made up to 100 ml. with benzene (Note 2). This solution remains usable for several months.

6. Ferrous sulphate solution for the standardization of dichlorophenolindophenol: About 1 g. of $FeSO_4,7H_2O$, analytical-reagent grade, is weighed out accurately, dissolved in water containing 2 ml. of concentrated hydrochloric acid and made up to 100 ml. (Note 3).

7. Pyrophosphoric acid solution: Pyrophosphoric acid (10 g. $H_4P_2O_7$) is dissolved in 100 ml. of methanol (Note 4).

8. Colorimeter: A photoelectric absorptiometer may be used in preference to a visual colorimeter. In the present work a Beckman spectrophotometer Model DU with 1-cm. cells was used.

9. Extraction bath: A bath as described by Hills & Thiel[2] is very useful for routine work. For odd determinations any hand-controlled water bath may be used.

10. Glassware etc.: Reasonable care is advisable in the cleaning of glassware. There is no need to extract iron from filter papers (Note 1).

Notes on the method

(1) Small amounts of ferric iron have no appreciable influence on the colour intensity of samples and blanks.

(2) The composition of solvents used in the preparation of ferrous chloride and dichlorophenolindophenol reagents has been so chosen that when 1 ml. of each reagent is used an over-all benzene : methanol ratio of 7 : 3 is obtained. This conforms with the solvent used for fat extraction. A solution containing 8 ml. of benzene–methanol mixture, 1 ml. of each reagent mentioned above and 1 drop of pyrophosphoric acid (reagent 7) constitutes the blank in the estimation of peroxides and should have a pink colour, which indicates a sufficient excess of dichlorophenolindophenol.

(3) The salt $FeSO_4,7H_2O$ used in the standardization of dichlorophenolindophenol gives results similar to those obtained with ferrous chloride solution of known strength.

(4) In the oxidation of ferrous salts with dichlorophenolindophenol, metaphosphoric acid

is usually employed to shift the equilibrium in favour of ferric iron. Because of its insufficient solubility in benzene–methanol, pyrophosphoric acid has been chosen instead.

The standardization of dichlorophenolindophenol

The aqueous $FeSO_4,7H_2O$ solution (5 ml., reagent 6) is diluted with a known volume of methanol to obtain approximately 56 μg. of Fe^{2+} in 1 ml. of the final solution. To 1 ml. of this solution (in a 10-ml. standard flask) is added 1 ml. of dichlorophenolindophenol solution (reagent 5) followed by 1·9 ml. of methanol and 1 drop of pyrophosphoric acid solution, and the mixture is made up to 10 ml. with benzene. A blank is prepared from 2 ml. of the diluted $FeSO_4,7H_2O$ solution in methanol, 1 ml. of dichlorophenolindophenol solution, 0·9 ml. of methanol and 1 drop of pyrophosphoric acid solution and making up to 10 ml. with benzene. The colour of the blank should be pink, otherwise the concentration of the dichlorophenolindophenol solution must be adjusted. From the colorimeter reading the extinction coefficient corresponding to 55·84 μg. of Fe^{3+} (= 1 micro-equiv. of peroxide oxygen) can be calculated, and in the present work it was 0·500 at 530 mμ (1·cm. cell). However, this coefficient is likely to vary for different lots of the dyestuff for reasons discussed below, whereas the relation between spectrophotometer readings and concentration of the dyestuff is, as a rule, linear. In the ferric thiocyanate method the extinction coefficient corresponding to 55·84 μg. of Fe^{3+} was found to be 1·436 at 510 mμ.

Fat extraction and estimation of peroxide values

The fat is extracted as in the Hills–Thiel procedure for milk powder[2] by refluxing a sample with a measured volume (15–20 ml.) of benzene–methanol mixture for 5 minutes at 60°. The size of the sample is chosen in accordance with the expected peroxide content and, if necessary, has to be determined by preliminary experiments. The fat solution is filtered into a narrow test-tube marked at 8 ml.; 1 ml. of ferrous chloride solution (reagent 3) is added, the tube shaken and set aside for 10 minutes at room temperature. Dichlorophenolindophenol solution (1 ml., reagent 5) is then added, followed by 1 drop of pyrophosphoric acid solution (reagent 7). The blank is prepared in a similar way, with 8 ml. of benzene–methanol mixture instead of the fat solution (see Reagents, Note 2), and the extinction is read without much delay, since the colour of dichlorophenolindophenol is not stable in an acid medium.

Results

Samples of ox and mutton fat, free from phospholipids, dried buttermilk, and meat and fish tissues were examined during the present work.

Peroxide values of fats free from phospholipids were determined by using the ferric thiocyanate procedure according to Hills & Thiel,[2] and the ferrous chloride–indophenol method described above. Comparative results are shown in Table I, which illustrates also the reproducibility and sensitivity of the new method.

Table I

Comparison of the ferric thiocyanate method with the ferrous chloride–indophenol method

Sample	Peroxide values in micro-equiv. per 1 g. of fat		
	Ferric thiocyanate method	Ferrous chloride–indophenol method	
		Range of spectrophotometer readings	
		E = 0·25–0·5	E = 0·05–0·1
Ox fat 1	0·42	0·43	0·43
	0·40	0·43	0·42
	0·42	0·44	0·46
Ox fat 2	7·95	8·16	8·20
	7·88	8·24	8·02
	7·82	8·18	8·28
Mutton fat (oxidized)	485	504	515
	492	516	521
	478	526	527

Meat and fish tissues were dried as described by Smith.[4] Parallel tests were run extracting each sample with benzene–methanol mixture, cold benzene and hot benzene respectively, and analysing for peroxides by both the ferric thiocyanate and the ferrous chloride–indophenol methods. The amount of fat extracted with each solvent was determined, and the peroxide values were calculated in micro-equivalents of peroxide oxygen per 1 g. of fat and 1 g. of dry material respectively. Results appear in Table II.

Table II

Peroxide values in micro-equiv. per 1 g. of fat and 1 g. of dry sample respectively

Sample	Ferric thiocyanate method			Ferrous chloride–indophenol method		
	Benzene at 20°	Benzene at 60°	Benzene–methanol at 60°	Benzene at 20°	Benzene at 60°	Benzene–methanol at 60°
Ox	7·42* (1·55)†	8·99 (2·34)	Nil	8·38 (1·75)	12·14 (3·16)	19·98 (6·16)
Chicken	7·24 (0·11)	6·06 (0·14)	Nil	11·20 (0·77)	12·99 (0·30)	30·85 (1·47)
Blue cod (*Parapercis colias*)	2·11 (0·06)	2·39 (0·10)	Nil	4·21 (0·12)	5·03 (0·21)	28·15 (3·14)
Ling (*Genypterus blacodes*)	7·36 (0·08)	9·23 (0·12)	Nil	10·12 (0·11)	18·48 (0·24)	27·97 (1·13)
Ling (oxidized)	13·62 (0·13)	14·62 (0·19)	Nil	13·40 (0·15)	23·10 (0·30)	170·40 (6·88)
Dried buttermilk	12·39 (0·31)	10·96 (0·40)	4·93 (0·52)	11·98 (0·30)	13·73 (0·50)	8·45 (0·89)

* Micro-equiv./g. of fat
† Micro-equiv./g. of dry sample

In an attempt to correlate the peroxide values with the phospholipid content of the extracted fat samples, the amount of lipid phosphorus in the various extracts was determined by means of the colorimetric method of Allen.[11] Table III shows the content of phospholipids in these fats, obtained by multiplying the percentage of phosphorus by a factor of 25.

Table III

Percentage of phospholipids in various fat extracts

Sample	Phospholipids, %, in fats extracted with		
	benzene at 20°	benzene at 60°	benzene–methanol at 60°
Ox	3·61	6·14	13·40
Chicken	16·53	27·94	46·37
Blue cod	50·36	55·21	48·24
Ling (oxidized)	35·67	31·53	29·55
Dried buttermilk	0·94	4·59	12·78

Discussion

The method described in this paper, although employing dichlorophenolindophenol, differs in principle from the dichlorophenolindophenol method suggested by Hartmann & Glavind.[8] In the latter method the reducing agent is the leuco-base of the dyestuff and the developed colour of the dye is the measure of the peroxide content; in the ferrous chloride–indophenol method dichlorophenolindophenol is used to determine the amount of ferric iron formed in the reaction between ferrous chloride and fat peroxides. The reasons for replacing the leuco-base of the dyestuff with the reduction–oxidation system comprising ferrous chloride and the dyestuff are as follows: Although free from interference by phospholipids, the dichlorophenol-indophenol method of Hartmann & Glavind gives less reproducible results than methods based on ferrous salts; its 'oxygen error' is higher and it is more susceptible to interference by traces of copper.[1] Further, the extinction coefficient of commercial dichlorophenolindophenol varies considerably from batch to batch, even after accounting for its apparent degree of purity

as determined iodometrically. This is probably due to the variable composition of the commercial dyestuff, the individual components having different light absorption. Since the extinction coefficient of the dyestuff forms the basis for calculating peroxide values there is a possibility of variable results being obtained. Thus, although the method might be used with advantage for some special purposes—it has been, for instance, adapted in this Laboratory for rapid estimation of fat peroxides in wet meat tissues[12]—its general applicability appears restricted.

In the method described in the present paper the extinction of dichlorophenolindophenol is calibrated against ferrous iron, and although this is necessary for each new lot of the dyestuff a uniform basis for calculating results is nevertheless provided. A 1-g. sample of the dyestuff suffices for some 1000 peroxide estimations. A further advantage is the lack of interference by traces of ferric iron, this interference being a major source of error in the ferric thiocyanate procedure.

When the new procedure was used to determine peroxides in fats free from phospholipids, higher values were obtained than with the ferric thiocyanate method, probably because ammonium thiocyanate employed in the latter method suppresses to some extent the autoxidation of ferrous iron. The use of ammonium thiocyanate in the ferrous chloride–indophenol method proved impracticable since the colour of the dyestuff was affected by the addition of this salt. However, the results obtained by the two methods differed by less than 10% (cf. Table I) and their reproducibility was similar. The sensitivity of the ferrous chloride–indophenol method is governed by the extinction coefficient of the dyestuff, and this, for a given peroxide content, is approximately one-third of the extinction produced by ferric thiocyanate. For most purposes the specific extinction coefficient of dichlorophenolindophenol should prove sufficient; in the examination of fats with very low peroxide values the sensitivity of the method could be increased by using indophenol or indamine derivatives with a higher specific extinction than that of dichlorophenolindophenol, e.g. phenol blue.

The use of the ferrous chloride–indophenol method made it possible to determine the extent of phospholipid interference in the ferric thiocyanate test when various solvents were employed for fat extraction. Table II shows that this interference was on the whole quite pronounced, even when the extraction was carried out with benzene at room temperature, as recommended by Smith.[4] This is understandable since, according to Table III, benzene extracted appreciable amounts of phospholipids, particularly from fish tissues. On the other hand, interference in the ferric thiocyanate test resulting from the use of benzene–methanol was too pronounced to be attributed solely to the amount of phospholipids extracted by this solvent; it might be due to the different composition of phospholipids. Incidentally, the use of acetone in the extraction of fish tissues produced more interference than the use of benzene.

Peroxide values obtained with the ferrous chloride–indophenol method showed an exactly opposite trend. They were, on the whole, much higher when fat extraction was carried out with benzene–methanol than with benzene alone, and this applied to values calculated per 1 g. of fat as well as per 1 g. of dry sample. Benzene–methanol appears therefore preferable as solvent, and this accentuates the need for taking account of phospholipids in the estimation of peroxides in animal tissues.

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References

[1] Lea, C. H., *J. Sci. Fd Agric.*, 1952, **3**, 586
[2] Hills, G. Loftus, & Thiel, C. C., *J. Dairy Res.*, 1946, **14**, 340
[3] Hills, G. Loftus, & Wilkinson, R., *J. Coun. sci. industr. Res. Aust.*, 1946, **19**, 430
[4] Smith, G. H., *J. Sci. Fd Agric.*, 1952, **3**, 26
[5] Snider, R. H., *J. biol. Chem.*, 1936, **116**, 503
[6] Tait, H., & King, E. I., *Biochem. J.*, 1936, **30**, 285
[7] Kaucher, M., Galbraith, H., Button, V., & Williams, H. H., *Arch. Biochem.*, 1943, **3**, 203
[8] Hartmann, S., & Glavind, J., *Acta chem. scand.*, 1949, **3**, 954
[9] Kolthoff, I. M., & Medalia, A. J., *Analyt. Chem.*, 1951, **23**, 595
[10] Vanossi, R., *An. Asoc. quim. argent.*, 1941, **29**, 48
[11] Allen, R. J. L., *Biochem. J.*, 1940, **34**, 858
[12] Hartman, L., Hooker, C. N., & Watt, H. E., *N.Z. J. Sci. Tech.*, 1954, [B] **35**, 307

THROUGH-CIRCULATION DRYING OF SEAWEED. IV.*—A Graphical Design Method for Continuous Multi-stage Driers

By R. G. GARDNER and T. J. MITCHELL

A graphical design method is proposed for calculating the drying time, throughput rates and specific evaporation for double- and triple-stage conveyor through-circulation driers. The method is illustrated with examples based on the unit wet-bulb depression drying curves for *Laminaria cloustoni* stipe and frond.

