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# The Phospholipids of the Housefly, *Musca domestica*

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Relatively few investigations have been reported on the phospholipid content of insects. A study of the lipids of bee brain was made by Patterson, Dumm & Richards (1945). More recently Wren & Mitchell (1959) have described the fractionation of the lipids of *Drosophila melanogaster*, Bieber, Hodgson, Cheldelin, Brookes & Newburgh (1961) the phospholipids of the blowfly, *Phormia regina*, and Fast & Brown (1962) the lipids of *Aedes aegypti* larvae. The incorporation of [$^{32}$P]orthophosphate into the phospholipids of *Arctia caia* moths has been studied by Chojnacki & Korzybski (1962). The present paper describes a study of the phospholipids of the housefly, *Musca domestica*, during which evidence was obtained for the presence of a novel phospholipid of the sphingomyelin type. It was possible to study the minor phospholipid constituents by labelling the insect phospholipids with $^{32}$P by techniques similar to those described by Winteringham, Bridges & Hellyer (1955) for the labelling of water-soluble phosphorus compounds. Because of the slow rate of turnover of some of the phospholipids it was not possible to use the distribution of $^{32}$P radioactivity as a measure of the distribution of phosphorus, so that this was determined by chemical methods. A preliminary account of the phospholipids of the housefly which are stable to both mild alkaline and mild acidic hydrolysis has been given by Crone & Bridges (1962), and the identification of the housefly phospholipids has been briefly reported by Bridges, Crone & Beard (1962).

## MATERIALS

*Radioactive chemicals.* L-[$\beta$-$^{14}$C]Serine (specific radioactivity 30 $\mu$c/mg.) and carrier-free [$^{32}$P]orthophosphate solution in dilute hydrochloric acid (radioactivity 5 mc/ml.) were obtained from The Radiochemical Centre, Amersham, Bucks.

*Other chemicals.* Silicic acid (100–200 mesh, chromatographic grade) was purchased from L. Light and Co. Ltd., Colnbrook, Bucks. Before use this was heated for 24 hr. at 120°. Sphingosine was prepared from sphingosine sulphate (L. Light and Co. Ltd.) by the method of Brady & Burton (1956). Glycerylphosphorylcholine was prepared from synthetic DL-$\alpha$-lecithin (L. Light and Co. Ltd.) by alkaline hydrolysis (Dawson, 1960), and a mixture of glycerylphosphorylserine and glycerylphosphorylethanolamine was obtained by a similar hydrolysis of kephalin from natural sources (L. Light and Co. Ltd.). Ethanolamine phosphate and *myo*inositol 2-phosphate were obtained from the California Corp. for Biochemical Research, Los Angeles, U.S.A. *myo*Inositol (under the name 'meso-inositol') was purchased from British Drug Houses Ltd., Poole, Dorset, and monomethylethanolamine from Kodak Ltd., London. L-$\alpha$-Glycerophosphate, as the sodium salt, was a gift from Dr R. W. Estabrook.

## METHODS

### *Labelling of housefly phospholipids* in vivo

For the preliminary experiments on the identification of the phospholipids in the housefly, *Musca domestica*, vicina type, groups of 25 1-day-old adult female flies were lightly anaesthetized with cyclopropane and placed in glass metabolism chambers containing 0·5 mc of 'carrier-free' [$^{32}$P]orthophosphate as described by Winteringham *et al.* (1955). The chambers were kept at 26–27°. After 24 hr. the flies were transferred to a 2·5 l. glass jar, supplied with glucose and water, and maintained at the same temperature for at least 24 hr., until they were taken for extraction of lipids. When a more precise knowledge of the time of introduction of the $^{32}$P was required, flies were injected intrathoracically with 1 $\mu$l. (5 $\mu$c) of [$^{32}$P]orthophosphate solution that had been brought to pH 6–7 with sodium hydrogen carbonate. Then the insects were allowed to feed on glucose and water as described above. Labelling with $^{14}$C was carried out by thoracic injection of 1 $\mu$l. (0·14 $\mu$c) of L-[$\beta$-$^{14}$C]serine and the flies were then treated as described above.

### *Extraction of phospholipids*

The method of Folch, Lees & Sloane-Stanley (1957) was followed. For the preparation of extracts of flies labelled with $^{32}$P or $^{14}$C, groups of 25 insects were killed by immersion in liquid nitrogen and extracted in glass Potter-type homogenizers with three successive 3 ml. portions of chloroform–methanol (2:1, v/v) at 0°. After each homogenization the solid was centrifuged down at 2000 *g* for 10 min. at 0°. The extracts were combined and washed with a volume of water equal to one-fifth of that of the chloroform–methanol extract. The mixture was allowed to reach room temperature and shaken vigorously, and the two layers were separated by gentle centrifuging. The water layer was removed and the surface of the organic layer washed with the upper layer from chloroform–methanol–water (8:4:3, by vol.). Drops of methanol were added to the washed extract to make it homogeneous.

Larger amounts of unlabelled phospholipid were obtained by a similar extraction of 20 g. live weight of flies. These were killed in liquid nitrogen and ground in a chilled mortar with appropriate quantities of extraction medium. The crude extract was washed as described above and then evaporated to dryness under reduced pressure at room temperature. The lipid was taken up in a small quantity of chloroform–methanol (2:1, v/v), leaving a proteinaceous residue. For column chromatography of unlabelled extracts a small amount of a similar radioactive extract was added to act as a marker. For experiments in which a measure of the specific radioactivity of the water-soluble phosphorus compounds was required, the extraction procedure of Hokin & Hokin (1958) was followed.

### *Fractionation of phospholipids on columns of silicic acid*

The method of Marinetti, Erbland & Kochen (1957) was used with minor modifications: 16 g. of silicic acid (100–200 mesh) was used in a column of 16 mm. internal diameter, and 2 ml. of chloroform containing not more than 100 mg. of the lipid extract was applied to the column and eluted with successive volumes of chloroform (80 ml.), chloroform–methanol (4:1, v/v) (160 ml.), chloroform–methanol (1:1, v/v) (240 ml.) and methanol (200 ml.).

