4. It is concluded that all observations are to be explained in terms of protein–ion interactions rather than true protein heterogeneity.

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Paper Chromatography of Saturated and Unsaturated Fatty Acids

BY P. E. BALLANCE AND W. MARY CROMBIE
Department of Botany, University of Southampton

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During an investigation of fat metabolism in the fungus *Trichoderma viride* it became desirable, in order to avoid culturing excessive quantities of mycelium, to find a method of separating micro-quantities of natural fatty acids. Such a method would also be useful for radioautographic purposes. In this communication the behaviour of over 40 pure fatty acids on several reversed-phase paper-chromatographic systems is examined: the systems are liquid paraffin/aqueous acetone (adapted from a column-chromatographic method, see Howard & Martin, 1950; Silk & Hahn, 1954; Crombie, Comber & Boatman, 1955), liquid paraffin/aqueous acetic acid (a system resembling that of Kaufmann & Nitsch, 1954; Wagner, Abisch & Bernhard, 1955; Seher, 1956; Leibnitz *et al.* 1957), castor oil/aqueous acetone (adapted from the column system of Savary & Desnuelle, 1953; Matic, 1956), castor oil/aqueous acetic acid, and polythene/aqueous acetone (adapted from the column method of Green, Howitt & Preston, 1955).

METHODS AND MATERIALS

Impregnation of papers

(1) *Liquid paraffin.* Whatman no. 1 chromatography papers (13 cm. × 45 cm.) were immersed in a solution of liquid paraffin (Nujol) (35 ml.) in benzene (65 ml.), blotted (10 min.) under an evenly distributed 18 lb. weight and dried by hanging in air at room temperature. Provided that the same grade of blotting paper is used, a constant weight of paraffin (25%) remains on each paper. It is important to avoid excess of paraffin since this results in loss of fatty acid during formation of the copper salts (for full details see below under 'Quantitative paper chromatography of fatty acids'). After removal of the top (11 cm.) and bottom (4 cm.) portions, each paper was cut into standard 30 cm. × 2 cm. lengths ('top' and 'bottom' refers to a paper hung vertically with the direction of machining pointing upwards).

(2) *Castor oil.* A solution of castor oil (B.P. grade) in ethyl ether was shaken three times with 4% (w/v) aq. K_2CO_3 to remove free fatty acids. After the solution had been washed three times with water and dried (Na_2SO_4) and the solvent evaporated, the neutral oil (35 ml.) was dissolved in benzene (65 ml.) and this solution used for impregnating papers as in (1) above.

(3) *Polythene.* Polythene powder (Schori Metallising Process Ltd.) was dissolved in xylene [3 or 6% (w/v) final solution] at 60° and papers impregnated with the hot solution. They were air-dried at 20° until solvent-free.

Application of fatty acids to the paper

Pure fatty acids or mixtures, dissolved in $CHCl_3$ (approx. 6 mg./ml.), were delivered (1–5 µl. at a time, total volume 10–20 µl.) on the starting line (5 cm. from the upper edge

of the paper) by means of an Agla micrometer syringe. After evaporation of the solvent the papers were equilibrated (4 hr.) in contact with vapour from the eluting solvent, before downward development.

Elution systems

(1) *Paraffin/acetic acid.* Fatty acids on paraffin-impregnated papers were eluted with 90% (system 1A), 95% (1B), 70% (1C) or 50% (1D) (v/v) aq. acetic acid, previously equilibrated with paraffin as described by Wagner *et al.* (1955). Elution was continued at 21° (±0·5°) for 16 hr.: the solvent front travelled from 16–18 cm. (1A) to 23–26 cm. (1D). After the position of the solvent front had been marked the papers were dried by hanging in air.

(2) *Paraffin/acetone.* Fatty acids on paraffin-impregnated papers were eluted with 90% (v/v) aq. acetone which had been previously equilibrated with paraffin by shaking and standing for 24 hr. (system 2). After elution for 8 hr. the solvent front had advanced 22–23 cm.

(3) *Castor oil/acetic acid.* Fatty acids on castor-oil-impregnated papers were eluted with 70% (system 3A) or 50% (3B), v/v, aq. acetic acid, which had been equilibrated with castor oil by the technique of (1) above. After elution for 16 hr. the solvent front had advanced 9–14 cm. (3A) or 17–20 cm. (3B).

(4) *Castor oil/acetone.* Fatty acids on castor-oil-impregnated papers were eluted with 50% (v/v) aq. acetone (system 4), which had been equilibrated with castor oil as in (1) above. After elution for 10 hr. the solvent front had travelled 14–15 cm.

(5) *Polythene/acetone.* Fatty acids on polythene papers were eluted with 95, 90, 80, 70 or 60% (v/v) aq. acetone (system 5), which had been equilibrated with polythene by the technique of (2) above. Elution times varied from 4 hr. (95% acetone) to 10 hr. (60% acetone).