Introduction

Drying of freshly harvested seaweed is desirable to reduce transport costs, to prevent decomposition during storage and to permit easy compounding with animal feeding-stuffs. Seaweed is frequently dried and ground as a preliminary stage in the extraction of algal chemicals and in all cases it is essential that drying is accomplished at a minimum cost.

As fresh seaweed has a high water content (5–7 lb. of water/lb. of bone-dry solids) it follows that evaporation must be efficient to reduce fuel costs. Large-scale tests on the three most promising types of drier indicated that through-circulation drying was the most satisfactory method for drying stipe and frond separately; the drying characteristics of seaweed were therefore further investigated on this type of drier.

Results of the comprehensive laboratory tests on *Laminaria cloustoni* stipe, *L. cloustoni* frond and *L. digitata* stipe and frond have been published in three earlier papers.[1–3] It was demonstrated in these tests that the drying rates of seaweed beds at average water contents of 5–0·2 lb. of water/lb. of bone-dry solids (B.D.S.) were directly proportional to the wet-bulb depression (W.B.D.) of the inlet air, and that a unit W.B.D. evaporation coefficient may be used to predict approximate drying times and rates. Correction factors for bed-loading and air velocity were given to enable the drying times to be adjusted for conditions that differed from those used in the standard curves.

The following examples are intended to illustrate how the W.B.D. evaporation coefficient may be applied to the design of single- double- and triple-stage through-circulation conveyor driers, with stipe or frond as the wet feed. They can also be used to explore the quantitative effects of operational changes on the output.

It may not be possible or economical to separate the stipe and frond, but it is normally desirable for efficient drying and for the subsequent extraction of any particular constituent. The separation of stipe or frond need not be absolute, however, since it is unlikely that small amounts of contamination would seriously affect the drying properties of either of the beds.

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Previous work

The designing of truck-and-tray tunnel dehydrators for vegetables has received full attention in the literature, largely owing to the need for drying vegetables for war-time use. Thus Brown & Kilpatrick[4] carried out a series of tests on the over-draught drying of potato strips and expressed their data as a nomograph. They presented a graphical method for estimating the water content/time relationship of the vegetable as it travelled through a tunnel drier.

Ede & Hales[5] made an extensive study of the drying of potatoes and carrots by the cross flow of air and found that the drying rates were directly proportional to the W.B.D. of the air stream. Ede & Hales devised unit drying time and rate curves for potato strips with air-speed and bed-loading correction factors.

Hendry & Scott[6] showed how the W.B.D. evaporation coefficients could be used to design both concurrent and counter-flow vegetable dehydrators. Van Arsdel[7] gave a summary of the principles of tunnel-dehydrator design arising from the war-time experience of the U.S. Department of Agriculture.

During the war, continuous through-circulation driers for vegetable and meat dehydration were being used increasingly as their operating characteristics became better known.[8]

Kraybill[9] described a process for dehydrating meat in a two-stage continuous through-circulation drier in which fresh minced meat (55% of water) was loaded to a depth of 1½ in. and dried to 34% of moisture, then reloaded to a depth of 4½–5 in. on a second belt and dried to 10%. This use of a greater bed-depth in the second stage was made possible by the increased porosity of the bed of partly dried material.

During the early part of the war, the Ministry of Food[10] carried out pilot-plant drying tests on a single-pass through-circulation drier with potato and carrot strips and cabbage shreds. It was found that carrots and potatoes could be dried satisfactorily if due attention was paid to the air flow and strip size, but the cabbage shreds were blown off the belt very easily. If the thickness of the cabbage layer was over 2 in., the bed soon developed blowholes and scorched patches, but the other vegetable strips rapidly formed a porous coherent bed which showed little shrinkage during drying. It was considered inadvisable to use multi-stage conveyors, as considerable fracturing of the strips was expected at the transfer point when the bed was being disintegrated and reformed.

A later test[10] was made on a full-size through-circulation drier which had three air-temperature zones of 160°, 180° and 210° F. The wet material passed through the high-temperature zone first, so that as drying proceeded the bed entered the cooler regions. The exhaust air from the coolest zone was mixed with hot gases from a furnace to form the supply air to the middle zone, and similarly this exhaust was recirculated through the high-temperature zone. On a test with potatoes cut into ridged cossettes 1 mm. thick, an evenly-dried product free from scorched patches was obtained.

Ede[11] presented data on the effect of bed depth on the through-circulation drying of carrot, potato and meat. Brown & Van Arsdel[12] presented nomographs for the through-draught drying of potato strips and demonstrated how these data could be used to calculate the water content/time relationship for heavily loaded beds by subdividing the bed into a number of thinner layers.

Marshall[13] provided data for the through-circulation drying of foodstuffs, in which he demonstrated the advantages to be gained by mixing and reloading vegetable beds during drying. In one instance, the constant-drying rate of a bed of diced beet was nearly doubled by mixing the bed when about 40% of the water had been removed.

Burton[14] recommended the use of multi-stage driers for vegetable dehydration, as this enabled the heating load at various stages of drying to be proportioned more accurately. He advocated the use of finishing bins where vegetables are to be dried to low final water contents.

Coles[15] summarized the various arrangements of through-circulation driers with reference to the drying of rayon fibre, and he also listed some of the desirable practical features in the design of continuous conveyor driers.

Simmonds, Ward & McEwen,[16] in recent work on through-circulation drying of wheat-grain, found that for shallow beds the drying rates were not proportional to the wet-bulb

depression of the air. They further showed[17] that the drying rate was proportional to the free water content of the grain, and presented a method of predicting the drying rate of deep beds for given air conditions.

Design of a drier for *L. cloustoni* stipe

Several factors to be considered in a drier design are apparent from the laboratory tests.

(*a*) The dry-bulb temperature (D.B.T.) of the air should not exceed 225° F for static beds of *L. cloustoni* stipe as scorching takes place at temperatures above this value.

(*b*) There is evidently little advantage to be gained by using air flows greater than 8–9 lb./(sq. ft.)(min.) for stipe beds, since the over-all drying time is not greatly shortened at higher air velocities, although it would probably be advantageous to use higher air velocities in the initial stages when the constant drying rate increases linearly with air velocity.

(*c*) The minimum bed-depth should be 3 in. (dry loading L_d = 1·28 lb. of B.D.S./sq. ft., where L_d is bed loading) as the output decreases rapidly below this value. The shrinkage of the bed appears to be virtually complete at a water ratio of 1–1·5, so that this may be a convenient point to deepen the bed to make more effective use of the drying air.

Flow sheets for some of the commoner types of continuous through-circulation driers are shown in Fig. 1.

Type I. Single-stage conveyor drier

Example 1.—A continuous through-circulation drier, 60 ft. long by 6 ft. wide, is to be designed to dry *L. cloustoni* stipe slices ($\frac{1}{8}$ in. thick) from 5·5 to 0·15 water ratio with an air-flow rate of 7·5 lb./(sq. ft.)(min.). The drying air at 220° F D.B.T. and 95° F W.B.T. (wet-bulb temperature) is to be heated from atmospheric air at 50° F D.B.T., 49° F W.B.T. If the seaweed loading on the belt is 3 lb. of B.D.S./sq. ft., determine (*a*) drying time, (*b*) output of C.D.S. (commercial dry solid) per h., (*c*) evaporation rate and (*d*) specific evaporation.

Total heat of air at 49° F W.B.T. = 12 B.Th.U./lb. and at 95° F W.B.T. = 54·9 B.Th.U./lb. (from Tables by Macey[18]).

(*a*) From the basic drying-time curve for *L. cloustoni* stipe slices,

$$\theta^{5}_{0\cdot15} = 170 \text{ h./° F W.B.D., where } \theta = \text{drying time, min.}$$

Loading factor for L_d, 3·0 lb. of B.D.S./sq. ft. = 0·72

Air-velocity factor for G [mass air flow, lb. of dry air/(min.)(sq. ft. of cross-sectional area of bed)], 7·5 lb./(sq. ft.)(min.) = 1·0

Hence for 125° F W.B.D. and L_d = 3·0 lb. of B.D.S./sq. ft.,

$$\text{drying time} = \frac{170 \times 60}{1\cdot0 \times 0\cdot72 \times 125} = 1\cdot89 \text{ h.} = 114 \text{ min.}$$

(*b*) $$\text{Output} = \frac{360 \times 3 \times 1\cdot15}{1\cdot89} = 656 \text{ lb. of C.D.S./h.}$$

(*c*) $$\text{Evaporation} = \frac{656 \times 5\cdot35}{1\cdot15} = 3060 \text{ lb./h.}$$

(*d*) $$\text{Heating load} = 7\cdot5 \times 60(54\cdot9 - 12) \times 60 \times 6$$
$$= 6{,}950{,}000 \text{ B.Th.U./h.}$$

$$\text{Therefore specific evaporation} = \frac{6{,}950{,}000}{3060} = 2270 \text{ B.Th.U./lb. of water evaporated.}$$

This example represents the simplest design of through-circulation drier (Fig. 1, Type I) and it can be solved by the direct application of the basic drying curve for stipe (Part I,[1] Fig. 18). It is assumed for the present calculations that there is no ' scale up ' factor involved in the calculations for the full-sized drier, and also that there are no heat losses. The specific evaporation will depend on the temperature and humidity of the atmospheric air, and practical values of specific evaporation must also include the thermal efficiency of the furnace or air heater. This lay-out of drier has a high output because the seaweed bed is supplied with hot dry air at all stages of drying, but has the disadvantage that the heat economy is correspondingly low.

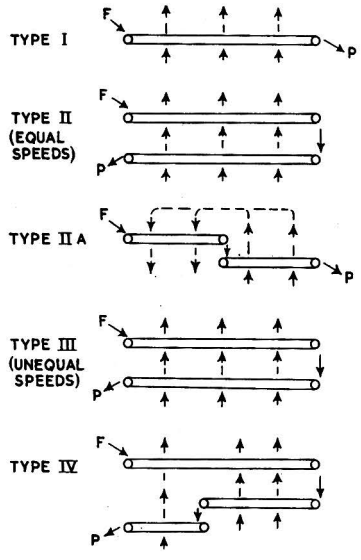


FIG. 1.—*Flow sheets for continuous through-circulation driers*
F = Feed; P = Product; --→ Air; ⟶ Solid

Type II. Double-stage conveyor drier

In this design, the 60-ft. belt is replaced by two superimposed belts 30 ft. long, the upper one discharging on to the lower (Fig. 1). The hot air leaving the dried seaweed at the discharge end of the drier passes through the fresh seaweed on the upper belt, thereby improving the thermal efficiency at the cost of a lower output.

Example 2.—A two-stage through-circulation drier with belts 30 ft. × 6 ft. is to be used to dry *L. cloustoni* stipe slices $\frac{1}{8}$ in. thick from 5·5 to 0·15 water ratio, with air conditions similar to those in Example 1. Both belts are to be operated at the same speed, and the loading is to be 3 lb. of B.D.S./sq. ft. Calculate (*a*) drying time, (*b*) water ratio at the change-over, (*c*) product rate, (*d*) evaporation rate and (*e*) specific evaporation.

The problem in this instance is to determine the total drying time such that the times in each stage are equal. This is complicated by the fact that the supply air to the upper belt is not constant as its humidity varies along the length of the drier.

A trial-and-error method can be used in which an assumed value for the water ratio at the turnover (T') is taken. From the basic curve, the time for the seaweed to dry from T' to 0·15 on the lower belt can be computed. From the average evaporation rate the average exhaust humidity can be evaluated by means of a moisture balance. By using the average W.B.D. from the bottom belt, the drying time for 5·5 to T' can then be found. This procedure is repeated until the two drying times are equal. To obviate the possibly tedious calculations, the following graphical design method has been devised, which is more positive in action.