The eluate was collected as fractions of about 10 ml. and the radioactivity of each assayed in a liquid-sample Geiger–Müller tube. The solvent was supplied to the top of the column by a simple siphon device without joints, which gave a solvent flow of 2·5 ml./min. with chloroform–methanol (4:1, v/v). The radioactivity was plotted as counts/min./ml. of eluate against volume of eluate (Fig. 2), and the fractions were combined into six main fractions. The solvent was removed under reduced pressure at room temperature and the residues were dissolved in chloroform–methanol (1:1, v/v). Samples were taken for chemical analysis.

### *Paper chromatography*

The following solvents were used. (1) Di-isobutyl ketone–acetic acid–water (40:30:7, by vol.); this was used with paper impregnated with silicic acid (Marinetti & Stotz, 1956). (2) Phenol saturated with water. (3) Phenol saturated with water–acetic acid–ethanol (50:5:6, by vol.) (Dawson, 1960). (4) Phenol (50 g.) dissolved in a mixture of 50 ml. of butan-1-ol, 3 ml. of 80% (v/v) formic acid and 5 ml. of water (Bremer, Figard & Greenberg, 1960). (5) Acetone–formic acid–water (30:7:13, by vol.) (Burrows, Grylls & Harrison, 1952). (6) Upper layer of butan-1-ol–acetic acid–water (4:1:5, by vol.). (7) Pyridine (Brady & Koval, 1958).

Whatman no. 1 paper was used throughout. For one-dimensional chromatograms the apparatus described by Winteringham *et al.* (1955) was used. Two-dimensional chromatograms were run on sheets 31 cm. square. Paper strips were washed in 2N-hydrochloric acid and then in water until the pH was above 5. When used with solvents 1 and 4 the strips were treated as detailed in the references.

### *Colour reactions on paper chromatograms*

*Ninhydrin.* Chromatograms were sprayed with a 0·1% (w/v) solution of ninhydrin in acetone and heated in an oven at 85° for 5–10 min.

*Sudan Black.* Chromatograms were soaked for 1 hr. in 0·1% (w/v) solution of Sudan Black in 50% (v/v) ethanol. They were then washed in 50% (v/v) ethanol to decrease the background colour.

*Osmic acid.* Chromatograms were exposed for 5 min. to osmic acid vapour in a desiccator.

*Dragendorff reagent.* This was prepared by the method of Bregoff, Roberts & Delwiche (1953) and used as a spray for detecting free choline, which gives a brick-red colour. With combined choline (e.g. glycerylphosphorylcholine) the colour is more yellow. Phosphatidylcholine and some other phospholipids give a yellow–orange reaction.

*Phosphate ester reagent.* This was as described by Bandurski & Axelrod (1951).

*Silver nitrate reagent.* This was as described by Partridge (1948).

*Aniline hydrogen phthalate.* This was as described by Partridge (1949).

*Fluorescein reagent for sphingosine.* This was as described by Saito (1960).

*Identification of compounds by paper chromatography.* Water-soluble compounds formed by hydrolysis of phospholipids were identified on paper chromatograms by colour reactions and co-chromatography with known compounds.

*Location of radioactive compounds*

Zones of radioactivity on one-dimensional chromatograms were located and assayed by the methods of Winteringham, Harrison & Bridges (1952). A $4\pi$-scanning apparatus with print-out counter was used for scanning chromatograms of low radioactivity (Winteringham, 1958). Radioautography on Kodak Crystallex X-ray film was used for the detection of $^{32}P$ on two-dimensional chromatograms.

*Hydrolytic procedures*

The mild alkaline (method 1), mild acid and strong acid hydrolyses described by Dawson (1960) were used except that a 15% (v/v) solution of conc. hydrochloric acid (sp.gr. 1·18) in methanol was used for the final hydrolysis. In one experiment, the effect of this hydrolysis method was compared with the results obtained with anhydrous methanolic 2N-hydrochloric acid. These hydrolysates were then evaporated to dryness, and each was dissolved in 0·5 ml. of water and extracted twice with 0·5 ml. of chloroform. A comparison was also made between hydrolysis with 15% conc. hydrochloric acid in methanol and the procedure of Carter, Smith & Jones (1958), who hydrolysed glycerol ether phosphatide by refluxing for 40 min. with 1N-sulphuric acid.

The presence of plasmalogens was investigated with the acetic acid-hydrolysis method of Marinetti, Erbland & Stotz (1959), and by hydrolysis of the dried lipid extract at room temperature for 18 hr. with acetic acid–saturated aq. mercuric chloride solution (5:1, v/v).

The phospholipid bases were released by hydrolysis at 100° for 48 hr. with 6N-hydrochloric acid in sealed tubes (Levine & Chargaff, 1951). The hydrolysate was extracted twice with equal volumes of chloroform, evaporated to dryness and redissolved in water.

*Chemical determinations*

*Phosphorus.* Samples containing 1–5 $\mu$g. of phosphorus were digested with 1 ml. of 72% (v/v) perchloric acid in 10 ml. graduated tubes on an electrically heated rack for 3 hr. This decreased the volume of acid to 0·7 ml. The blue phosphomolybdate colour was developed by the heating method of Bartlett (1959). The phosphorus contents of individual phospholipids were determined by determination of the phosphorus in hydrolytic products separated by paper chromatography. These were detected by radioautography or colour tests, and the corresponding areas of paper were cut out, cut into small pieces and digested as described above. Similar areas of paper from blank chromatograms were digested to correct for the phosphorus content of the paper. This procedure was satisfactory provided that each area of paper did not exceed 15 cm.$^2$, and that the tubes were heated intermittently at first to avoid explosions. Determinations of the phosphorus of phospholipids separated on silicic acid papers, either by direct digestion or by elution and subsequent determination, gave poor recoveries.