Detection of fatty acid spots

After the papers had been dried in air at room temperature, copper salts of the fatty acids were formed by immersing the papers in cupric acetate solution (20 ml. of a saturated solution in 1 l. of water) for 20 min. (Wagner *et al.* 1955). Excess of copper was removed by washing for 15 min. in dilute acid (0·2 ml. of acetic acid/l.). If quantitative results are not required a further 5–10 min. wash in fresh dilute acetic acid improved the differentiation of acid spots from the background, but causes loss of copper, particularly from acids of high R_F, e.g. linolenic acid or lauric acid. After drying, the copper was detected by immersing the papers (30 sec.) in 0·03% (w/v) dithio-oxamide (rubeanic acid) in ethanol. This method produces light-stable dark-green spots against a lighter background and can detect 0·1 μg. of copper (Vaeck, 1955). Excess of dithio-oxamide is removed by washing for 2 min. in ethanol.

Sources of fatty acids

Saturated *normal* even-numbered acids of chain length C_{12}–C_{20} were obtained from Professor N. K. Adam, F.R.S., the C_{10} acid from British Drug Houses Ltd. and the C_{22} acid from Hess Products Ltd. The C_{11} *normal* acid was obtained from Dr L. Crombie, *normal* odd-numbered acids (C_{13}–C_{19} inclusive) from Dr F. B. Shorland and the branched-chain C_{19} (tuberculostearic) acid from Dr B. C. L. Weedon. Oleic, linoleic and linolenic acids came from the California Foundation for Biochemical Research, U.S.A., *erythro*- and *threo*-dihydroxystearic acid, elaidic acid, petroselinic acid, tariric acid and erythrogenic acid from Dr B. C. L. Weedon, ximenynic acid from Dr S. P. Ligthelm, *cis*- and *trans*-octadec-2-enoic acid from Dr G. S. Myers and the remaining acids from Dr L. Crombie. Details on the purity of some of these specimens have already been recorded (Crombie *et al.* 1955), and each sample was tested for purity chromatographically. The melting points of the highly unstable conjugated octadecatrienoic acids were checked immediately before use and corresponded exactly with those reported for the pure acids (Crombie & Jacklin, 1957).

Spores of *Trichoderma viride* were obtained from cultures grown on agar slopes on a 20% glucose medium in the light. Separation was achieved by shaking each tube with water, pouring the suspension through a sieve (to remove fragments of mycelium) and centrifuging. Spore-free mycelium was grown in a 20% glucose liquid medium in the dark and separated from the culture fluid by filtration. Material was killed by immersion in boiling ethanol and the lipids were extracted with ethanol and diethyl ether. A crude separation into phosphatides and glycerides was effected with ice-cold acetone, and the fatty acids were extracted from each fraction by standard techniques (Hardman & Crombie, 1958).

RESULTS

Qualitative separation of fatty acids in different reversed-phase systems

Polythene/aqueous acetone (*system* 5). Satisfactory separation of *normal* even-numbered saturated acids of chain length C_{14}–C_{18} was achieved, although it proved impossible to prevent 'tailing'. Further development of this system was discontinued when it became apparent that it was less promising than others described below.

Paraffin/aqueous acetone (*system* 2). Separation of *normal* saturated acids (C_{14}–C_{20}) was achieved (Table 1), but the fatty acid spots were diffuse: the solvent front travels rapidly, which may account for the effect.

Paraffin/aqueous acetic acid (*system* 1 *A–D*). This system gave excellent separations of the *normal* even-numbered saturated fatty acids (C_{10}–C_{22}) (Table 1). Acids of medium chain length (C_{10}–C_{14}) separate best in more aqueous solvents (system 1C), and long-chain acids (C_{18}–C_{22}) in the less aqueous solvents (1B). The acids usually encountered in plant tissues (C_{14}–C_{20}) are most conveniently separated in 90% acetic acid (1A). These results agree with those of Kaufmann & Nitsch (1954), who used a similar system with paraffin of lower boiling point. Acids of chain length less than C_{10} cannot be detected by the copper/dithio-oxamide method owing to the solubility of their copper salts. R_F values are recorded in Table 1 as mean values, but, although these serve as a useful guide, the only decisive test of separability is chromatography of appropriate mixtures. Thus some of the acids with closely similar R_F values do in fact separate.

Table 1. *Separation of saturated fatty acids on different paper-chromatographic systems*

Systems: 1A, paraffin/90% acetic acid; 1B, paraffin/95% acetic acid; 1C, paraffin/70% acetic acid; 2, paraffin/90% acetone; 3A, castor oil/70% acetic acid.