Graphical design method for double-stage drier

(1) From the basic drying curve calculate the water content/time curve of the lower belt under the given conditions and plot it with a reversed time scale, i.e. with zero time at 0·15 water ratio.

(2) Select suitable equal time-increments and interpolate the terminal water ratios for each increment from the curve.

(3) Calculate the water picked up by the air passing through each increment of the bed (by using equation 1 below).

(4) Add the water pick-up to the inlet-air humidity to give the humidity of the air leaving each section of the lower belt. Assume a constant W.B.T. for the hot air and obtain the D.B.T. and hence the W.B.D. from a psychrometric chart.

(5) From the basic drying curve and the appropriate W.B.D. compute the water content of the seaweed on the top belt as it reaches the end of each successive time-increment.

(6) Plot the water ratio/time curve for the upper belt until it intersects the corresponding curve for the lower belt. This intersection gives the water content at the turnover and the drying time on each belt (which are equal in this case).

The water pick-up in lb. of water per lb. of dry air is given by:

$$\frac{L_d(T_0 - T_1)}{G\theta}$$

where T_0 and T_1 = terminal water ratios for each increment
θ = incremental time, min.
G = air mass velocity, lb/(sq. ft.)(min.)

The calculations for Example 2 can be conveniently set out in tabular form with a 10-minute time interval (Table I), and Fig. 2 shows the graphical solution to the problem.

Table I

Increment, min.	Terminal water ratios	ΔT	Water pick-up, lb./lb.	Exhaust air from lower belt		
				Humidity, lb./lb.	D.B.T., °F	W.B.D., °F
0–10	0·15–0·20	0·05	0·0020	0·0092	211	116
10–20	0·20–0·28	0·08	0·0032	0·0104	206	111
20–30	0·28–0·40	0·12	0·0048	0·0120	200	105
30–40	0·40–0·65	0·25	0·0100	0·0172	177	82
40–50	0·65–1·05	0·40	0·0160	0·0232	151	56
50–60	1·05–1·60	0·55	0·0220	0·0292	125	30
60–70	1·60–2·20	0·60	0·0240	0·0312	116	21
70–80	2·20–2·90	0·70	0·0280	0·0352	99	4

The time scale of the basic chart for stipe is graduated in 10-hour divisions for unit W.B.D., so that, from a knowledge of the W.B.D. and the relevant loading factors, the number of these divisions corresponding to the 10-minute interval can be calculated, and the water ratio at the end of the increment can then be read off from the curve.

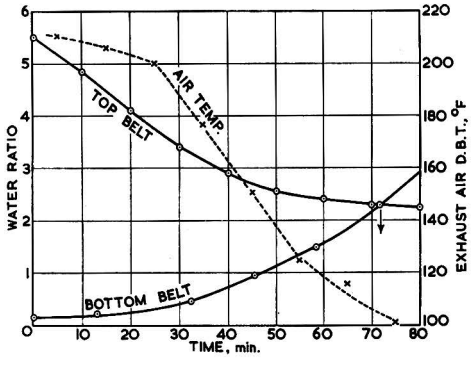


FIG. 2.—*Double-stage drier, Example 2 :* L. cloustoni *stipe (sliced)*

This graphical design method may also be used in conjunction with other methods of correlating drying times (e.g. Brown & Van Arsdel,[12] Simmonds *et al.*[16, 17]), if it is assumed that drying is adiabatic.

(*a*) and (*b*) By interpolation from Fig. 2, the drying time on each belt is 72 min. and the water ratio at the turnover is 2·3.

(*c*) Product rate $= \dfrac{180 \times 3 \times 1{\cdot}15 \times 60}{72} = 517$ lb. of C.D.S./h.

(*d*) Evaporation rate $= \dfrac{180 \times 3 \times 5{\cdot}35 \times 60}{72} = 2410$ lb./h.

(*e*) Heating load $= 450(54{\cdot}9 - 12) \times 180$ B.Th.U./h.
$= 3{,}480{,}000$ B.Th.U./h.

Specific evaporation $= \dfrac{3{,}480{,}000}{2410} = 1440$ B.Th.U./lb.

It can thus be seen that the heating load is halved by superimposing the belts, and that the air is much more effectively used than in a single-pass drier which must discharge relatively dry air when the bed is nearly dry. The two-stage drier is operating with an equivalent bed-loading of 6 lb. of B.D.S./sq. ft.

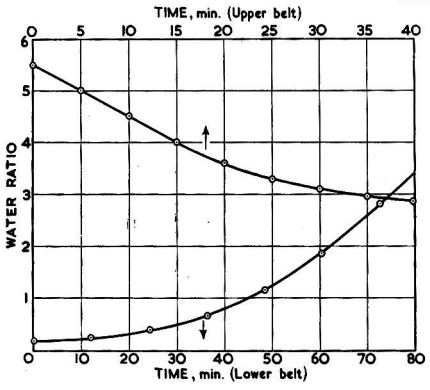


FIG. 3.—*Double-stage drier (unequal belt-speeds), Example 4:* L. cloustoni *stipe (sliced)*

An alternative way of achieving the same result is to arrange the two belts in series, so that the exhaust air from the second half of the drying cycle is blown through the wet fresh seaweed (Fig. 1, Type IIA). The same graphical method can be adapted to the design of this type of drier, although the tandem layout would be less compact.

Example 3.—For comparison, the data have been calculated for a drier identical to that of Example 2 except that the bed loading on each belt was reduced to 1·28 lb. of B.D.S./sq. ft.

The corresponding data for Example 3 are: drying time (total) 92 min., product rate 345 lb./h., evaporation rate 1600 lb./h., specific evaporation 2180 B.Th.U./lb.

This shows clearly the advantage of using the deepest convenient bed for stipe. A drier with intermediate characteristics can be obtained if the lower belt is operated at half the speed of the top belt, thus doubling the bed loading on the lower band.

Type III. Double-stage conveyor drier (unequal belt-speeds)

Example 4.—If the drier of Example 2 is run so that the top-belt loading is 1·28 lb. of B.D.S./sq. ft. and the lower is 2·56, calculate (*a*) time on each belt, (*b*) output rate, (*c*) evaporation rates and (*d*) specific evaporation.

The problem is approached in a similar manner to Example 2 for the calculation of the times on the bottom belt.

The time axis for the upper belt is now plotted on a scale twice that of the lower belt (Fig. 3) since, for a given increment of drier length, the seaweed on the upper belt will be exposed to the air for only half the time of the material on the lower belt. The curve for the top belt is plotted in the usual way until it intersects the curve for the lower belt. The output can be calculated from the B.D.S. flow/h. on either belt by using the appropriate bed-loading and residence time, since the flow of B.D.S. through the drier must be constant. The output and evaporation rates for this arrangement are respectively 408 and 1900 lb./h. and the specific evaporation is 1830 B.Th.U./lb.

The substitution of minced stipe for sliced stipe would increase the output when a single-stage drier is used, but for multi-stage driers the drying time has a diminishing influence on the output. As the efficiency of evaporation is increased, the output will eventually depend on the water-uptake capacity of the drying air.

Design of a drier for *L. cloustoni* frond

Some of the drying characteristics of *L. cloustoni* and *L. digitata* frond are summarized below.

(*a*) To prevent scorching of the frond, the air D.B.T. should not exceed 225° F.

(*b*) Air velocities above approximately 8 lb./(sq. ft.)(min.) result in a comparatively small reduction in drying time.

(*c*) There is an optimum bed-loading for frond which appears to vary with the particle size and the time of year when the plant was harvested.

(*d*) Agitation of the frond bed during drying brings about a marked reduction in drying time, particularly if the bed is broken up in the later stages of drying.

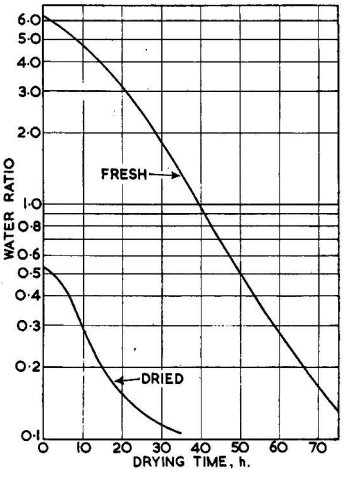
(*e*) The drying times for frond show a substantial seasonal variation.

Type I. Single-stage conveyor drier

Example 5.—If the drier described in Example 1 is loaded with minced *L. cloustoni* frond,

FIG. 4.—L. cloustoni *frond : basic drying-time curves for unit W.B.D.,* ° F

	L_d	G
Fresh	0·382	7·2
Dried	3·64	8·1



under similar air conditions, determine (*a*) drying time, (*b*) production rate, (*c*) evaporation rate and (*d*) specific evaporation.

It has been shown[2] that there is an optimum bed-loading for frond which gives the maximum output. The experimental run that was closest to the peak in Fig. 2, Part II[2] was selected to form the basic drying curve for *L. cloustoni* frond, and it is plotted on a semilog graph in Fig. 4. It should be noted that this curve applies strictly only to Oban seaweed harvested in May, because of the seasonal variation previously observed.

The optimum bed-loading for minced frond for May was 0·382 lb. of B.D.S./sq. ft. with air at 156° F, but it will be assumed that the optimum loading will be the same if air at 220° F is used. An air flow of 7·2 lb./(sq. ft.)(h.) has been adopted as standard.

(*a*) $\theta^{5}_{0.15} = 67$ h./° F (from Fig. 4)
Therefore for 125° F W.B.D., drying time = 32·1 min.
(*b*) Output = 301 lb./h.
(*c*) Evaporation rate = 1140 lb./h.
(*d*) Heat input = 6,680,000 B.Th.U./h.
Specific evaporation = 5860 B.Th.U./lb.

It is evident that the efficiency of drying frond is much lower than that of stipe, largely owing to the comparatively shallow bed that must be used. The position can be improved by using a two-stage drier.

Type II. Double-stage conveyor drier

Example 6.—If the drier in Example 5 is replaced by a double-belt drier (30 ft. × 6 ft.) determine the corresponding drying data. The belt speeds are to be equal. By using the design method described in Example 2 the total drying time of 34 min. was obtained. The output was only slightly smaller (279 lb./h.), but the specific evaporation was markedly reduced to 2510 B.Th.U./lb.

It has been demonstrated that much heavier bed-loadings may be employed when the frond is pre-dried to about 0·5 water ratio. No data are available for this material when being dried at intermediate water ratios, e.g. starting from 1·5–2·0, but it seems probable that the bed loading could be progressively increased as the frond became less sticky, resulting in improved efficiency of drying. Moreover the transfer points at the end of the belts will give some degree of agitation to the bed and will promote more rapid drying.

A three-stage drier can, however, be designed in which the drying from $T = 0{\cdot}5$ to $T = 0{\cdot}15$ is carried out at the heavy bed-loading, so that the last belt is in effect a finishing bin.

Type IV. Triple-stage conveyor drier

Example 7.—A three-stage drier for minced *L. cloustoni* frond is to be designed as shown in Fig. 1, Type IV, such that the loading on the final belt is to be increased tenfold. The bed loading for the first two stages is 0·382 lb. of B.D.S./sq. ft., and the air conditions are identical to those in Example 5. If the drier is 30 ft. long by 6 ft. wide, evaluate (*a*) drying time in each stage, (*b*) length of the last belt, (*c*) product and evaporation rates and (*d*) specific evaporation.

The example can be solved by first computing the W.B.D. of the exhaust air from the

deepest bed (belt 3) and hence calculating the moisture content of the frond on the top belt as it leaves the zone above the third stage. The remainder of the problem now becomes similar to that of Example 6, except that the terminal water content is different.

(*a*) From the basic curve for frond at $L_d = 3{\cdot}82$ (Fig. 4) the drying time for $T = 0{\cdot}5$ to $T = 0{\cdot}15$ is 11·5 min. for 125° F W.B.D. The slight variations of the bed depth and air velocity may be neglected in this region of low water content.

The average water pick-up in this time is 0·0762 lb./lb. of air, corresponding to an exhaust D.B.T. of 150° F. As the top belt is in contact with this air for only 1·15 min. the loss of water will be small and has been neglected.

The graphical solution for the range 5·5 to 0·5 water ratio is given in Fig. 5, from which it can be seen that the drying time is 12·5 min. Hence the time on the top belt is 12·5 + 1·15 = 13·65 min., and on the second and third belts 12·5 and 11·5 min. respectively.