*Choline.* Lipid samples were hydrolysed with 6N-hydrochloric acid in sealed tubes at 100° for 48 hr. The hydrochloric acid was removed *in vacuo*, fatty acids were extracted with light petroleum (b.p. 60–80°), and the choline was determined by the method of Smits (1957).

*Ethanolamine.* After hydrolysis as for the choline determinations, the ethanolamine was determined by the method of Axelrod, Reichenthal & Brodie (1953).

*Serine.* Lipid samples were first separated by paper chromatography with solvent 1. The corresponding zones to peak VI (Fig. 1) were eluted with methanol, and the eluates were evaporated to dryness and hydrolysed as for the choline determination. The hydrolysates were applied to Whatman no. 1 paper sheets together with known weights of serine and chromatographed overnight in solvent 6. The sheets were dried, dipped in a 0·5% (w/v) solution of ninhydrin in 1% (v/v) acetic acid in acetone, and heated at 90° for 30 min. The colour zones corresponding to the serine, which were completely separated from those of the ethanolamine, were cut from the paper and the sections were each extracted with 4·0 ml. of 60% (v/v) ethanol and centrifuged. The colours were read at 570 m$\mu$ against a blank prepared by extraction of a similar blank area of paper. Extinction was proportional to the amount of serine up to 15 $\mu$g. A similar procedure for the determination of ethanolamine was not satisfactory.

*Inositol.* Lipid samples containing 20–50 $\mu$g. of inositol were hydrolysed as described for the choline determination. Inositol was separated from glycerol by paper chromatography with butan-1-ol saturated with water as solvent. The area containing the inositol was cut from the strip, and the inositol was eluted with water and determined by the periodate method of Agranoff, Bradley & Brady (1958). Known weights of inositol were similarly treated.

*Measurement of specific radioactivity*

After chemical assay for phosphorus, a sample of the solution of the phosphomolybdate complex was diluted to 10 ml. with water and the radioactivity assayed in a liquid-sample Geiger–Müller tube. Normally a minimum of 1000 counts was recorded. Corrections for background, probe-unit quench-time (400 $\mu$sec.) and decay were made in the usual manner.

## RESULTS

*Chromatographic behaviour of phospholipids.* A typical distribution pattern of $^{32}P$-labelled phospholipids from houseflies after separation on a paper chromatogram is shown in Fig. 1. All radioactive zones except zone I could be stained with

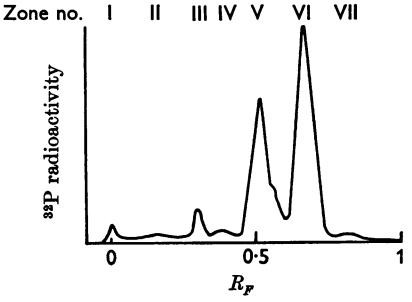


Fig. 1. Radiochromatogram of $^{32}P$-labelled phospholipids extracted from houseflies fed with [$^{32}P$]orthophosphate. Experimental details are given in the text.

osmic acid and Sudan Black. Zones IV and VI gave positive reactions with ninhydrin. Zone I was water-soluble.

Fig. 2 shows the distribution of $^{32}P$ radioactivity in the phospholipids from a housefly-lipid extract separated on a column of silicic acid. The separation of fractions 2 and 3 varied in different experiments. Table 1 shows the correlation of the column fractions with the zones separated on paper, and also the bases identified in the fractions.

*Mild alkaline hydrolysis of lipid extracts.* This hydrolysis was performed on the total lipid extract and on the separated column fractions. Between 90 and 95 % of the $^{32}P$ radioactivity became water-soluble. Chromatography in solvent 5 of the water-soluble hydrolysate of the total lipid extract and also of column fraction 4 separated one radioactive zone ($R_F$ 0·12) from the main zone of activity ($R_F$ 0·17–0·40). The former zone coincided with added *myo*inositol 2-phosphate. The water-soluble products were also chromatographed two-dimensionally in solvents 3 and 5 (Fig. 3). On the basis of colour reactions, of $R_F$ values of known compounds and of the identity of the constituent bases, the products of alkaline hydrolysis of the separated column fractions are given in Table 2. Column fraction 1 contained no nitrogenous base, and the water-soluble product after alkaline hydrolysis was not α-glycerophosphate since it had $R_F$ values in solvents 3 and 5 of 0·30 and 0·35 respectively, compared with 0·45 and 0·60 for α-glycerophosphate in the same solvents. Traces of other $^{32}P$-labelled compounds in addition to those listed in Table 2 were resolved by two-dimensional chromatography (zones *d*, *f*, *g* and *i* in Fig. 3). Similar chromatography in solvents 3 and 4 of the alkaline hydrolysate of the total lipid extract showed that only two zones moved in the second solvent. One proved to be glycerylphosphorylcholine, which had $R_F$ 0·46 in solvent 4, and the other separated from the glycerylphosphorylethanolamine and had $R_F$ 0·26 in the second solvent.

*Detection of plasmalogens in phospholipids.* When the total lipids were subjected to mild alkaline hydrolysis and then hydrolysed with trichloroacetic acid (Dawson, 1960), less than 5 % of the $^{32}P$ radio-

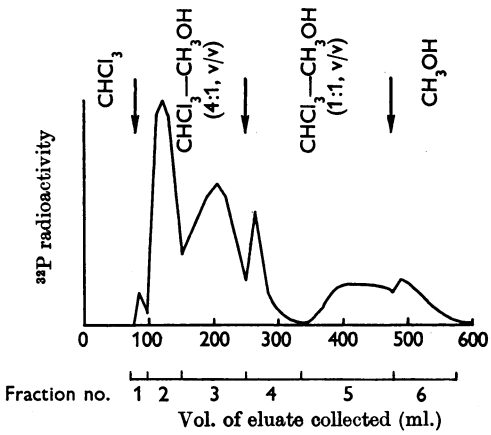


Fig. 2. Elution of $^{32}P$-labelled phospholipids from the housefly from a silicic acid column. The phospholipids were eluted with increasing proportions of methanol in chloroform, the points of solvent change being indicated by arrows. Fractions of approx. 10 ml. were collected and counted in a Geiger–Müller liquid-counter tube. They were then combined into the main fractions shown at the bottom of the Figure.