Fatty acid	Structure	System	No. of chromatograms	Mean R_F	Standard error
Capric	C_{10} *normal* chain	1C	2	0·57	0·020
Undecanoic	C_{11} *normal* chain	1C	10	0·45	0·029
Lauric	C_{12} *normal* chain	1C	4	0·33	0·087
		1A	12	0·53	0·014
Tridecanoic	C_{13} *normal* chain	1A	5	0·60	0·020
Myristic	C_{14} *normal* chain	1A	12	0·38	0·015
		2	2	0·69	0·000
		3A	2	0·29	0·015
Pentadecanoic	C_{15} *normal* chain	1A	5	0·42	0·022
Palmitic	C_{16} *normal* chain	1A	25	0·29	0·012
		2	2	0·58	0·005
		3A	2	0·12	0·010
Margaric	C_{17} *normal* chain	1A	5	0·27	0·019
Stearic	C_{18} *normal* chain	1A	43	0·16	0·007
		1B	3	0·34	0·018
		2	2	0·45	0·010
		3A	4	0·00	0·000
Nonadecanoic	C_{19} *normal* chain	1A	5	0·11	0·060
Tuberculostearic	$CH_3\cdot[CH_2]_7\cdot CHMe\cdot[CH_2]_8\cdot CO_2H$	1A	4	0·12	0·008
Arachidic	C_{20} *normal* chain	1A	10	0·10	0·010
		2	2	0·33	0·010
Behenic	C_{22} *normal* chain	1A	3	0·06	0·004
		1B	1	0·14	—

The *normal* odd-numbered saturated acids (C_{11}–C_{19}) were completely separable, again confirming that a difference in chain length of $\cdot[CH_2]_2\cdot$ is sufficient for successful chromatography. When odd- and even-numbered acids are mixed, the success of separation depends on the chain length, e.g. C_{10}, C_{11}, C_{12}, C_{13}, C_{14} and C_{15} acids form discrete spots, but satisfactory separation of the C_{16}, C_{17}, C_{18} and C_{19} acids could not be achieved. Rather similar results have been obtained on radial chromatograms (Leibnitz *et al.* 1957). Branching of the acid chain, as in tuberculostearic acid, had little effect, this acid behaving similarly to the C_{19} *normal* acid. Such results are closely similar to those obtained with a paraffin/aqueous acetone column system (Howard & Martin, 1950; Silk & Hahn, 1954; Crombie *et al.* 1955; Garton & Lough, 1957).

Incorporation of an ethylenic linkage increases the R_F, but the degree of separation from a *normal* saturated acid of the same chain length is dependent on the position of unsaturation. Among the C_{18} monoethylenic acids the octadec-2-enoic acids (both *cis*- and *trans*-) are inseparable from octadecanoic (stearic) acid; octadec-6-enoic (petroselinic) acid separates moderately well and both *cis*- and *trans*-octadec-9-enoic acids (oleic and elaidic) are completely separable from stearic acid (Table 2). When the double bond is well removed from the carboxyl group the effect is to increase the R_F to such an extent that the acid is eluted with the *normal* saturated acid of chain length $\cdot[CH_2]_2\cdot$ less: e.g. erucic (C_{22} monoene) is eluted with arachidic (C_{20}) acid; petroselinic acid, oleic acid and elaidic acid (C_{18} monoene) are eluted with palmitic (C_{16}) acid. *cis*- and *trans*-Isomers (e.g. *cis*- and *trans*-octadec-2-enoic acids and *cis*- and *trans*-octadec-9-enoic acids) are not separable.

The R_F of a monoethenoid fatty acid is increased further by incorporating extra double bonds in the centre of the chain; e.g. linoleic (C_{18} diene) is eluted with myristic (C_{14}) acid and linolenic (C_{18} triene) acid is eluted with lauric (C_{12}) acid. Each additional ethylenic linkage increases the R_F to approximately the same extent as the removal of $\cdot[CH_2]_2\cdot$ from a saturated *normal* acid. Conjugated octadecatrienoic (α- and β-elaeostearic and -punicic) acids have similar R_F values to that of a non-conjugated octadecatrienoic (linolenic) acid, but the former are too unstable for accurate paper chromatography. When the normal procedure of equilibrating for 4 hr. before eluting was followed, these acids decomposed to gums which remained on the starting line. When they were applied to the papers immediately before elution, or chromatographed in an inert atmosphere, breakdown was considerably reduced, but there were still traces of

Table 2. *Separation of ethylenic and acetylenic acids on different paper-chromatographic systems*

See Table 1 for key to systems.