(*b*) Let the length of the third belt = X ft. and the speed of the second belt = Y ft./min.
Hence $X = 11{\cdot}5 \times 0{\cdot}1Y$ and also $X = 30 - 12{\cdot}5Y$;
thus $1{\cdot}15Y = 30 - 12{\cdot}5Y$, and $Y = 2{\cdot}2$ ft./min. and $X = 2{\cdot}53$ ft.

(*c*) Product rate = 349 lb. of C.D.S./h.
Evaporation rate = 1625 lb./h.

(*d*) Heat input = 3,340,000 B.Th.U./h.

Hence, specific evaporation = 2060 B.Th.U./h.

It may be possible to reduce this specific evaporation figure still further by recirculation of the air.

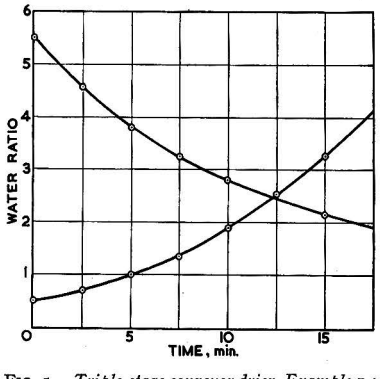


FIG. 5.—*Triple-stage conveyor drier, Example 7 :* L. cloustoni *frond (minced)*

As has been shown in Part II, the drying time of frond varies with the season, so that the output of dried product will likewise fluctuate. The belt speeds of the drier should be capable of control within wide limits to enable the drier to be adapted to the prevailing conditions.

Driers of larger capacity than those in the examples can be designed by increasing the length and breadth of the conveyors. The largest belt-width used in normal practice is about 10 ft.

It is recommended that pairs of spiked rollers be installed at the end of the belts to ensure that the frond particles in the partly dried bed are separated and do not form wet knots on the following belt. Shredding of the frond, if practicable on the large scale, should give a bed with greater porosity, resulting in lower fan-power requirements.

Discussion of scale factors

The preceding calculations have neglected one factor—the elusive ' scale up ' factor which is required to allow for differences between laboratory and commercial operating conditions. This factor consists of a number of components which are summarized below.

Uneven air distribution is probably the major inherent cause of error in through-circulation drying. Ede[11] found that beds of vegetable material had a tendency to develop weak spots through which the air passed more readily than the remainder. The resultant increased drying and shrinkage made the blowhole worse and preserved the conditions which Ede described as ' auto catalytic drying '. This condition was not serious for the laboratory tests on stipe since the slight movement of the basket when it was removed for weighing tended to fill up any large voids. The relatively deep beds used for stipe should lessen the chances of any channel's persisting from the bottom to the top of the layer.

Edge effect—the shrinkage of the bed away from the basket walls—was noticeable with frond, but the maladjustment of the air was possibly minimized by the relatively high resistance

of the gauze on the basket floor. In full-size practice the edge effect would be very much smaller owing to the bed perimeter per unit area being lower. The drying rate for a given velocity on the small drier will therefore be conservative if the large drier is supplied with air uniformly distributed. If the air flow is uneven, the predicted drying times can be in serious error.

Conduction from the basket walls will tend to increase the drying rates for the laboratory runs, but radiation errors should be negligible, since the surface exposed to radiation is a small fraction of the total surface available for mass transfer, especially where deep beds are involved.

It appears, therefore, that the dual effects of edge effect—the increase of drying rate by conduction and the decreased rate by the air short-circuiting the bed—are in opposition. It is probable that the air leakage will be the predominant factor, but their relative magnitudes can be evaluated only by tests on different sizes of driers.

The use of the proportionality of the drying rate to W.B.D. for calculating drying rates at higher temperatures may involve an error as the basic curve was derived from a run at a moderate temperature. This error should also be conservative since higher temperatures will tend to shorten the drying times towards the end of the drying run. The bed-loading and air-velocity factors should be used cautiously since they are average values for the total drying time and may not hold for drying rates at intermediate water contents.

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References

[1] Gardner, R. G., & Mitchell, T. J., *J. Sci. Fd Agric.*, 1953, **4**, 113

[2] Gardner, R. G., & Mitchell, T. J., *J. Sci. Fd Agric.*, 1953, **4**, 237

[3] Gardner, R. G., & Mitchell, T. J., *J. Sci. Fd Agric.*, 1953, **4**, 364

[4] Brown, A. H., & Kilpatrick, P. W., *Trans. Amer. Soc. mech. Engrs*, 1943, **65**, 837

[5] Ede, A. J., & Hales, K. C., *Spec. Rep. Fd Invest. Bd, Lond.*, No. 53, 1948

[6] Hendry, R., & Scott, A. W., *J. Inst. Fuel*, 1950, **23**, 285

[7] Van Arsdel, W. B., 'Principles of the Drying Process with Special Reference to Vegetable Dehydration', U.S. Dept. Agric. A.I.C.—300, 1951

[8] Ramage, W. D., & Rasmussen, C. L., *Food Ind.*, 1943, **15**, (7), 64

[9] Kraybill, H. R., *Industr. Engng Chem. (Industr.)*, 1943, **35**, 46

[10] 'Dehydration', D.S.I.R. & Min. of Food, Sect. VI, Part 3, 1943 (London: H.M.S.O.)

[11] Ede, A. J., 'Dehydration', D.S.I.R. & Min. of Food, Sect. X, Part 2, 1943 (London: H.M.S.O.)

[12] Brown, A. H., & Van Arsdel, W. B., 'Drying Rate Nomographs. III. White Potato Strips—Vertical Air Flow', U.S. Dept. Agric. A.I.C.—31—III, 1944 (Reissued 1951)

[13] Marshall, W. R., *Heat. Pip. Air Condit.*, 1942, **14**, 724

[14] Burton, L. V., *Food Ind.*, 1943, **15**, (5), 51

[15] Coles, W. V., *J. Inst. Fuel*, 1952, **25**, 60

[16] Simmonds, W. H. C., Ward, G. T., & McEwen, E., *Trans. Instn chem. Engrs, Lond.*, 1953, **31**, 265

[17] Simmonds, W. H. C., Ward, G. T., & McEwen, E., *Trans. Instn chem. Engrs, Lond.*, 1953, **31**, 279

[18] Macey, H. H., 'Drying in the Heavy Clay Industries', Min. of Works, 1950 (London: H.M.S.O.)

Note

In Part I of this series (*J. Sci. Fd Agric.*, 1953, **4**, 113), for '$dW/d\theta = 0{\cdot}525[L_d]^{-1{\cdot}12}$' in the 3rd line of p. 122 read '$dW/d\theta = 5{\cdot}25[L_d]^{-1{\cdot}12}$'.

In Part III of the series (*J. Sci. Fd Agric.*, 1953, **4**, 364), for '$\theta = 296 \log_{10}(L_d + 2{\cdot}02) - 0{\cdot}102$' in the second line from the bottom of p. 368, read '$\theta = 296 [\log_{10}(L_d + 2{\cdot}02) - 0{\cdot}102]$'.

DIFFERENCES IN ESTERASES FROM INSECT SPECIES: TOXICITY OF ORGANO-PHOSPHORUS COMPOUNDS AND *IN VITRO* ANTI-ESTERASE ACTIVITY

By K. A. LORD and C. POTTER

Two types of esterase, the one capable of hydrolysing phenyl acetate and not acetylcholine, the other able to hydrolyse acetylcholine, have been examined in adults of four species of insect—*Tenebrio molitor* L., *Tribolium castaneum* Hbst., *Dysdercus fasciatus* Sign. and *Blatella germanica* L.

The *in vitro* inhibition of both types of enzyme from each species has been examined with five organo-phosphorus compounds: tetraethyl pyrophosphate, paraoxon, parathion, *OO*-diethyl *S*-*p*-nitrophenyl phosphorothiolate and *OS*-diethyl *O*-*p*-nitrophenyl phosphorothiolate. Both types of esterase were inhibited by high dilutions of inhibitors. Susceptibility of both types of enzyme appeared to vary from species to species and may provide a basis for specific differences in resistance to insecticides.

There is no consistent correlation between the toxicity of the compounds examined and the *in vitro* inhibition of either kind of esterase. Parathion did not inhibit any of the esterases. The other four substances were, in general, less potent inhibitors of the esterases that hydrolysed acetylcholine than those that did not. Therefore both types of esterase are worthy of further investigation since there are insufficient data to decide which type of esterase is involved in the insecticidal action of organo-phosphorus compounds.

Introduction

It is generally accepted that the mammalian toxicity of organo-phosphorus compounds is related to their anticholine-esterase activities. In this field a considerable body of evidence, both biochemical and pharmacological, showing this to be the case, was reviewed by Webb[1] as early as 1948. A number of workers, among them Dubois & Mangun,[2] Metcalf & March[3] and Chamberlain & Hoskins,[4] have suggested that the insecticidal action of organo-phosphorus compounds results from the inhibition of choline-esterase activity associated with nervous tissue, in a manner closely analogous to the mode of toxic action of these substances in mammals. The evidence of insecticidal action is, however, not conclusive at present. For example, Chadwick & Hill[5] and Roeder[6] showed that the insecticidal action of certain compounds closely paralleled the power to inhibit an enzyme that hydrolysed acetylcholine *in vitro*. The enzyme was associated with the nervous system, but they were unable to show that acetylcholine was involved in the nervous activity of the American roach. In addition, acetylcholine and some of its analogues did not appear to increase the action of the choline-esterase-inhibiting substances they used. In work of this nature, negative evidence does not exclude the inhibition of choline-esterase as the mode of insecticidal action of organo-phosphorus compounds, but it does introduce an element of doubt. This doubt is reinforced, not only by the gross differences between the structure of insects and mammals, but also by the different chemicals, and consequently enzymes, which are known to be used by different living organisms to perform similar over-all functions. The variety of respiratory pigments known, and the utilization of creatine phosphate by vertebrates and arginine phosphate by invertebrates in muscle metabolism are but two examples of this diversity of method.[7] The certain identification of acetylcholine[8] in one species of insect, when taken in conjunction with other evidence, supports the theory that organo-phosphorus insecticides may act by inhibition of choline-esterases. However, it does not exclude the possibility that acetylcholine may be absent from some species, although some of them may contain enzymes that hydrolyse acetylcholine *in vitro*. In addition it is possible that death may be caused in some species that utilize acetylcholine by the inhibition of enzymes other than choline-esterase.

Until further evidence is available, use of the term ' choline-esterase ' may be misleading. We prefer to use the more general term ' esterase capable of hydrolysing acetylcholine ' where it is necessary to distinguish these enzymes from those esterases that do not hydrolyse acetylcholine to any significant extent *in vitro*. In these circumstances the inhibition of enzymes other than those that hydrolyse acetylcholine is worthy of investigation. This suggestion has already been advanced by Lord & Potter,[9] who demonstrated the inhibition, by organo-

phosphorus compounds, of an esterase from *Tenebrio molitor* L. larvae that did not hydrolyse acetylcholine. This view has received further support from the work of Hopf,[10] who studied the effects of injecting acetylcholine and analogous esters both alone and with TEPP (tetraethyl pyrophosphate) and eserine on *Locusta migratoria migratoriodes* R. & F. Nevertheless Hopf concluded that there was insufficient evidence to arrive at any definite conclusions.

For these reasons we decided to follow up our preliminary results[9] by working on the inhibition of insect esterases, including those that hydrolyse acetylcholine, by parathion (*OO*-diethyl *O*-*p*-nitrophenyl thionphosphate), two of its isomers (*OO*-diethyl *S*-*p*-nitrophenyl phosphorothiolate and *OS*-diethyl *O*-*p*-nitrophenyl phosphorothiolate), paraoxon (diethyl *p*-nitrophenyl phosphate) and TEPP. Two types of esterase were studied: the one hydrolyses phenyl acetate rapidly but not acetylcholine to any significant extent, the other hydrolyses acetylcholine. The enzyme preparations were not highly purified, so that more than one enzyme might have been hydrolysing either of the substrates.

The first objective of the investigation was to compare the inhibition *in vitro* of these two types of esterase by organo-phosphorus compounds, since, if all else is equal, the poisons would have their first action on the more susceptible enzyme. The two types of enzyme were studied in each of four species of insect in order to determine what variation, if any, occurs between species. The second objective was to determine if there was any correlation between the toxicity of the compounds studied and their capacity to inhibit *in vitro* either type of enzyme activity. This comparison was also made with the same four species of insect. It was thought that if there were a correlation between toxicity and the inhibition of one type of esterase, but not the other, it would be an indication as to the kind of enzyme that is of primary importance to the action of the poisons. There are, however, many factors, such as penetration to the site of action and metabolism or decomposition *en route*, that are likely to affect *in vivo* toxicity, so that an obvious correlation cannot be expected.