Table 1. *Relation of phospholipid fractions from the housefly separated by column chromatography to zones separated by paper chromatography, and the identity of bases liberated from the fractions by hydrolysis in 6N-hydrochloric acid*

Experimental details are given in the text. The column fraction nos. are as in Fig. 2 and the zone nos. on paper chromatograms as in Fig. 1.

| Column fraction no. | Zone no. on paper chromatograms | Bases identified in fraction |
|---|---|---|
| 1 | VII | None found |
| 2 | VI | Ethanolamine |
| 3 | VI | Ethanolamine + serine |
| 4 | III, IV, V, VI | Ethanolamine + serine |
| 5 | V | Choline |
| 6 | V | Choline |

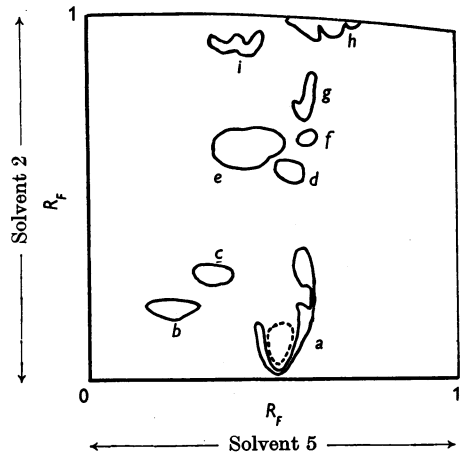


Fig. 3. Tracing of a radioautograph from a two-dimensional chromatogram of the water-soluble products of alkaline hydrolysis. The technique is described in the text. The broken line encloses the salt spot visible on the wet chromatogram. The main spots are those given below, and the others are mentioned in the text. *b*, Glycerylphosphorylinositol and phosphorylinositol; *c*, glycerylphosphorylserine; *e*, glycerylphosphorylethanolamine; *h*, glycerylphosphorylcholine.

activity remaining soluble in the organic-solvent extract from the first treatment became water-soluble after the second hydrolysis. Paper chromatography of similar aqueous extracts after mild acid hydrolysis of the separated column fractions revealed glycerylphosphorylethanolamine, derived from the phospholipids in column fractions 2, 3 and 4, glycerylphosphorylserine from fractions 3 and 4, and glycerylphosphorylcholine from fractions 4, 5 and 6. The small amounts of these compounds could have arisen from a slight contamination of the organic extract with the aqueous extract after alkaline hydrolysis and does not necessarily indicate the presence of plasmalogens. Acetic acid hydrolysis of the total lipid extract resulted in changes in the distribution of $^{32}P$ radioactivity on chromatograms run in solvent 1 (Table 3). The water-soluble material in zone I of these chromatograms was eluted and rechromatographed in solvents 3 and 5. Inositol monophosphate or glycerylphosphorylinositol, α-glycerophosphate and the glycerylphosphoryl derivatives of ethanolamine, serine and and choline were present. Increasing the time of hydrolysis for two further periods of 20 min. caused the amount of water-soluble radioactivity to increase from 12·6 % to 15·6 % (at 40 min.) and 17·6 % (at 60 min.). The amounts of all the glycerylphosphoryl derivatives, apart from that of inositol, increased with the longer times of hydrolysis. Hydrolysis of the total lipid extract in the presence of mercuric chloride resulted in changes in the distribution of $^{32}P$ radioactivity on chromatograms run in solvent 1 (Table 3). These changes indicate a small increase in the lysophosphatides of choline and ethanolamine (zones III and IV respectively).

*Phospholipids remaining unhydrolysed by mild alkali and mild acid.* The phospholipid remaining unhydrolysed after successive treatments with mild alkali and mild acid was equivalent to approx. 7 % of the total $^{32}P$ radioactivity of the phospholipid extract, or 4·5 % of the total lipid phosphorus. Paper chromatography of this fraction in solvent 1 separated it into two main zones of radioactivity both giving positive tests with ninhydrin. Only traces of radioactive material remained unhydrolysed after treatment of column fractions 1, 5 and 6 with mild alkali and mild acid, and these were not investigated further. The major part of this 'stable' fraction was concentrated in column fraction 4.

The rates of hydrolysis of this fraction by 15 % (v/v) conc. hydrochloric acid in methanol and by 1 N-sulphuric acid were compared by following the appearance of water-soluble, and fall in chloroform-soluble, $^{32}P$ radioactivity (Fig. 4). The specific radioactivity of the phosphorus that remained chloroform-soluble at the end of the hydrolysis was only 40 % of that of the phosphorus rendered water-soluble The water-soluble products obtained from the 'stable' fraction by hydrolysis with 15 % (v/v) conc. hydrochloric acid in methanol and with

Table 2. *Chromatographic behaviour and identity of the water-soluble products of alkaline hydrolysis of phospholipid fractions from the housefly*

Experimental details are given in the text. The column fraction nos. are as in Fig. 2 and the zone nos. on paper chromatograms as in Fig. 3.