Fatty acid	Structure	System	No. of chromatograms	Mean R_F	Standard error
Monoethylenic					
Undec-10-enoic	$CH_2{=}CH\cdot[CH_2]_8\cdot CO_2H$	1C	6	0·57	0·024
Oleic	$CH_3\cdot[CH_2]_7\cdot CH\overset{c}{=}CH\cdot[CH_2]_7\cdot CO_2H$	1A	34	0·26	0·010
Elaidic	$CH_3\cdot[CH_2]_7\cdot CH\overset{t}{=}CH\cdot[CH_2]_7\cdot CO_2H$	1A	6	0·33	0·014
Petroselinic	$CH_3\cdot[CH_2]_{10}\cdot CH\overset{c}{=}CH\cdot[CH_2]_4\cdot CO_2H$	1A	8	0·21	0·014
Octadec-*cis*-2-enoic	$CH_3\cdot[CH_2]_{14}\cdot CH\overset{c}{=}CH\cdot CO_2H$	1A	4	0·20	0·003
Octadec-*trans*-2-enoic	$CH_3\cdot[CH_2]_{14}\cdot CH\overset{t}{=}CH\cdot CO_2H$	1A	5	0·19	0·006
Erucic	$CH_3\cdot[CH_2]_6\cdot CH\overset{c}{=}CH\cdot[CH_2]_{12}\cdot CO_2H$	1A	1	0·12	—
		1B	3	0·25	0·010
Diethylenic					
Linoleic	$CH_3\cdot[CH_2]_4\cdot CH\overset{c}{=}CH\cdot CH_2\cdot CH\overset{c}{=}CH\cdot[CH_2]_7\cdot CO_2H$	1A	31	0·40	0·013
		2	2	0·72	0·020
Triethylenic					
Linolenic	$CH_3\cdot CH_2\cdot CH\overset{c}{=}CH\cdot CH_2\cdot CH\overset{c}{=}CH\cdot CH_2\cdot CH\overset{c}{=}CH\cdot[CH_2]_7\cdot CO_2H$	1A	22	0·53	0·012
α-Elaeostearic	$CH_3\cdot[CH_2]_3\cdot CH\overset{t}{=}CH\cdot CH\overset{t}{=}CH\cdot CH\overset{c}{=}CH\cdot[CH_2]_7\cdot CO_2H$	1A	4	0·46	0·012
β-Elaeostearic	$CH_3\cdot[CH_2]_3\cdot CH\overset{t}{=}CH\cdot CH\overset{t}{=}CH\cdot CH\overset{t}{=}CH\cdot[CH_2]_7\cdot CO_2H$	1A	3	0·37	0·022
Punicic	$CH_3\cdot[CH_2]_3\cdot CH\overset{c}{=}CH\cdot CH\overset{t}{=}CH\cdot CH\overset{c}{=}CH\cdot[CH_2]_7\cdot CO_2H$	1A	4	0·46	0·002
Acetylenic					
Undec-10-ynoic	$CH{\equiv}C\cdot[CH_2]_8\cdot CO_2H$	1C	6	0·76	0·013
Stearolic	$CH_3\cdot[CH_2]_7\cdot C{\equiv}C\cdot[CH_2]_7\cdot CO_2H$	1A	12	0·45	0·011
Tariric	$CH_3\cdot[CH_2]_{10}\cdot C{\equiv}C\cdot[CH_2]_4\cdot CO_2H$	1A	6	0·40	0·005
Ximenynic	$CH_3\cdot[CH_2]_5\cdot CH{=}CH\cdot C{\equiv}C\cdot[CH_2]_7\cdot CO_2H$	1A	4	0·59	0·005
Erythrogenic	$CH_2{=}CH\cdot[CH_2]_4\cdot C{\equiv}C\cdot C{\equiv}C\cdot[CH_2]_7\cdot CO_2H$	1A	5	0·71	0·034
		1C	4	0·47	0·015
Octadec-11-ene-9:13-diynoic	$CH_3\cdot[CH_2]_3\cdot C{\equiv}C\cdot CH{=}CH\cdot C{\equiv}C\cdot[CH_2]_7\cdot CO_2H$	1A	2	0·51	0·015
		1C	3	0·35	0·021

gum on the starting line and traces of another acid of higher R_F, presumably another breakdown product. Such decomposition of these very unstable acids is avoided by column chromatography (Crombie *et al.* 1955). Difficulty is likely to be considerable in other paper-chromatographic methods which involve heating to remove paraffin from the papers.

The introduction of an acetylene linkage has a marked effect on R_F; octadec-9-ynoic (stearolic) acid and octadec-6-ynoic (tariric) acid are completely separable from the corresponding octadecanoic acids (oleic acid and petroselinic acid), the R_F being increased to approximately the same extent as by the addition of two ethylenic linkages or the removal of $\cdot[CH_2]_4\cdot$ from a saturated chain (Table 2, Fig. 1). An extra ethylenic linkage, conjugated with the acetylene linkage as in ximenynic acid, increases the R_F still further and makes possible a separation from the acetylenic acid. Extra acetylenic linkages also increase the R_F (e.g. erythrogenic acid and octadec-11-ene-9:13-diynoic acid), but the effect of two conjugated acetylenic linkages with the ethylenic linkage in the ω-position (as in erythrogenic acid) is greater than that of an yn-en-yn conjugated system and the two acids are completely separable.