Because of the very different methods required to obtain the results on toxicity and enzyme inhibition, the experimental techniques have been separately described, but the results cannot be so considered if maximum use is to be made of them.

Experimental

Biological

Four insect species were used throughout the tests, *Tribolium castaneum* Hbst., *Tenebrio molitor* L., *Dysdercus fasciatus* Sign. and *Blatella germanica* L. These species were chosen since they represent three different orders of insects, and are readily reared in quantity under standard conditions. Both male and female adults of *T. castaneum* and *T. molitor* were used, but only male adults of *D. fasciatus* and *B. germanica* because it is found that more reproducible toxicological results are obtained in this way.

Rearing of insects

T. castaneum were reared on National Wholemeal Flour at constant temperature (28–30°) in 2-lb. jam jars.

T. molitor were reared in dust-bins on a mixture of bran, chicken meal and poultry yeast with slices of raw potato. No attempt was made to control the temperature, which would normally be between 18 and 24°, although wider temperature variations were possible.

D. fasciatus were reared on black cottonseed with moistened cotton wool in cages kept at 28–30°.

B. germanica were reared at 28–30° on a diet consisting of rolled oats and dried skimmed milk with water supplied by drinking fountains.

The same stages and sexes were used both for the biological assay and the preparation of enzyme extracts.

Bio-assay

As far as possible measured amounts of solutions of known strength were applied to individual insects, in order to avoid the uncertainty in the actual amount of poison applied to an insect which necessarily occurs when a spraying technique is used. When a poison is sprayed

its physical and chemical properties will affect to some extent the actual dose received by an insect. Only for *T. castaneum* was a spraying method used. The insects were at once removed from the treated surfaces to eliminate, as far as possible, the effects of pick-up from these surfaces. Such effects will vary from poison to poison, since the stability and other physical and chemical properties of the poison may be expected to exert considerable influence on the dose picked up by an insect in long contact with a treated surface.

Table I

Toxicity of five organo-phosphorus compounds to four species of insects

	Log(concn., %) to give 50% kill Direct spray	Log(dose, mg./kg.) to give 50% kill Topical application		
	T. castaneum	*T. molitor*	*B. germanica*	*D. fasciatus*
TEPP	$\bar{2}\cdot67 \pm 0\cdot036$	$0\cdot76 \pm 0\cdot039$	$0\cdot64 \pm 0\cdot034$	$0\cdot41 \pm 0\cdot057$
Paraoxon	$\bar{3}\cdot79 \pm 0\cdot010$	$\bar{1}\cdot46 \pm 0\cdot024$	$0\cdot18 \pm 0\cdot033$	$0\cdot05 \pm 0\cdot034$
Parathion	$\bar{3}\cdot59 \pm 0\cdot026$	$\bar{1}\cdot71 \pm 0\cdot050$	$0\cdot52 \pm 0\cdot024$	$0\cdot61 \pm 0\cdot194$
OS-Diethyl *O*-*p*-nitrophenyl phosphorothiolate	$\bar{2}\cdot48 \pm 0\cdot033$	$0\cdot30 \pm 0\cdot025$	$1\cdot03 \pm 0\cdot048$	$0\cdot90 \pm 0\cdot038$
OO-Diethyl *S*-*p*-nitrophenyl phosphorothiolate	$\bar{1}\cdot18 \pm 0\cdot047$	$0\cdot61 \pm 0\cdot019$	$0\cdot93 \pm 0\cdot075$	$1\cdot21 \pm 0\cdot050$
	Slope of probit lines			
TEPP	$3\cdot13 \pm 0\cdot31$	$4\cdot42 \pm 0\cdot93$	$5\cdot26 \pm 0\cdot67$	$2\cdot97 \pm 0\cdot61$
Paraoxon	$3\cdot34 \pm 0\cdot30$	$6\cdot60 \pm 0\cdot60$	$5\cdot75 \pm 0\cdot90$	$5\cdot28 \pm 0\cdot94$
Parathion	$5\cdot36 \pm 0\cdot62$	$2\cdot55 \pm 0\cdot64$	$4\cdot17 \pm 0\cdot95$	$1\cdot54 \pm 0\cdot87$
OS-Diethyl *O*-*p*-nitrophenyl phosphorothiolate	$3\cdot39 \pm 0\cdot30$	$5\cdot48 \pm 0\cdot76$	$3\cdot77 \pm 0\cdot85$	$4\cdot97 \pm 0\cdot43$
OO-Diethyl *S*-*p*-nitrophenyl phosphorothiolate	$2\cdot55 \pm 0\cdot52$	$5\cdot50 \pm 0\cdot52$	$2\cdot47 \pm 0\cdot90$	$3\cdot22 \pm 0\cdot60$
Mean weight of individuals	—	0·12 g.	0·045 g.	0·05 g.

After treatment all insects were kept at 20° and 70–75% r.h. The results obtained were examined by the method of probits. In Table I only one set of experimental results has been quoted for each species. In each case duplicate determinations were carried out and the results were not statistically different from those quoted and agreed with those from preliminary tests.

T. castaneum were sprayed, in Petri dishes covered with filter paper, by means of the spraying tower described by Potter[11] with a medium consisting of 0·1% of technical sodium lauryl sulphate and 10% of acetone in water. After being sprayed, the insects were transferred to clean 9-cm. Petri dishes covered with 9-cm. circles of No. 1 Whatman filter paper so that only the direct-contact effect of the substances was measured. Forty-five insects were used for each concentration. The apparatus was set to deposit approximately 5·5 mg. of spray fluid per square cm.

T. molitor, *D. fasciatus* and *B. germanica* were treated by the method of topical application, the poisons being applied in the form of acetone solutions by means of a micrometer syringe,[12] 1 mm.^{3} to each insect. In each case the insects were anaesthetized with carbon dioxide to facilitate handling and the accurate placement of drops. At each concentration 24 insects were used. *T. molitor* were treated by placing the drop of solution between the pronotum and anterior of the elytra in the scutellum and membrane; after treatment they were kept in a Petri dish with the base covered with filter paper. *B. germanica* and *D. fasciatus* were treated on the ventral side between the second pair of legs, after treatment the *B. germanica* were kept in Petri dishes, whose base was lined with filter paper, and *D. fasciatus* were kept in 100-ml. beakers covered with muslin.

T. castaneum and *T. molitor* were inspected four days after treatment. *B. germanica* and *D. fasciatus* were inspected one day after treatment and it is the results of these examinations that are quoted. A second examination after two days showed no significant change in mortality for any substance, except parathion, when mortality increased. With *D. fasciatus* it was found that deaths were liable to occur in the controls after more than one day.

Biochemical

Extracts for inhibition studies

Studies on enzyme inhibition are best carried out on preparations that show low or zero activity in the absence of added substrates. According to Lord & Potter,[13] crude extracts of insects show considerable production of acid in the absence of added substrates which, for some stages and species of insects, is of the same order or greater than the increase in acid production on the addition of acetylcholine. In order, therefore, to be able to make reliable inhibition studies on the hydrolysis of acetylcholine by insect extracts some degree of purification may be necessary.

Once a method of making a preparation with a suitably low blank has been worked out, it is convenient to prepare a considerable bulk of material and to store it, so that a reproducible and constant preparation may be used for a series of inhibition studies. It was found possible to preserve the acetylcholine-hydrolysing enzymes by freeze-drying the preparations once they were made. In this way stable preparations were made from which reproducible extracts could be prepared, as required, simply by stirring a weighed amount of powder with water. With the acetylcholine-hydrolysing enzyme from *T. castaneum* it was more convenient to prepare the extract freshly each day rather than to make a dried enzyme preparation.

Acetone powders of *T. castaneum*, *T. molitor*, *B. germanica* and *D. fasciatus* provided a convenient source of esterases that hydrolyse phenyl acetate. The processes used for making the acetone powders not only produced stable enzyme preparations but also ensured low blanks in the enzyme estimations. The enzyme extracts were prepared by stirring the acetone powders with water and centrifuging. The clear supernatant liquid was used, in each case, as the source of esterase activity, and showed a negligible blank when the activity was measured by the technique used for the inhibition studies.

Preparations that hydrolyse phenyl acetate.—A similar technique was used for the preparation of these esterases from all the species of insects used. Acetone powders of the insects were prepared by homogenizing batches of insects with cold acetone in a Waring Blendor. The solids were filtered off through a Buchner funnel, washed with more acetone, and finally dried in a current of air at room temperature, 18–22°. Extracts of the dried materials were prepared, as required, by stirring with water and centrifuging off the solids. The clear supernatant fluids containing the enzyme activity were used for the inhibition studies. Convenient extracts for use in inhibition studies were prepared by using the following amounts of acetone powders with 10 ml. of water: *B. germanica* 0·015 g., *T. castaneum* 0·085 g., *D. fasciatus* 0·01 g. and *T. molitor* 0·02 g.

Preparations that hydrolyse acetylcholine.—From *T. molitor*, 100 g. of adults were ground in a mortar with 200 ml. of water, and strained through muslin. The solids retained by the muslin were rejected and the extract was freeze-dried. The freeze-dried materials were extracted with two 100-ml. portions of ethanol, followed by three 100-ml. portions of ether. (This treatment was used to remove substances that give a high rate of acid production in the absence of added substrate.) A convenient extract of this material was prepared by stirring it with water (0·2 g./ml.) and incubating at 25° for 1–2 hours before use to further reduce the blank.

From *B. germanica:* adult males were ground with water and strained through muslin. The solids retained by the muslin were rejected and the extract was freeze-dried. A convenient extract was prepared by mixing the freeze-dried material with water (0·02 g./ml.).

From *T. castaneum:* extracts of whole *T. castaneum* adults do not appear to hydrolyse acetylcholine, although extracts of heads and thoraxes do.[13] The heads and thoraxes were removed from adults and homogenized in ice-cold water freshly each day. A convenient extract contained the heads and thoraxes from about 100 insects in one ml. (100 insects weighed 0·15 g.).

From *D. fasciatus:* batches of 400 (approximately 20 g.) whole adult male *D. fasciatus* were homogenized in a Waring Blendor with 50 ml. of ice-cold water to which 5 ml. of M-sodium bicarbonate solution had been added. The homogenate was strained through muslin, the solids

were rejected, and the extract dialysed, with stirring, against 3000 ml. of ice-cold 0·033M-sodium bicarbonate solution for six hours. This was followed by dialysis, without stirring, overnight (about 16 hours) against 5000 ml. of 0·0015M-disodium hydrogen phosphate. (Dialysis in this way virtually eliminated acid production by the extract in the absence of added substrate.[13] The material was then freeze-dried. Several such preparations were made and the products bottled. To ensure uniformity, the powder was ground in a mortar before use. A convenient extract was prepared by stirring the freeze-dried material with water (0·06 g./ml.).

Properties of extracts.—Heating to 70° for 5 minutes destroyed both types of esterase activity derived from all four species of insect.

Extracts of the acetone powders of *D. fasciatus*, *T. molitor* and *T. castaneum*, prepared by stirring with water, did not hydrolyse acetylcholine chloride when tested by the standard Warburg procedure.[14] The Warburg manometer vessels contained 1 ml. of extract, 0·5 ml. of 1% sodium bicarbonate solution, 1·2 ml. of water and 0·3 ml. of 0·5% acetylcholine chloride (placed in side-bulb before tipping). The manometers were gassed with a mixture of 95% of nitrogen and 5% of carbon dioxide. The reaction was carried out at 25°.

Extracts of acetone powder prepared from *B. germanica* by stirring with water hydrolysed acetylcholine. When the extract was centrifuged, the clear supernatant fluid did not hydrolyse acetylcholine (when tested as above); the solids, resuspended in water, hydrolysed acetylcholine.

The freeze-dried preparations all hydrolysed phenyl acetate when tested in the Warburg apparatus. The conditions were as described above for the hydrolysis of acetylcholine, except that the 0·5% solution of acetylcholine was replaced by a 0·5% solution of phenyl acetate in 5% ethanol.