| Column fraction no. | $R_F$ in solvent 3 | Zone no. on paper chromatograms | Compounds identified |
|---|---|---|---|
| 1 | 0·30 | *a* | Not identified |
| 2 | 0·67 | *e* | Glycerylphosphorylethanolamine |
| 3 | 0·40, 0·67 | *c*, *e* | Glycerylphosphorylserine, glycerylphosphorylethanolamine |
| 4 | 0·20, 0·40, 0·67 | *b*, *c*, *e* | Glycerylphosphorylinositol + inositol monophosphate, glycerylphosphorylserine, glycerylphosphorylethanolamine |
| 5 | 0·90–1·00 | *h* | Glycerylphosphorylcholine |
| 6 | 0·90–1·00 | *h* | Glycerylphosphorylcholine |

Table 3. *Hydrolysis of lipid extracts from the housefly by acetic acid or mercuric chloride: changes in the distribution of $^{32}P$ radioactivity between the phospholipid zones separated by paper chromatography in solvent* 1

Experimental details are given in the text. The zone nos. on paper chromatograms are as in Fig. 1.

| | Change in radioactivity of zone as percentage of total radioactivity on chromatogram | | | | |
|---|---|---|---|---|---|
| Hydrolysis procedure | Zone I | Zone III | Zone IV | Zone V | Zone VI |
| Acetic acid (20 min. at 100°) | +12·6 | −2·8 | +2·7 | −2·7 | −9·8 |
| Mercuric chloride and acetic acid (18 hr. at room temperature) | + 2·1 | +0·5 | +2·4 | +2·4 | −7·4 |

anhydrous methanolic 2N-hydrochloric acid were chromatographed in solvents 2 and 6. Two main peaks of activity, both associated with ninhydrin-positive material, were found in each case. These had $R_F$ 0·45 and $R_F$ 0·8–1·0 in solvent 2, and $R_F$ 0·10 and $R_F$ 0·75 in solvent 6. The radioactive material at $R_F$ 0·45 in solvent 2 ($R_F$ 0·10 in solvent 6) could not be separated from carrier ethanolamine phosphate in solvents 2, 3, 5 or 6, and it gave orthophosphate and ethanolamine after hydrolysis with 6N-hydrochloric acid. The radioactive material at $R_F$ 0·8–1·0 in solvent 2 was isolated by two-dimensional chromatography in solvents 2 and 6, eluted from the paper and hydrolysed in 6N-hydrochloric acid. Ethanolamine was one product; attempts to demonstrate the presence of any sphingosine-like compound in this hydrolysate failed, possibly because there was insufficient material. Free ethanolamine was also present in the water extracts of the methanolic-hydrochloric acid hydrolysates.

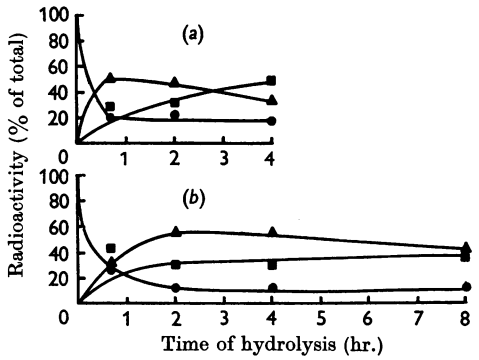


Fig. 4. Effect of two kinds of acidic hydrolysis on the phospholipid fraction which is stable to mild alkali and mild acid. The graphs show the changes in the distribution of $^{32}P$ radioactivity between the hydrolysis products after increasing times of hydrolysis. Experimental details are given in the text. ▲, Ethanolamine phosphate; ■, other water-soluble compounds; ●, chloroform-soluble compounds. After partitioning of the dried hydrolysate between chloroform and water, the ethanolamine phosphate was separated from the other water-soluble products by chromatography in solvent 5. (*a*) Hydrolysis by 1N-$H_2SO_4$ under reflux. (*b*) Hydrolysis by 15% (v/v) conc. HCl in methanol at 102°.

The chloroform-soluble material remaining after the two types of methanolic-hydrochloric acid hydrolysis was chromatographed in solvent 1. Two radioactive peaks were found in each hydrolysate. The $R_F$ values were 0·32 and 0·5–0·6, with some activity remaining at the origin. The radioactive peak of $R_F$ 0·32 was eluted and found to give $R_F$ 0·75 in solvent 6. Conversely, the radioactive peak at $R_F$ 0·75, from a chromatogram of the water extract in solvent 6, had $R_F$ 0·32 in solvent 1, indicating that one substance had become partitioned between the two layers. The amount of this radioactivity was much decreased in the extract from the anhydrous-methanolic-hydrochloric acid hydrolysis. The effects of the 'wet' and 'dry' methanolic-hydrochloric acid hydrolyses are contrasted in Table 4.

Hydrolysis of the 'stable' fraction with 6N-hydrochloric acid gave ethanolamine as a main product, together with a trace of ethanolamine phosphate. A third ninhydrin-positive compound was found, which had $R_F$ 0·55 in solvent 6 and $R_F$ 0·05 in solvent 7. Chromatography of sphingosine gave $R_F$ values of 0·7–0·8 and 0·8 respectively.

The 'stable' fraction was also hydrolysed with 5% (v/v) sulphuric acid in methanol and extracted for sphingosine in the manner described by Saito (1960). The final extract contained a zone of $R_F$

Table 4. *Comparison of the effects of hydrolysis with* 15% *(v/v) conc. hydrochloric acid in methanol ('wet') and with anhydrous methanolic* 2N-*hydrochloric acid ('dry') on the phospholipid from the housefly which is resistant to hydrolysis with mild alkali and mild acid*

Experimental details are given in the text. The values for P content show phosphorus as percentages of total phosphorus in the initial extract. The specific radioactivities are expressed relative to the lowest expressed as unity.

| | After 'wet' hydrolysis | | After 'dry' hydrolysis | |
|---|---|---|---|---|
| | P content (% of total P) | Relative specific radioactivity | P content (% of total P) | Relative specific radioactivity |
| Starting material (alkali and mild-acid stable) | 4·3 | 1·9 | 4·3 | 1·9 |
| Total in water layer | 2·6 | 2·1 | 2·7 | 2·3 |
| Ethanolamine phosphate in water layer | 1·9 | 2·1 | 2·5 | 2·2 |
| Sphingosylphosphorylethanolamine in water layer (by difference) | 0·7 | — | 0·2 | — |
| Total in chloroform layer | 1·3 | 1·3 | 1·2 | 1·0 |
| Sphingosylphosphorylethanolamine in chloroform layer* | 0·55 | — | 0·2 | — |

* Values obtained from activity distributions on solvent 1 chromatograms.