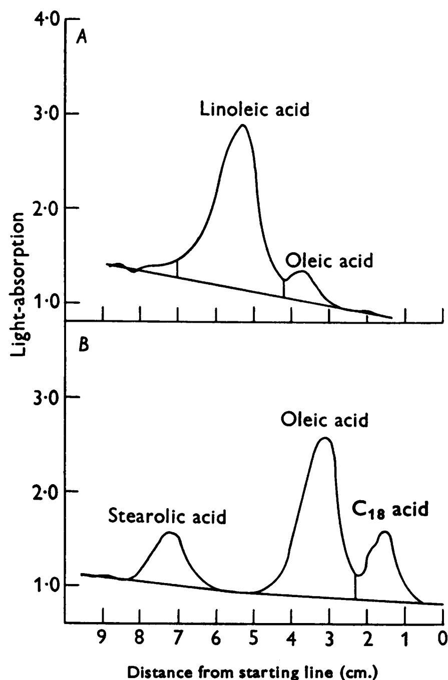

Fig. 1*A*. Use of paper chromatography to indicate acid purity. The sample is a commercial sample of linoleic acid (not from the California Foundation for Biochemical Research). Analysis by paper chromatography shows linoleic acid (88 %), oleic acid (12 %); figures from column chromatography (Crombie *et al.* 1955) were 87·5 and 12·5 %. Linoleic acid content determined by ultraviolet spectrophotometry (Brice, Swain, Herb, Nichols & Riemenschneider, 1952) was 86 %. Similar results for a commercial sample of linoleic acid were obtained by Wagner *et al.* 1955. *B*, Separation of stearolic acid, oleic acid and stearic acid. Molar percentage composition (weighed), stearolic acid 20, oleic acid 60, stearic acid 20; molar percentage composition (determined by paper chromatography), stearolic acid 19, oleic acid 62, stearic acid 19.

The presence of a hydroxyl group increases the R_F considerably (Table 3); e.g. both 8- and 12-hydroxystearic acids are completely separable from stearic acid, ricinoleic acid and ricinstearolic acid from oleic acid and stearolic acid, and α-kamlolenic acid from α-elaeostearic acid. When two hydroxyl groups are present, as in the dihydroxystearic acids, there is no separation from monohydroxystearic acids.

An oxo group also increases the R_F but to a somewhat smaller extent than a hydroxyl group, e.g. 12-hydroxystearic acid and 12-oxostearic acid are partially separable in 90 % acetic acid. The presence of both an oxo and a hydroxy group (e.g. 12-hydroxy-10-oxostearic acid or 12-hydroxy-9-oxostearic acid) increases the R_F still further.

Castor oil/aqueous acetic acid (systems 3 A and B) and castor oil/aqueous acetone (system 4). Since mono- and di-hydroxystearic acid were inseparable on paraffin systems, two castor-oil systems were investigated for this purpose. The more successful was castor oil/aqueous acetic acid (systems 3 A and 3 B, Tables 1 and 3), and by using this good separations of mono- and di-hydroxystearic acid were achieved. This system should be of value for separating cuticle hydroxy acids and for separating the hydroxylation products of mixed unsaturated acids. For mixtures containing only monohydroxy acids, however, the paraffin system is preferred, since this can be used with eluting solvents containing up to 95 % of acetic acid, whereas castor-oil papers are limited to more aqueous mixtures because of the solubility of castor oil in aqueous acetic acid stronger than 75 % (v/v).

Quantitative paper chromatography of fatty acids

Standard mixtures. Quantitative methods for estimating micro amounts of fatty acids on either paraffin- or silicone-impregnated paper have been described (Wagner *et al.* 1955; Perila, 1956; Seher, 1956; Gellerman & Schlenk, 1956; Schlenk, Gellerman, Tillotson & Mangold, 1957; Bernhard, Abisch & Wagner, 1957; Leibnitz *et al.* 1957). Direct photometric measurement of the light-absorption of suitable derivatives on the paper is the method generally used, and indicators are of

Table 3. *Separation of hydroxy and oxo acids on different paper-chromatographic systems*

See Table 1 for systems: 1 D, paraffin/50% acetic acid; 3 B, castor oil/50% acetic acid; 4, castor oil/50% acetone.