Inhibition studies

As far as possible the inhibition studies were carried out in a standard manner, in order to avoid variations that are liable to arise from the presence of interfering substances in the relatively crude enzyme preparations used. For this reason stable preparations of each enzyme were used and the concentration of each enzyme from each source was kept approximately constant throughout the experiments. In each case (apart from the enzyme hydrolysing acetylcholine from *T. castaneum* adults) the material was taken from a single sample which was well mixed before use. These precautions also reduce possible errors that may occur when the rate of hydrolysis is not strictly proportional to the amount of extract used.

Inhibition was determined by comparison of enzyme activity in the presence of inhibitor with the activity of controls run at the same time under similar conditions. The controls differed from the other treatments only by the absence of inhibitor. Each treatment was arranged so that the reaction mixture contained the same concentration of buffer, solvent (ethanol), substrate and enzyme extract.

In all cases the concentration required to inhibit 50% of enzyme activity was determined at 25° by using a series of dilutions arranged to give between 0 and 100% inhibition. The ratio between successive dilutions of inhibitor was equal to, or less than, two, and all dilutions were prepared in 5% ethanol. The final concentration of ethanol in all determinations (including blanks and controls) was 1·5% for the study of the inhibition of hydrolysis of phenyl acetate and 1·25% for acetylcholine. Each estimation was carried out on at least two separate occasions. The standard error of all determinations lay between $\pm$ 0·1 and $\pm$ 0·05. The experimental results are summarized in Table II.

Inhibition of the hydrolysis of phenyl acetate.—Estimation of the residual esterase activity was carried out colorimetrically by the following method: 0·5 ml. of a suitable dilution of inhibitor in 5% ethanol (or 0·5 ml. of 5% ethanol for control and blanks) was added to a mixture of 0·5 ml. of 0·1M-phosphate buffer, pH 6·5, and 0·5 ml. of aqueous enzyme preparation, at 25°. Five minutes later, 0·5 ml. of substrate (0·02% phenyl acetate in 1% ethanol was added and the enzyme was allowed to react for 10 minutes at 25°. The reaction was then stopped and the amount of phenol liberated was determined by the addition of 1 ml. of a diazotized solution of 0·03% *p*-nitroaniline in 0·1N-hydrochloric acid, followed by the addition of 2 ml. of 0·1M-sodium carbonate solution. The colour produced was read off at 470 mμ one minute after the addition of the sodium carbonate solution. This method of estimating

phenol is a modification of a method described by Collins.[15] The readings obtained were corrected for blanks arising from colour-producing materials in the insect extracts and non-enzymic hydrolysis of phenyl acetate.

Inhibition of the hydrolysis of acetylcholine.—Residual activity was determined at 25° with Warburg manometers. The enzyme preparation (1·0 ml.), 0·5 ml. of 1% sodium bicarbonate solution and 0·45 ml. of water were placed in the main compartment of the manometer vessel.

Table II

The inhibition of esterases from four species of insect by organo-phosphorus compounds

pI_{50} [= $-\log_{10}$(molar concentration of inhibitor to produce 50% inhibition)]

	T. castaneum	*T. molitor*	*B. germanica*	*D. fasciatus*
Hydrolysis of phenyl acetate				
TEPP	6·7	7·8	8·4	6·1
Paraoxon	6·6	8·6	8·6	6·6
Parathion	No inhibition at log(molar concn.) 5·4			
OS-Diethyl *O*-*p*-nitrophenyl phosphorothiolate	6·9	8·9	9·0	7·0
OO-Diethyl *S*-*p*-nitrophenyl phosphorothiolate	5·9	7·7	8·6	7·6
Hydrolysis of acetylcholine chloride				
TEPP	7·6	6·7	7·8	7·2
Paraoxon	6·7	6·5	7·1	6·7
Parathion	No inhibition at log(molar concn.) 5·4			
OS-Diethyl *O*-*p*-nitrophenyl phosphorothiolate	6·1	5·7	6·4	5·9
OO-Diethyl *S*-*p*-nitrophenyl phosphorothiolate	6·2	6·0	6·8	5·9

Substrate solution (0·3 ml. 0·5% of acetylcholine chloride) was placed in the side arm. A suitable dilution of the inhibitor (0·75 ml.) in 5% ethanol (or 5% ethanol for controls and blanks) was then added to the enzyme solution and the manometer was gassed with a mixture of 5% of carbon dioxide and 95% of nitrogen. Thirty minutes after the inhibitor had been added to the enzyme preparation the substrate was tipped from the side arm into the main body of the vessel and the remaining enzyme activity determined.

The pH of each determination was 7·3 except for that of *T. molitor* extracts. The high buffering capacity of the *T. molitor* extract, a property not possessed by the other preparations, reduced the pH in the manometers to 7·0.

Discussion

Esterases studied

When enzyme preparations from similar living material act on a range of substrates there is a possibility that only one enzyme may be responsible. This is especially so when the substrates are attacked at a common grouping, such as an ester link. In the studies reported here, on the inhibition by organo-phosphorus compounds of the hydrolysis of two esters (acetylcholine and phenyl acetate) by extracts obtained from a number of insect species, the results would indicate that two groups of enzymes are involved. The two kinds of esterase differ in their ability to hydrolyse acetylcholine. By destroying the enzyme that hydrolyses acetylcholine or by removing it by physical processes it was possible to obtain preparations that hydrolysed phenyl acetate alone. On the other hand, all the extracts that hydrolysed acetylcholine also hydrolysed phenyl acetate. However, all studies on the inhibition of the hydrolysis of phenyl acetate were carried out with preparations that did not hydrolyse acetylcholine, so there would appear to be little doubt that the inhibition of two kinds of esterase activity has been studied.

The inhibition results in Table II also indicate that two different kinds of esterase have been studied. If in any one species the same enzyme were responsible for the hydrolysis of both phenyl acetate and acetylcholine then it might be expected that the same concentration of each inhibitor would be required to stop the hydrolysis of both substrates. This is clearly not the case for any species we have tested (Table II). In general, the enzymes hydrolysing phenyl acetate only are inhibited by lower concentrations of inhibitor. In only three cases

are similar concentrations of an inhibitor required to give a 50% inhibition of the two types of esterase activity in the same species. Thus the same concentration of paraoxon inhibits equally both the hydrolysis of phenyl acetate and of acetylcholine by extracts of *D. fasciatus* and *T. castaneum*. The two kinds of esterase from *T. castaneum* are also about equally susceptible to inhibition by *OO*-diethyl *S-p*-nitrophenyl phosphorothiolate. Apart from these minor exceptions the evidence available therefore indicates that the inhibition of two kinds of esterase has been studied and not the hydrolysis of two substrates by one enzyme.

The enzyme preparations were all crude, although in each case they had been submitted to various treatments that may be expected to effect some degree of purification. However, it is not possible to rule out the possibility that some of the organo-phosphorus inhibitors were inactivated or activated by constituents of some of the extracts used. This must be remembered when examining the inhibition results, although it is not considered that any general conclusions drawn from them are likely to be invalidated on this account. We consider that unequivocal inhibition data can be obtained only if highly purified enzyme preparations are used. Such material is also necessary to determine substrate spectra and other properties required for the characterization of the individual esterases.

Work is at present in progress to isolate and characterize the esterases referred to in this paper. It is hoped also to obtain a knowledge of their location in the tissues of the insect and to study their inhibition *in vivo* as well as *in vitro*.

Inhibition and toxicological results

The enzyme inhibition results (see Table II), taken alone, suggest that the enzymes we have studied vary from species to species. Thus for example, widely different concentrations of *OO*-diethyl *S-p*-nitrophenyl phosphorothiolate are required to inhibit the esterases that hydrolyse phenyl acetate and not acetylcholine, according to the species from which the enzyme preparations are derived. The ratios of the concentrations required for the inhibition of the enzymes from *T. castaneum, T. molitor, B. germanica* and *D. fasciatus* respectively are 500 : 10 : 1 : 10.

Differences of a similar nature occur with each of the inhibitors we have tested. It would seem that the esterases that do not hydrolyse acetylcholine differ according to the species from which they are derived, and this appears to be equally true of the enzymes that hydrolyse acetylcholine. The results therefore leave little doubt that esterases from different species of insect differ in their susceptibility to organo-phosphorus compounds. It must therefore be inferred that both types of esterase may differ from species to species, a finding that is in line with the observations of Myers[16] on the specificity pattern of mammalian and avian ψ-choline-esterases, and also with the findings of Mounter & Whittaker,[17] Metcalf & March[18] and Vincent & Lagreu.[19]

If the primary insecticidal action of organo-phosphorus compounds is due to their anti-esterase activity, the differences in susceptibility of the enzymes from one species to another could at least partly account for the differences of resistance that occur between species. Our results do not show any relationship between toxicity and power to inhibit either type of esterase *in vitro*.

If only one insect species is considered and the *in vivo* toxicity (Table I) of the various compounds is compared with the *in vitro* inhibition of the enzymes (Table II) derived from the same species, no relationship can be observed. A similar result was reported by Aldridge & Barnes[20] for mammals. A number of reasons may be put forward to account for this lack of correlation. Thus it is known that parathion which is not an active esterase-inhibitor (see Table II, also Diggle & Gage[21]) is converted both by mammals[22, 23] and by insects[24, 25] into an active esterase-inhibitor. Apart from metabolism, the conditions of an *in vivo* toxicity test and *in vitro* inhibition experiment are widely different. *In vitro*, the inhibitors are placed in more or less immediate contact with the enzymes being used in a uniform solution or suspension, but, *in vivo*, the poison is placed on the outside of, or in a limited locality within, an insect and must then reach its site of action, which in this case is assumed to be an enzyme that is inhibited. It seems unlikely that either the enzyme or inhibitor will be uniformly distributed throughout any insect, and therefore the amount of poison necessary to kill an

insect will not be a simple function of enzyme-inhibiting power, but will be a complex function dependent upon the properties of both poison and insect. Thus the distribution of two poisons in one insect species could not be expected to be identical owing to the differences, however slight, in the physical properties of the substances, so that the relative concentration (amount or proportion) of each poison at the site of action would not be the same. As a result, *in vivo* enzyme inhibition and hence toxicity would not be simply related to the amount of poison administered, and consequently toxicity would not be directly related to *in vitro* inhibition.

If only one poison is considered and its *in vivo* toxicity (Table I) (to the three species *D. fasciatus*, *T. molitor* and *B. germanica* in which known doses were administered) is compared with *in vitro* enzyme inhibition (Table II) no relationship between the inhibition of either type of enzyme or toxicity can be detected. The distribution of any one poison within an insect is dependent upon the structure of the insect and so may be expected to vary from one species to another. Thus when a poison is applied to an insect the proportion that reaches its site of action may be expected to vary from species to species, so vitiating any simple relationship between *in vivo* toxicity and *in vitro* enzyme inhibition. Nevertheless, it seems likely that differences in the susceptibility of enzymes (Table II) may play a considerable part in determining the relative resistance of insect species to insecticides, although the rates of metabolism (both activation and detoxification) and excretion of a poison may be expected to vary from species to species.

In the absence of any correlation between the inhibition of either kind of esterase (*in vitro*) and toxicity (*in vivo*) our experiments do not provide any strong additional evidence that the toxic action of organo-phosphorus insecticides is due to their anti-esterase activity. But if the anti-esterase (including anticholine-esterase) activity of the organo-phosphorus insecticides is accepted as their mode of *in vivo* action, then our results show that two types of esterase (one that hydrolyses acetylcholine and one that does not) are inhibited by organo-phosphorus compounds in low dilution. In addition, if both kinds of enzyme are equally accessible *in vivo* to the inhibitor and are equally vital to the insect, then the group of esterases that do not attack acetylcholine would appear in most cases to be the enzymes primarily affected. However, susceptibility to *in vitro* inhibition is not a sufficient criterion to judge whether or not an enzyme is involved in the toxic action of a compound. Other factors, such as inhibitor and enzyme distribution and function, may also be involved. In mammals, extensive work has led to the conclusion that the inhibition of choline-esterase is the mode of toxic action of organo-phosphorus insecticides, in spite of the susceptibility of other esterases to inhibition by organo-phosphorus compounds. In insects, sufficient data are not available to draw any general conclusions, and in view of differences that exist between insect and mammalian physiology the possibility that esterases other than those that hydrolyse acetylcholine may be important in the action of organo-phosphorus insecticides cannot be dismissed from consideration.