Table 5. *Distribution of* $^{32}P$ *radioactivity between the main phospholipid zones separated by paper chromatography from lipid extracts made at various times after feeding houseflies on* [$^{32}P$]*orthophosphate for 24 hr., and the specific radioactivities of the total phospholipid, phosphatidylethanolamine and phosphatidylcholine phosphorus relative to that of the water-soluble phosphorus compounds*

Experimental details are given in the text. The zone nos. are as in Fig. 1. The results are the means of duplicate determinations.

| Time after giving [$^{32}P$]phosphate (days) | Radioactivity in each zone as percentage of total of the four zones | | | | Specific radioactivity of P in fraction relative to that of water-soluble P | | |
|---|---|---|---|---|---|---|---|
| | Zone III | Zone IV | Zone V | Zone VI | Total phospholipid | Phosphatidylethanolamine | Phosphatidylcholine |
| 0 | 11·3 | 7·8 | 27·7 | 53·2 | 0·2 | 0·2 | 0·3 |
| 1 | 9·0 | 7·6 | 28·2 | 55·2 | 0·6 | 0·6 | 0·6 |
| 2 | 6·7 | 3·4 | 31·7 | 58·2 | 0·6 | 0·6 | 0·9 |
| 3 | 6·5 | 3·1 | 31·6 | 58·8 | 0·7 | 0·7 | 1·0 |
| 7 | 5·8 | 2·9 | 31·3 | 60·0 | 0·8 | 0·7 | 1·1 |
| 14 | 5·3 | 2·9 | 32·0 | 59·8 | 0·9 | 0·9 | 1·2 |

Table 6. *Specific radioactivities of phosphorus-containing compounds present in the mild-alkaline hydrolysates of phospholipid extracts prepared from houseflies* 1 *and* 14 *days after injection with* [$^{32}P$]*orthophosphate solution*

Experimental details are given in the text. The compounds were separated one-dimensionally in solvent 3. The zone nos. on paper chromatograms are as in Fig. 3. The results are the means of duplicate determinations.

| Compound | Zone no. on paper chromatograms | Specific radioactivity of zone expressed relative to that of the P in the whole hydrolysate | |
|---|---|---|---|
| | | 1 day after injection with [$^{32}P$]phosphate | 14 days after injection with [$^{32}P$]phosphate |
| Glycerylphosphorylinositol, inositol monophosphate, glycerylphosphorylserine and others | *b*, *c* and *a* (?) | 0·69 | 0·77 |
| Glycerylphosphorylethanolamine and others | *d*, *e* and *f* | 0·87 | 0·92 |
| Glycerylphosphorylcholine and one other | *h* and *i* | 1·51 | 1·21 |

0·7–0·8 in solvent 6, which was ninhydrin-positive, gave an orange colour with 0·25 % fluorescein and was coincident with added sphingosine. Sphingosine gave a red colour with the fluorescein test.

Hydrolysates obtained with 15 % (v/v) conc. hydrochloric acid in methanol were examined for hexoses with the aniline hydrogen phthalate and silver nitrate reagents on chromatograms in solvent 6. No positive tests were obtained, with a sensitivity that should show cerebroside present at values above 1 % of the total lipids of the initial extract.

*Rate of incorporation of* [$^{32}P$]*orthophosphate into the phospholipids of the housefly.* The distribution of $^{32}P$ radioactivity in the main phospholipid fractions separated by paper chromatography in solvent 1 is given in Table 5. The lipid extracts were made at various times after the flies had been fed for 24 hr. on the [$^{32}P$]phosphate solution. Table 5 includes values for the specific radioactivity of the phosphorus in the total lipid extract and in the phosphatidylcholine and phosphatidylethanolamine, expressed as a fraction of the specific radioactivity of the water-soluble phosphorus extracted from the flies. The specific radioactivities of the two phospholipids were obtained from measurements made on their glycerylphosphoryl derivatives separated from mild-alkaline hydrolysates by chromatography in solvent 3. Table 6 summarizes the results obtained when flies were injected with [$^{32}P$]orthophosphate solution and left for 1 and 14 days before the lipids were extracted. Specific radioactivities of the phosphorus in the phospholipid fractions were obtained after separation of the glycerylphosphoryl compounds in solvent 3, and are expressed as a fraction of the specific radioactivity of the phosphorus in the total lipid extract.

*Quantitative analysis of the phospholipids in the housefly.* Table 7 (*a*) gives the distribution of phosphorus in the phospholipid fractions separated by column chromatography from housefly lipids, and also the percentage of the total phosphorus in an extract that had been hydrolysed by successive treatments with mild alkali, mild acid and 15 % (v/v) conc. hydrochloric acid in methanol. Table 7 (*b*) shows the distribution of phosphorus among the individual phospholipids. The values for the alkali-labile compounds were obtained after separation of the hydrolysis products by two-dimensional chromatography in solvents 2 and 5 and deter-

Table 7. *Distribution of phosphorus in a phospholipid extract prepared from houseflies of both sexes 2 days after emergence*

(*a*) Distribution of phosphorus among fractions eluted from a silicic acid column, expressed as a percentage of the total lipid phosphorus. (*b*) Distribution of phosphorus among individual phospholipids, expressed as a percentage of the total lipid phosphorus. The total lipid phosphorus was 0·75 mg. of phosphorus/g. wet wt. of fly. Experimental details are given in the text. The column fraction nos. are as in Fig. 2. The results are the means of duplicate determinations.