Fatty acid	Structure	System	No. of chromato-grams	Mean R_F	Standard error
8-Hydroxystearic	$CH_3\cdot[CH_2]_9\cdot CH(OH)\cdot[CH_2]_6\cdot CO_2H$	1 A	4	0·77	0·031
12-Hydroxystearic	$CH_3\cdot[CH_2]_5\cdot CH(OH)\cdot[CH_2]_{10}\cdot CO_2H$	1 A	3	0·88	0·003
		1 C	2	0·74	0·000
		3 A	6	0·15	0·002
threo-9:10-Dihydroxystearic	$CH_3\cdot[CH_2]_7\cdot CH(OH)\cdot CH(OH)\cdot[CH_2]_7\cdot CO_2H$	1 A	1	0·89	—
		1 C	2	0·87	0·005
		3 A	6	0·38	0·011
		4	1	0·05	—
erythro-9:10-Dihydroxystearic	$CH_3\cdot[CH_2]_7\cdot CH(OH)\cdot CH(OH)\cdot[CH_2]_7\cdot CO_2H$	1 A	2	0·91	0·002
		1 C	1	0·91	—
		3 A	6	0·38	0·013
Ricinoleic	$CH_3\cdot[CH_2]_5\cdot CH(OH)\cdot CH_2\cdot CH{=}CH\cdot[CH_2]_7\cdot CO_2H$	1 A	2	0·83	0·005
		1 C	1	0·79	—
		1 D	4	0·55	0·015
		3 A	2	0·78	0·000
		3 B	2	0·03	0·005
		4	1	0·03	—
α-Kamlolenic	$CH_2OH\cdot[CH_2]_3\cdot CH{=}CH\cdot CH{=}CH\cdot CH{=}CH\cdot[CH_2]_7\cdot CO_2H$	1 A	2	0·93	0·026
		1 C	2	0·97	0·016
12-Hydroxydodec-10-ynoic	$CH_2OH\cdot C{\equiv}C\cdot[CH_2]_8\cdot CO_2H$	1 A	Soluble copper soap		
Ricinstearolic	$CH_3\cdot[CH_2]_5\cdot CH(OH)\cdot CH_2\cdot C{\equiv}C\cdot[CH_2]_7\cdot CO_2H$	1 A	2	0·83	0·005
		1 C	1	0·72	—
		1 D	3	0·00	0·000
		3 B	2	0·00	0·000
		4	1	0·04	—
Homoricinstearolic	$CH_3\cdot[CH_2]_4\cdot CH(OH)\cdot CH_2\cdot C{\equiv}C\cdot[CH_2]_8\cdot CO_2H$	1 C	3	0·83	0·015
Oxo acids					
12-Oxostearic	$CH_3\cdot[CH_2]_5\cdot CO\cdot[CH_2]_{10}\cdot CO_2H$	1 A	4	0·81	0·010
		3 A	2	0·63	0·005
12-Hydroxy-9-oxostearic	$CH_3\cdot[CH_2]_5\cdot CH(OH)\cdot[CH_2]_2\cdot CO\cdot[CH_2]_7\cdot CO_2H$	1 C	1	0·87	—
12-Hydroxy-10-oxostearic	$CH_3\cdot[CH_2]_5\cdot CH(OH)\cdot CH_2\cdot CO\cdot[CH_2]_8\cdot CO_2H$	1 C	1	0·89	—
		3 A	2	0·87	0·020

various types, e.g. iodine vapour and α-cyclodextrin (Schlenk *et al.* 1957), silver salts (Perila, 1956), mercuric acetate derivatives (Inouye, Noda & Hirayama, 1955), or estimation of the copper present in the copper salts after conversion into copper ferrocyanide (Wagner *et al.* 1955; Seher, 1956; Leibnitz *et al.* 1957).

The green complex formed by the reaction of copper soaps with dithio-oxamide, which is used to detect fatty acids in the present work, is also suitable for quantitative assay of copper by direct photometry (Vaeck, 1955; Lacourt & Hendryckx, 1956), but has not previously been applied to the quantitative determination of copper in the copper soaps of fatty acids. Photometric measurements were made by advancing paper strips past the photoelectric cell in a Scanner (Evans Electroselenium Ltd., red filter) and determining light-absorption at 1 mm. intervals. The slit dimensions were 1 mm. × 17 mm. and translucency was improved by soaking the papers for 2 hr. in liquid paraffin (Takahara & Taniguchi, 1955). The areas of the light-absorption peaks obtained were measured with a planimeter. A rather high but regular background line, horizontal or slightly sloping, was normally obtained. Peak areas were estimated by dropping verticals to this line from the lowest points between peaks (Fig. 1) as recommended by Crook, Harris, Hassan & Warren, 1954; Vaeck, 1955.