The inhibition experiments have clearly shown that enzymes hydrolysing the same substrates derived from different insect species differ largely in their susceptibility to inhibitors. This may not be the sole factor in determining the resistance of the living insect to an insecticide, but it must play an important role. The variations in esterases from species to species strongly suggest that more specific organo-phosphorus insecticides may be developed. Some of these may not inhibit mammalian enzymes and may thus be less toxic to mammals than the currently used organo-phosphorus insecticides.

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References

[1] Webb, E. C., *Proc. Symp. Biochem. Soc.*, 1948, **2**, 50
[2] Dubois, K. P., & Mangun, G. H., *Proc. Soc. exper. Biol., N.Y.*, 1947, **64**, 137
[3] Metcalf, R. L., & March, R. B., *J. econ. Ent.*, 1949, **42**, 721
[4] Chamberlain, W. F., & Hoskins, W. M., *J. econ. Ent.*, 1951, **44**, 177
[5] Chadwick, L. E., & Hill, D. L., *J. Neurophysiol.*, 1947, **10**, 235
[6] Roeder, K. D., *Johns Hopk. Hosp. Bull.*, 1949, **83**, 587
[7] Baldwin, E., 'An Introduction to Comparative Biochemistry', 1948 (Cambridge University Press)
[8] Lewis, S. E., *Nature, Lond.*, 1953, **172**, 1004
[9] Lord, K. A., & Potter, C., *Ann. appl. Biol.*, 1951, **38**, 495
[10] Hopf, H. S., *Ann. appl. Biol.*, 1952, **39**, 193
[11] Potter, C., *Ann. appl. Biol.*, 1952, **39**, 1
[12] Needham, P. H., private communication
[13] Lord, K. A., & Potter, C., *Nature, Lond.*, 1953, **172**, 679
[14] Umbreit, W. W., Burris, R. H., & Stauffer, J. F., 'Manometric Techniques & Tissue Metabolism', 1949 (Minneapolis, U.S.A.: Burgess Publishing Co.)
[15] Collins, E., *Analyst*, 1945, **70**, 326
[16] Myers, D. K., *Biochem. J.*, 1953, **55**, 67
[17] Mounter, L. A., & Whittaker, V. P., *Biochem. J.*, 1953, **54**, 551
[18] Metcalf, R. L., & March, R. B., *J. econ. Ent.*, 1950, **43**, 670
[19] Vincent, D., & Lagreu, R., *C.R. Soc. Biol., Paris*, 1950, **144**, 641
[20] Aldridge, W. N., & Barnes, J. M., *Nature, Lond.*, 1952, **169**, 345
[21] Diggle, W. M., & Gage, J. C., *Biochem. J.*, 1951, **49**, 491.
[22] Diggle, W. M., & Gage, J. C., *Nature, Lond.*, 1951, **168**, 998
[23] Gage, J. C., *Biochem. J.*, 1953, **54**, 426
[24] Metcalf, R. L., & March, R. B., *Ann. ent. Soc. Amer.*, 1953, **46**, 63
[25] Lord, K. A., unpublished

CHEMICAL INVESTIGATION OF SEED OIL OF *SESBANIA AEGYPTICA*

By M. O. FAROOQ, M. S. AHMAD and M. A. MALIK

The composition of the mixed fatty acids from the seed oil of *Sesbania aegyptica*, as determined by the thiocyanometric and fractionation methods, has been found to be oleic 24·4, linoleic 36·3, linolenic 10·9, palmitic 8·98, stearic 17·53 and lignoceric 1·89%. The saturated acids were obtained by the Bertram oxidation method.

Sesbania aegyptica (Leguminosae), Hindustani 'Ravasin', is a small tree found wild and cultivated as a hedge plant in almost all parts of India. The seeds have been reported to contain: fat 4·8, albuminoids 33·7, carbohydrates 18·2, cellulose 28·3 and ash 4·2%.[1] Various parts of the plant are used in medicine: seeds and bark for diarrhoea, excessive menstrual flow and skin diseases, and leaves in the form of a poultice are used for suppuration of boils, absorption of hydrocele and inflammatory rheumatic swellings.[1,2]

The composition of the fat has not been reported, and a systematic chemical examination was therefore carried out.

During the present analysis of the fatty acid composition the liquid acids were found to be composed of oleic, linoleic and linolenic acids. The presence of the triethenoid (linolenic) acid has been established by the isolation of the hexabromide derivative (m.p. 177–179°). The presence of this acid has rendered the fractionation method of analysis of the liquid acid esters unworkable, as it is known that the fractionation method does not give the desired results for liquid acid mixtures containing oleic, linoleic and linolenic acids.[3] Therefore a modified method of procedure has been found helpful in their estimation. The composition of the unsaturated acids was determined by using the thiocyanogen and iodine values of the mixed fatty acids and the quantity of the total saturated acids estimated by Bertram's method. The saturated acid composition was later ascertained by isolating a pure sample of saturated acids (I.V. nil,

acid value 189·6) by Bertram's oxidation method and then subjecting their methyl esters to fractionation. It is further interesting to note that a higher saturated acid (C_{24}, lignoceric, m.p. 80–82°; *M* 366·6) has been found to accompany the more common acids (palmitic and stearic, these being the major components) the intermediate acids being absent.

Experimental

The seeds used in the present investigation were collected locally. Dry and powdered seeds on extraction with light petroleum (b.p. 40–60°) yielded 5·3% of a greenish yellow oil, with the physical and chemical constants shown in Table I:

Table I

Characteristics of seed oil of Sesbania aegyptica

Yield, %	5·3	Iodine value (Hanus)	112·4
Sp. gr. at 20°	0·9241	SCN value	71·6
Refractive index at 20°	1·4805	Unsaponifiable, %	3·3
Sap. value	193·2	Protein in defatted seeds, %	38·4
Acid value	3·0	Ash in defatted seeds, %	3·35
Acetyl value	23·0		

The oil, on saponification with alcoholic potash and subsequent decomposition, and removal of unsaponifiable matter, yielded 88·0% of the total fatty acids, with the following constants: mixed fatty acids, I.V. 118·5; SCN value, 74·0; sap. value 197·7.

The mixed fatty acids were then resolved into their liquid and solid components by Twitchell's lead salt–alcohol method, as modified by Hilditch,[4] which gave 27% of solid acids (I.V., 5·15; sap. value 188·2) and 73% of liquid acids (I.V., 154·8; sap. value 200·35). The amount of saturated acids as determined by Bertram oxidation was found to be 28·4%.

Bromination of the liquid acids gave hexabromostearic acid, m.p. 177–179°, and tetrabromostearic acids, m.p. 114–115°. From the formation of these derivatives and the result of the oxidation of liquid acids by potassium permanganate in alkaline medium (cold), the presence of oleic, linoleic and linolenic acids was established.

The percentage composition of the mixed fatty acids, as determined by the thiocyanometric[5] method and Bertram oxidation, is: oleic acid 24·4, linolenic acid 10·9, linoleic acid 36·3 and saturated acids 28·4.

Composition of saturated acids

The proportions of the unsaturated acids having been ascertained, a pure sample of saturated acids (I.V. nil; acid value 189·6) was obtained by oxidation (Bertram's method) of the mixed fatty acids. The saturated acids were then converted into their methyl esters and fractionally distilled under reduced pressure. The saponification equivalent (S.E.) of each fraction was determined and the composition ascertained by using S.E. figures in conjunction with qualitative data. Results are shown in Table II.

Table II

Methyl esters of saturated acids

Fraction No.	Wt., g.	B.p., °c/ 2·5 mm.	S.E.	Calculated composition		
				Palmitate	Stearate	Lignocerate
S_1	8·6	< 162	279·8	5·522	3·078	—
S_2	11·2	162–64	290·9	2·790	8·410	—
S_3	8·2	164–66	291·7	1·810	6·390	—
S_4	4·0	Residue	337·9	—	1·872	2·128
Total 32·0				10·122	19·750	2·128
	As esters, %			31·631	61·718	6·651
	As acids, %			31·63	61·72	6·65

Identification of saturated acids

Palmitic, stearic and lignoceric acids were identified by their melting and mixed melting points from the following fractions:

S_1–S_3: Palmitic 59–61°; stearic 70–72°
S_4: Stearic 70–72°; lignoceric 80–82°, mean mol. wt. 366·6.

The calculated percentage composition of the total fatty acids was: oleic 24·4, linoleic 36·3, linolenic 10·9, palmitic 8·98, stearic 17·53, lignoceric 1·89.

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References

[1] 'Indian Materia Medica-Nadkarni', 1926, p. 790 (Bombay, 4: A. K. Nadkarni)
[2] Chopra, R. N., 'Indigenous Drugs of India', 1933, p. 527 (Calcutta: The Art Press)
[3] Hilditch, T. P., 'The Chemical Constitution of Natural Fats', 1947, 2nd edn., p. 464 (London: Chapman & Hall Ltd.)
[4] Hilditch, T. P., 'The Chemical Constitution of Natural Fats', 1947, 2nd edn., p. 469 (London: Chapman & Hall Ltd.)
[5] B.S. 684: 1950, p. 41 (London: British Standards Institution)

THE ASSAY OF 'PYRETHRIN' AND ALLETHRIN CONCENTRATES WITH 2:4-DINITROPHENYLHYDRAZINE

By B. P. MOORE

A new technique for the assay of 'pyrethrins' and allethrin, based upon the reaction with 2:4-dinitrophenylhydrazine, is described. Comparative assays on a series of standards and concentrates indicate that the new method is consistent and accurate, but that the A.O.A.C. (Association of Official Agricultural Chemists) method gives seriously high results.

It is now established[1] that the insecticidal activity of pyrethrum is attributable to four closely related, rather labile esters of differing potency, termed collectively 'pyrethrins'. Of these, 'pyrethrins I' comprise the esters of the keto-alcohols pyrethrolone and cinerolone with chrysanthemic acid (chrysanthemum-monocarboxylic acid), and 'pyrethrins II' are the corresponding esters of pyrethric acid (chrysanthemumdicarboxylic acid monomethyl ester). It appears that the four esters invariably occur together and, combined, they seldom amount to more than about 20% by weight of pyrethrum oleoresin. The balance of inactive material comprises 'polypyrethrins',[2, 3] pyrethrolone and cinerolone esters of fatty acids,[4] free organic acids, and many other chemical entities of (mainly) undetermined constitutions and properties. Clearly, therefore, an unequivocal chemical method of assay would need (*a*) to be highly specific and (*b*) to estimate each active ester separately. No such method has yet been devised.

The two methods in current use, the Seil[5] and the mercury-reduction (A.O.A.C.)[6] methods, together with that put forward recently by Mitchell,[7] depend on titrimetric determination of the chrysanthemum-mono- and -di-carboxylic acids obtained by alkaline cleavage of the total esters. The assumption is made that these acids are derived solely from active material and the results are calculated accordingly as pyrethrin I and pyrethrin II. These methods lack specificity, and a recent survey has shown[5] that, in general, inter-laboratory errors are high. Further, the relationship between the results obtained and the true 'pyrethrin' contents has been a matter for conjecture. Recently, however, the 'pyrethrins' have been separated and purified by chromatographic techniques[8, 9] and a crystalline form of the synthetic analogue,

allethrin, has become available.[10] These substances provide the necessary standards for a direct assessment of the various analytical methods.

The question of allethrin assay is more satisfactory, as the two accepted methods appear to be accurate. These, the hydrogenolysis[11] and the ethylenediamine[12] methods, depend on cleavage of the ester link, followed by titrimetric estimation of the liberated chrysanthemic acid. Unfortunately, an elaborate series of blank corrections for free acid, acid chloride and acid anhydride render the methods tedious. A colorimetric method[13] is also available, but the accuracy attainable is not of a high order.

In recent years, 2 : 4-dinitrophenylhydrazine (D.N.P.) has come into prominence as a reagent for the detection and estimation of many carbonyl compounds, and there seemed good reason to suppose that it would be applicable to the present problem. Preliminary experiments soon indicated that the reagent reacted quantitatively with allethrin under certain conditions, and that the resulting 2 : 4-dinitrophenylhydrazone could be freed from concomitant substances by chromatography on alumina. The 'pyrethrins' behaved similarly, and a satisfactory photometric method for estimating, collectively, the mixed dinitrophenylhydrazones has now been developed.

A series of standards and concentrates have been assayed by the new method and the results, together with comparative figures for the A.O.A.C. method[6] (modified[14]), are given in Table I. For the purpose of calculating 'pyrethrins' values, mean molecular weights have been employed as follows: 'pyrethrins I', 322; 'pyrethrins II', 366; total 'pyrethrins', 340. These values assume average pyrethrin/cinerin ratios of 1 : 1 and 'pyrethrin I'/'pyrethrin II' ratios of approximately 6 : 4.