(*a*)

| | P content (% of total lipid P) | | | | | |
|---|---|---|---|---|---|---|
| Fraction no. ... ... ... ... ... ... ... ... | 1 | 2+3 | 4 | 5 | 6 | Total |
| Phosphorus in fraction | 5·0 | 72·1 | 6·0 | 12·3 | 4·6 | 100 |
| Hydrolysed by mild alkali | 4·9 | 68·5 | 4·5 | 11·7 | 4·4 | 94 |
| Hydrolysed by mild acid | 0·05 | 0·8 | 0·1 | 0·1 | 0·1 | 1·2 |
| Hydrolysed by 15% (v/v) conc. HCl in methanol | — | 1·9 | 1·2 | 0·4 | 0·1 | 3·6 |
| Phosphorus remaining soluble in chloroform after the series of hydrolyses | — | 0·9 | 0·1 | 0·1 | 0·0 | 1·1 |

(*b*)

| | P content (% of total lipid P) |
|---|---|
| Phosphatidylethanolamine* | 65·2 |
| Phosphatidylcholine | 17·2 |
| Phosphatidylserine | 3·5 |
| Polyglycerophosphatide† | 5·0 |
| Phosphoinositide | 3·2 |
| Plasmalogens‡ | 1·3 |
| Ethanolamine sphingolipid | 3·5 |
| Glycerol ether phosphatide | 1·1 |

* Includes two minor zones (*d* and *f* in Fig. 3) separated by two-dimensional chromatography.
† Column fraction no. 1.
‡ Phosphorus rendered water-soluble by mild acid but not by mild alkali.

Table 8. *Nitrogenous bases and inositol released by hydrolysis in* 6N-*hydrochloric acid from the phospholipids of the housefly*

Experimental details are given in the text. The phosphorus contents were calculated by assuming a 1:1 molar ratio of compound to phosphorus. The results are given ±S.D., with the numbers of determinations in parentheses.

| | Amount released (mg./g. wet wt. of fly) | P content (% of total lipid P) |
|---|---|---|
| Ethanolamine | 0·98 | 68·0±4·9 (11) |
| Choline | 0·58 | 19·9±0·9 (11) |
| Serine | 0·05 | 1·5±0·3 (4) |
| Inositol | 0·12 | 3·1±0·6 (6) |

Table 9. *Elution of a* $^{14}$C-*labelled lipid extract of houseflies from a silicic acid column*

The extract was prepared 5 hr. after injection of the flies with L-[$\beta$-$^{14}$C]serine. Experimental details are given in the text. Radioactivity was assayed by applying samples of the fractions to filter paper and counting under a thin end-window Geiger–Müller tube, under standard conditions of geometry and sample thickness.

| Eluting solvent | Radioactivity in eluate (% of total $^{14}$C radioactivity recovered) |
|---|---|
| Chloroform | 52 |
| Acetone | 1 |
| Chloroform–methanol (4:1, v/v) | 3 |
| Chloroform–methanol (1:4, v/v) | 40 |
| Methanol | 4 |

mination of phosphorus in the spots. Table 8 summarizes the distribution of the nitrogen-containing bases and of inositol.

*Incorporation of* L-[$\beta$-$^{14}$C]*serine into phospholipids of the housefly.* At 24 hr. after injection of [$^{14}$C]-serine into houseflies 30% of the total $^{14}$C radioactivity present in the flies was recovered in the lipid extract. Of this 30%, about two-thirds was present in the phospholipid fraction. Examination of the alkaline hydrolysate of the lipid extract by chromatography in solvents 3 and 4 showed considerable labelling of glycerylphosphorylserine and glycerylphosphorylethanolamine, but no labelling of the glycerylphosphorylcholine. The fraction of the hydrolysate remaining lipid-soluble was applied to a small silicic acid column and the fatty acids were eluted with chloroform. The remaining material was then eluted with methanol and hydrolysed with 15% (v/v) conc. hydrochloric acid in methanol. Sphingosine was added to the hydrolysate, and the water and chloroform extracts of this were chromatographed in solvent 6. The added sphingosine was detected mainly on the chromatogram of the chloroform layer, coincident with a zone of $^{14}$C radioactivity. A $^{14}$C-labelled lipid extract prepared 5 hr. after injection of the [$^{14}$C]serine was chromatographed on a small silicic acid column, with the result shown in Table 9. The glycerylphosphorylethanolamine zone separated

from the mild-alkaline hydrolysate in solvent 3 was hydrolysed with N-hydrochloric acid at 100° for 16 hr. After removal of the acid the hydrolysate was run in solvent 4. A radioactive zone separated from the main activity, the latter remaining at the point of application. The $R_F$ of the mobile zone was 0·28, compared with 0·30 for monomethylethanolamine.