By using standard cupric sulphate solution it was confirmed that 0·1 μg. of copper was detectable and that peak area is proportional to the weight of copper in the range 0·1–7·0 μg. (corresponding to approximately 1–63 μg. of fatty acid/spot). Seher (1956) showed that fatty acids, both saturated and containing ethylenic linkages, react stoicheiometrically with cupric acetate and, in the present experiments, similar results were obtained with fatty acids containing one or more non-terminal acetylenic linkages. The method is therefore applicable to a wide range of unsaturated fatty acids. It is not suitable for quantitative work on acids of chain length below C_{12} owing to the solubility of their copper soaps.

When paraffin is present on the paper the amount of copper fixed per weight of fatty acid is less than the theoretical. We attribute this effect to 'lifting' of fatty acid in the paraffin layer when the papers are immersed in cupric acetate solution. Acids of low R_F, i.e. those preferentially soluble in the paraffin phase, retain less copper than those of high R_F (Table 4). Careful control of the weight of paraffin in the paper reduces this effect, but for quantitative work additional accuracy was obtained by correction from a standard mixture of approximately the same composition as the unknown sample. This was chromatographed on an adjacent paper strip on the same occasion. Schlenk *et al.* (1957) also had to resort to this expedient in their quantitative work on silicone-impregnated papers. At least three replicates of each mixture were used.

Analysis of natural mixtures of fatty acids. Fatty acids from natural sources contain both saturated and unsaturated components and, as shown above, these are not all separable chromatographically, e.g. palmitic acid and oleic acid have similar R_F values and so have linoleic acid, palmitoleic acid and myristic acid. From a chromatogram of the original mixture, however, the total quantity of acid in each peak can be estimated. Several methods were considered for the analysis of these mixed peaks. Formation and separation of lead salts into ethanol- or acetone-soluble (unsaturated) and -insoluble (saturated) fractions, followed by chromatography of the two fractions, has been used by Wagner *et al.* (1955). However, complete separation is difficult to achieve in the lead-salt method and, further, unsaturated acids of similar R_F values are not separable. Halogenation with Hanus iodine (Kobrle & Zahradnik, 1954) proved unsuitable for use with paraffin-impregnated papers, since the R_F values of halogenated acids were similar to those of the original unsaturated acids.

Hydrogenation (in acetic acid over Adams platinum oxide catalyst), followed by chromatography, was finally used to determine the total amount of acid of each chain length. If only one unsaturated component is present in each peak, direct computation from the results of the original and hydrogenated chromatograms can be used to determine acid composition. Results for the analysis, by this method, of two fatty acid mixtures from *Trichoderma viride* are shown in Table 5.

Table 4. *Quantitative analysis of standard mixtures without correction*

Plain figures, molar % (found); figures in parentheses, molar % (theoretical). Each figure is the mean of three replicates.

	Linolenic acid	Linoleic acid	Oleic acid + C_{16}		C_{18}	C_{22}
Mixture 1	—	49 (48)	51 (52)	—	—	—
2	7 (7)	36 (39)	44 (46)	—	10 (6)	3 (2)
3	—	—	—	23 (16)	77 (84)	—
4	—	—	—	20 (9)	76 (87)	—

Table 5. *Quantitative analysis of* Trichoderma viride *fatty acids by comparison of composition before and after hydrogenation*

u, Uncorrected molar %; c, corrected molar %; d, molar % analysis by column chromatography (Crombie *et al.* 1955).

		Linolenic acid	Linoleic acid	Oleic acid	C_{16}	C_{18}	C_{22}
Spore phosphatide acids	(u)	—	50	29	21	—	—
	(c)	—	49	36	15	—	—
	(d)	—	48	36	16	—	—
Mycelium glyceride acids	(u)	7	35	25	17	13	3
	(c)	8	37	37	8	8	2
	(d)	7	39	35	11	6	2

Correction from standard mixtures was applied both to the original chromatogram and to that of the hydrogenated acids: it is particularly important with the latter, which contains a high proportion of stearic acid. The results are compared with an analysis by the well-tested method of reversed-phase column chromatography (Crombie *et al.* 1955) and show good agreement.

If there is more than one unsaturated component in an acid peak, a further set of data can be obtained by destroying the unsaturated components by Bertram oxidation (strong alkaline permanganate at room temperature for 16–18 hr.) and chromatographing the remaining saturated fatty acids (the plant acids usually encountered have degradation products of high R_F which are completely separable from acids of chain length above C_{12}). Comparison of such a chromatogram with an original one and one after hydrogenation will indicate if there is more than one unsaturated acid in any mixed peak, and appropriate computation can be made.