Experimental

Materials

All solvents were of A.R. quality; those used for the chromatographic steps were dried by distillation. Carbonyl-free ethanol was prepared according to the general directions of Lappin & Clark.[15]

The alumina was type 'O' supplied by Peter Spence & Sons, Ltd.; it was neutralized by washing with dilute hydrochloric acid and water, dried in an oven at 80°, and standardized to grade III activity on the Brockmann scale.[16] Commercial 2 : 4-dinitrophenylhydrazine was recrystallized once from butanol before use.

Preparation of standards and samples

Pyrethrum oleoresin was freshly prepared from powdered flowers by warm extraction with light petroleum (b.p. $< 40°$) in a Soxhlet apparatus. The extract was chilled overnight, filtered, and evaporated *in vacuo*.

Purified oleoresin was prepared by the nitromethane technique[17] and further refined by re-extraction with light petroleum (b.p. $< 40°$), whereby a considerable quantity of 'polypyrethrins' was eliminated. Evaporation and final drying *in vacuo* afforded sample 2.

Sample 1 was prepared by admixture of accurately weighed quantities of purified 'pyrethrins I' and 'pyrethrins II', obtained by chromatography on grade III alumina of sample 2 (cf.[9]), a pilot run having indicated that the two fractions were eluted by 1 : 1 and 4 : 1 benzene–hexane respectively. These materials were used without delay.

Sample 6 was prepared by heating crude oleoresin in an oil bath at 200–220° until the knock-down activity to adults of the black fungus beetle, *Alphitobius laevigatus* F., had fallen to about 2% of the original value; this required about ten hours' heating. The material was then extracted with boiling light petroleum (b.p. 40–60°) and filtered. Evaporation of the filtrate afforded a brown oleoresin which, on the basis of further bio-assay, contained less than 1% by weight of 'pyrethrins'.

Crystalline α-(±)-*trans*-allethrin was obtained by cooling a commercial sample of allethrin and was recrystallized from light petroleum to constant melting point (50·5–51°).

Preparation of the reference curve

About 20 mg. of α-allethrin was accurately weighed into a small conical flask and dissolved

by the addition of 10% (v/v) sulphuric acid in carbonyl-free ethanol (15 ml.). Powdered 2 : 4-dinitrophenylhydrazine (80 mg.) was added and the mixture swirled gently to complete the dissolution. The flask was corked and placed in an oven at 80° for 20 minutes. The reaction mixture was then cooled, transferred quantitatively, by means of ether (20 ml.), to a 100-ml. separating funnel and shaken with distilled water (15 ml.). The aqueous layer was separated and the washing continued successively with 15 ml. and 10 ml. of water. The combined washings were re-extracted with ether (20 ml.) and the ether layer was washed with 2 × 10 ml. portions of water. The process was repeated with a further 20-ml. portion of ether.

Table I

Comparative analytical figures of 'pyrethrin' and allethrin standards and concentrates

Sample No.	Nature of sample	New (D.N.P.) method : total 'pyrethrins' (allethrin), %	A.O.A.C. method : 'pyrethrins' (allethrin), %		
			I	II	Total
1	'Pyrethrins I' 64·4% + 'pyrethrins II' 35·6%	100 101	70·6	38·3	108·9
2	Purified oleoresin (nitromethane method)	85·3 84·9	61·4 61·2	34·7 34·1	96·1 95·3
3	Aged sample 2 (6 months at 4°)	64·1 64·8	48·5 47·0	35·6 34·5	84·1 81·5
4	Crude oleoresin in refined kerosene	12·9 13·0	10·9 10·6	7·1 7·4	18·0 18·0
5	Crude oleoresin	17·4 17·2	15·5 15·9	10·5 10·2	26·0 26·1
6	Heat-treated oleoresin (activity equivalent to < 1% 'pyrethrins')	0·65	2·1	3·9	6·0
7	Sample 6 + pure α-allethrin (2 : 1)	(99·5 net)	(101 net)	(8·4 net)	(109·4 net)
8	Pure α-allethrin	(99·7)	(99·3)	(8·4)	(107·7)
9	Commercial allethrin (94·3% by hydrogenation)	(94·6) (94·6) (94·6)	Not determined		
10	Commercial allethrin (nominal 75%)	(74·9) (75·5) (75·6)	Not determined		

The combined ether layers were evaporated to small bulk over a simmering-water bath and the residue was diluted with dry benzene (25 ml.). Distillation was continued until dry benzene began to pass over, when the mixture was cooled and diluted with an equal volume of dry light petroleum (b.p. 40–60°).

A column was prepared by pouring a slurry of the grade III alumina (50 g.) in 1 : 1 benzene–light petroleum into a suitable tube of diameter 2·5 cm. When the packing was even, the dinitrophenylhydrazone solution was added and the chromatogram developed with 1 : 1 solvent. Free 2 : 4-dinitrophenylhydrazine was fixed as a dark band at the top, and the orange allethrin derivative moved slowly down the column (see below, Note 1). When the allethrin derivative neared the bottom of the column, it was eluted with 2 : 1 benzene–light petroleum. The orange-yellow eluate was freed from the bulk of the petroleum *in vacuo* and made up to 100 ml. with pure benzene. An aliquot (10 ml.) was pipetted and diluted to 100 ml. with benzene. The optical density was then determined on a Hilger Spekker photometer, with 1-cm. cells, a blue-green filter (Note 2), and benzene as reference liquid.

This technique was repeated with further quantities of α-allethrin, ranging from 30 to 60 mg.; the results were plotted graphically and gave a good straight line passing through the origin (Fig. 1).

Assay of pyrethrin and allethrin concentrates (*Note* 3)

Quantities of the concentrates estimated to contain about 40 mg. of active material were subjected to the general procedure described above, but larger portions (120–150 mg.) of 2 : 4-dinitrophenylhydrazine were used (Note 4). The chromatograms derived from impure 'pyrethrins' in particular were naturally more complex, but no difficulty was experienced

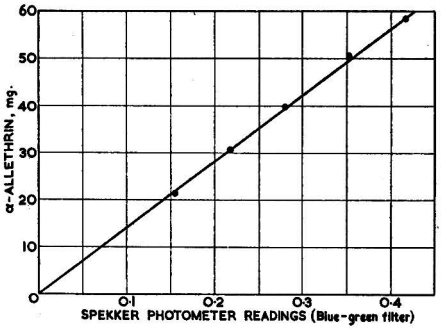

FIG. 1.—*Reference curve for α-allethrin*

in detecting and isolating the appropriate band. Polymerized material remained as a brown band above the 2 : 4-dinitrophenylhydrazine at the top of the column, and other impurities (probably the pyrethroids derived from palmitic and linoleic acids) passed through rapidly as a pale-yellow forerun.

The optical densities of the final aliquots normally fell within the optimum limits (25–45 mg. of allethrin on the curve); when they did not, an appropriate adjustment of the final dilution was made and a further reading taken. Allethrin equivalents were thereby obtained directly from the curve and were convertible into 'pyrethrins' equivalents by multiplying by a molecular-weight factor of 1·07 (Note 5).

A single assay could be completed in considerably less than a full working day.

Notes

1. The 2 : 4-dinitrophenylhydrazone obtained from the α-(±)-*trans*-allethrin experiments crystallized readily, but was not entirely homogeneous. Thus a satisfactory reference curve could not be derived from simple dilution of aliquots of the pure crystalline derivative (m.p. 128°).

2. The use of a violet filter, which more nearly corresponds to the absorption maxima of the 2 : 4-dinitrophenylhydrazones, was not advantageous since the resulting increase in sensitivity was more than offset by the greater degree of dilution required.

3. Extracts containing large amounts of heavy mineral oil cannot be determined directly as they do not dissolve sufficiently in the reaction medium. A preliminary chromatographic isolation of the active material would be necessary.

4. Crude 'pyrethrin' concentrates contain variable quantities of extraneous ketonic material and therefore call for a considerable excess of 2 : 4-dinitrophenylhydrazine. Whenever possible, duplicate assays were run, with an additional quantity (30 mg.) of the reagent.

5. This calculation involves the assumption that, mole for mole, the various 'pyrethrin' dinitrophenylhydrazones and the corresponding allethrin compounds have similar optical absorptions. This assumption is reasonable since the compounds are closely related and contain the same chromophore; it is borne out by the results obtained.

Discussion

Before any new technique can become established, adequate inter-laboratory trials and corroboration are essential. Nevertheless, the present results indicate that the new (D.N.P.)

method has proved remarkably accurate and consistent in the author's hands, and shows promise for both 'pyrethrins' and allethrin estimations. By contrast, the A.O.A.C. method has given high results, the margin of error ranging from about 10% on pure materials to 50% or more on crude oleoresins. It seems clear that these errors result largely from the effect on the final value of considerable quantities of 'polypyrethrins' (which are more soluble in light petroleum than has been generally supposed), together with smaller amounts of other extraneous materials. The values for 'pyrethrins II' suffer particularly in this respect and appear always to be appreciable, even when no genuine material is present (cf. samples 6, 7 and 8). Evidently the alkaline cleavage of the 'pyrethrins I' gives rise to more than one equivalent of organic acid. Studies on a similar cleavage of allethrin have already demonstrated[18] that the reaction is not a straightforward hydrolysis.

The above conclusions on the A.O.A.C. method are in general agreement with those derived recently by Oiwa *et al.*[19] from polarographic studies. However, these authors do not report such high errors for crude oleoresins, and it seems probable that their polarographic technique also gives high results with crude extracts, possibly owing to inclusion of the inactive pyrethroids which are usually present.[4] The evidence put forward by these authors in support of their method, namely the (approximately) correct assay by difference of pure allethrin and 'pyrethrins' added to crude extracts, is not really diagnostic, since ground errors are largely compensated by such a procedure. This phenomenon is illustrated by the A.O.A.C. results in the Table for samples 6 and 7.

It is to be hoped that other laboratories will test and comment on the new method.

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References

[1] LaForge, F. B., & Barthel, W. F., *J. org. Chem.*, 1944, **9**, 242; 1945, **10**, 106, 114

[2] Mitchell, W., *Pyrethrum Post*, 1949, **1**, (3), 7

[3] Brown, N. C., Phipers, R. F., & Singleton, K. G., *Pyrethrum Post*, 1954, **3**, (3), 3

[4] Acree, F., & LaForge, F. B., *J. org. Chem.*, 1937, **2**, 308

[5] Report of the Standing Sub-Committee on methods of Analysis of Vegetable Insecticides on the World-Wide Collaborative Analysis of Pyrethrum Flowers, 1948–49, 1950 [London: Colonial Products Advisory Bureau (Plant and Animal)]

[6] 'Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists', 1950, 7th edn., p. 72 (Washington, D.C.: Association of Official Agricultural Chemists)

[7] Mitchell, W., *J. Sci. Fd Agric.*, 1953, **4**, 278

[8] Lord, K. A., Ward, J., Cornelius, J. A., & Jarvis, Mary W., *J. Sci. Fd Agric.*, 1952, **3**, 419

[9] Ward, J., *Chem. & Ind.*, 1953, p. 586

[10] Schechter, M. S., LaForge, F. B., Zimmerli, A., & Thomas, J. M., *J. Amer. chem. Soc.*, 1951, **73**, 3541

[11] Schechter, M. S., Konecky, M., Storherr, R. W., Green, M., & LaForge, F. B., unpublished

[12] Hogsett, J. N., Kacy, H. W., & Johnson, J. B., *Analyt. Chem.*, 1953, **25**, 1207

[13] Cueto, C., & Dale, W. E., *Analyt. Chem.*, 1953, **25**, 1367

[14] *J. Ass. off. agric. Chem. Wash.*, 1952, **35**, 64 (Items 2, 3 and 4)

[15] Lappin, G. R., & Clark, L. C., *Analyt. Chem.*, 1951, **23**, 541

[16] Brockmann, H., & Schodder, H., *Ber. dtsch chem. Ges.*, 1941, **74**, 73

[17] Barthel, W. F., Haller, H. L., & LaForge, F. B., *Soap, N.Y.*, 1944, **20**, (7), 121

[18] LaForge, F. B., Green, N., & Schechter, M. S., *J. Amer. chem. Soc.*, 1952, **74**, 5392

[19] Oiwa, T., Shinohara, T., Takeshita, Y., & Ohno, M., *Botyu-Kagaku*, 1953, **18**, 142

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