The method is obviously not suitable for the analysis of highly complex mixtures such as those of fish oils, nor for those containing appreciable amounts of short-chain acids such as palm-kernel oil. It is, however, applicable to a wide range of natural plant fatty acids and has been used successfully with fats from *Ricinus*, *Pterocarya*, *Onguekoa* etc. It can also be used to test the purity of acid samples and to determine the success of semihydrogenation of acetylenic acids (Crombie, 1955; Fig. 1). The amounts of material required are small (less than 5 mg. is sufficient for a complete analysis involving both oxidation and hydrogenation) and the method is therefore particularly useful with material containing only a low percentage of fat, or with material difficult to obtain in quantity, such as fungal spores. The method has obvious possibilities for radioautographic work with ^{14}C-labelled fatty acids.

SUMMARY

1. The separation of over 40 pure fatty acids on several reversed-phase paper-chromatographic systems is described, and the relationship between structure and R_F is discussed. For acids of chain length C_{12}–C_{22} (saturated and unsaturated) the best results were obtained with a paraffin/aqueous acetic acid system. For acids containing more than one hydroxyl group a castor oil/aqueous acetic acid system gives satisfactory results.

2. The method is made quantitative by photometric estimation of the copper (as the dithiooxamide complex) retained in the paper by the copper soaps of the fatty acids. Amounts of acid from 1 to 70 μg. in each spot and total quantities of up to 140 μg. of mixed fatty acids can be estimated.

3. A method is described for the analysis of natural mixtures of saturated and unsaturated acids by comparison of chromatograms of: (1) the original mixture, (2) the mixture after hydrogenation, and (3) the mixture after oxidation with alkaline permanganate. For a full analysis 5 mg. of fatty acids is sufficient.

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Effects of Methyl Bromide on Phosphorus Metabolism in the Adult Housefly, *Musca domestica* L.

BY F. P. W. WINTERINGHAM, G. C. HELLYER AND M. A. McKAY
Department of Scientific and Industrial Research, Pest Infestation Laboratory, London Road, Slough, Bucks

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The distribution and biochemical significance of the acid-soluble phosphorus compounds of insect tissues closely resemble those of higher animals. For example, the principal intermediates which accumulate in rat muscle (LePage, 1948) are also those found in the thoracic muscle of the housefly (Winteringham, Bridges & Hellyer, 1955*a*). Chefurka (1954) has demonstrated in homogenates of housefly tissue many of the glycolytic enzymes of the Embden–Meyerhof scheme. Oxidative phosphorylation has been demonstrated with sarcosomes isolated from blowfly muscle by Lewis & Slater (1954). Adenosine triphosphate (ATP) recovered from various insect species appears to be identical with that recovered from vertebrates (Albaum & Kletzkin, 1948; Calaby, 1951). It also appears to play a comparable role in muscle contraction (Gilmour & Calaby, 1953*a*; Maruyama, 1954*a*, *b*) and in the synthesis of acetylcholine (Smallman, 1956). There are, however, some differences between the phosphorus metabolism of insects and higher animals which might be significant in the mechanisms of toxic action in the intact animal. For example, Sacktor (1955) and Zebe & McShan (1957) have observed a remarkably low lactic acid dehydrogenase activity in some insect-muscle preparations and have suggested that lactic acid may not accumulate as an end product of glycolysis as it does in vertebrate skeletal muscle. An earlier intermediate such as a triose phosphate may enter the Krebs cycle more directly. Arginine phosphoric acid (Arg-P) plays the part of the phosphagen of insects, in common with other invertebrates (Baldwin & Needham, 1934), instead of the creatine phosphoric acid of vertebrates. There is evidence that certain of the glycolytic enzymes of insect and vertebrate tissues may differ in their sensitivity to inhibitors. For example, Chefurka (1954) was unable to find any inhibition of housefly triose phosphate dehydrogenase by 0·1 mM-iodoacetate, which inhibits the mammalian enzyme (Cori, Slein & Cori, 1948). This paper describes a quantitative study, by means of the labelled-pool technique (Winteringham, 1956), of the effects of methyl bromide on the phosphorus metabolism of the adult housefly, *Musca domestica* L., *in vivo*. For a preliminary communication see Winteringham & Hellyer (1954).

METHODS AND MATERIALS

Labelling and extraction. Techniques (Winteringham *et al.* 1955*a*) for labelling with ^{32}P the soluble intermediates of adult houseflies *in vivo*, and for their extraction, have been used with minor modifications. Entire thoraces (minus wing and leg appendages) or abdomens have been homogenized and extracted three times with 1·05 ml. portions of ethanolic formic acid instead of 1·8 ml. portions as originally described, and concentration of the extracts *in vacuo* has been omitted. This modification was without significant effect on either recovery or distribution of the labelled compounds. Entire heads have similarly been extracted three times with 0·55 ml. portions of ethanolic formic acid.

Paper-radiochromatographic techniques. The techniques described earlier (Winteringham *et al.* 1955*a*) have been used with slight modification. Resolution of the ATP–adenosine diphosphate (ADP) fraction in the aq. acetic acid–acetone solvent originally described has been omitted in the