## DISCUSSION

The diacylphosphatides of ethanolamine, serine and choline, and a form of phosphoinositide, are present in the housefly. One phospholipid fraction (zone VII in Fig. 1; fraction 1 in Fig. 2) is possibly a polyglycerophosphatide as it contains no nitrogenous base and did not yield α-glycerophosphate on alkaline hydrolysis, as would be expected for phosphatidic acid. The latter compound is believed to occur in trace amounts because a $^{32}P$-labelled lipid fraction having the chromatographic properties of phosphatidic acid has been found in housefly extracts after short-term $^{32}P$-labelling experiments. This fraction reached its peak of radioactivity within 1 hr. of the introduction of the $^{32}P$ label (R. G. Bridges, unpublished work). Trace amounts of other phosphorus-containing compounds could be separated out by two-dimensional chromatography of the phospholipid fraction hydrolysed by mild alkali. One of these (zone *f* in Fig. 3), from its chromatographic behaviour in the system of Bremer *et al.* (1960), could be a monomethylethanolamine derivative. From the $^{14}C$-labelling experiments a $^{14}C$-labelled fraction with similar properties to monomethylethanolamine was obtained. These phospholipids, which are all hydrolysed by mild alkali, account for approx. 94% of the total phospholipids extractable from the housefly, the two main constituents phosphatidylethanolamine and phosphatidylcholine accounting for 82% of the total. The plasmalogen content is low, since only 1·3% of the total lipid phosphorus is labile to trichloroacetic acid hydrolysis after hydrolysis with mild alkali. This is possibly an overestimate of the true plasmalogen content, since there may be contamination with material carried over from the alkali-labile fraction. The results from hydrolysis with mercuric chloride are, however, consistent with the presence of small amounts of phosphatidylethanolamine and phosphatidylcholine in the extracts. The determination of plasmalogens as their lysophosphatides after hydrolysis with acetic acid (Marinetti *et al.* 1959) gave results that were difficult to interpret, as small changes were masked by a general degradation of all the phospholipids.

The fraction of the housefly-phospholipid extract which was stable to both mild alkali and mild acid treatment was not sphingomyelin. From its chromatographic properties, its behaviour on methanolic-hydrochloric acid hydrolysis and from determinations of specific radioactivity, the fraction contained at least two compounds. The low concentration of plasmalogens found in the housefly extracts make it unlikely that any significant amounts of cyclic acetal compounds could arise from lysoplasmalogens during the mild-acid hydrolysis (Davenport & Dawson, 1962; Pietruszko & Gray, 1962). It is also unlikely that cyclic acetal compounds occur naturally in the initial lipid extracts, since most of the phosphorus in the aqueous extracts of the anhydrous-methanolic-2N-hydrochloric acid hydrolysates can be accounted for as ethanolamine phosphate. There is no trace of α-glycerophosphate or derivatives in the aqueous extracts of these hydrolysates.

The results of the various hydrolyses performed on this fraction, which is stable to mild alkaline and mild acid hydrolyses, are consistent with the presence of a sphingomyelin-type compound containing ethanolamine and of a glycerol ether phosphatide also containing ethanolamine, in the approximate ratio of 3:1. The products of hydrolysis of the former compound in methanolic hydrochloric acid are ethanolamine phosphate and a compound similar to sphingosylphosphorylethanolamine. The relative proportion of the latter product is decreased when anhydrous acid is used, in a manner comparable with the fall in sphingosylphosphorylcholine content observed in similar hydrolysates from brain sphingomyelin (Ansell & Spanner, 1961*a*). The acid hydrolysates also contain a compound chromatographically similar to, but not identical with, sphingosine. This material was labelled in the $^{14}C$ experiment. The Saito (1960) procedure alone gave a reasonable identification for sphingosine, suggesting that methanolic hydrochloric acid may degrade this compound. The possibility that cerebrosides provide a source of sphingosine is excluded by the failure to detect significant amounts of hexoses in the extracts or to elute $^{14}C$-labelled material (Table 9) from the silicic acid column with acetone (cf. Smith & Freeman, 1959).

The presence of ethanolamine phosphate in strong-acid hydrolysates of phospholipids has been reported by Dittmer, Feminella & Hanahan (1958), and small amounts were found by Carter *et al.* (1958) in hydrolysates of the glycerol ether phosphatide from egg yolk. However, ethanolamine phosphate is a major product of methanolic-hydrochloric acid hydrolysis of the 'stable fraction' from houseflies, so that it is unlikely that this compound could arise from a glycerophosphatide. We know of no reports in the literature of this suggested form of phospholipid, i.e. a sphingomyelin

in which ethanolamine replaces choline, apart from the suggestion of Weiss (1956) that such a compound occurs in brain tissue. Weiss did not, however, investigate the possible presence of glycerol ether phosphatides in his extracts which could explain his results. Both Dawson (1960) and Ansell & Spanner (1961*a*) found unidentified water-soluble material containing phosphorus in methanolic-hydrochloric acid hydrolysates of their 'stable' phospholipid fraction from various mammalian tissues. From the published chromatographic behaviour this material could be ethanolamine phosphate, which possibly indicates a more widespread occurrence of the ethanolamine-containing sphingolipid than is at present realized.

The second component of the 'stable fraction' is identified as a glycerol ether phosphatide because some phosphorus-containing material remains chloroform-soluble after hydrolysis with methanolic hydrochloric acid or with sulphuric acid. The faster-moving of the two spots separated out in solvent 1 chromatograms of the chloroform extracts from the hydrolysates must represent this compound with the nitrogenous base and any esterified fatty acid removed. The slower spot at $R_F$ 0·32 represents some of the sphingosylphosphorylethanolamine from the other lipid. The conclusion that the nitrogenous base of the ether phosphatide is ethanolamine is based on the observation that the water extracts of the various types of acid hydrolysate always contain this compound. Phospholipids of this type have been reported by Carter *et al.* (1958) in egg yolk, by Svennerholm & Thorin (1960) and Ansell & Spanner (1961*b*) in mammalian brain extracts and by Hanahan & Watts (1961) in erythrocytes.

Isotopic equilibrium between the $^{32}P$ introduced by injection or feeding and the phosphorus in the phospholipids is not attained even after 14 days, during which time the insects were fed on a phosphorus-free diet (see Tables 5 and 6). Therefore the introduction of $^{32}P$ into the adult fly does not enable the phospholipid distribution to be deduced from the radioactivity distribution, as was initially hoped. Davison & Dobbing (1960) have demonstrated that part of the phospholipid in rat brain is metabolically inert. This position could be even more marked in the housefly because up to 75 % of the phospholipid content of the adult fly is present in the pupa before emergence (R. G. Bridges, unpublished work). Thus any structural phospholipid may have been laid down before the introduction of the $^{32}P$ in the present experiments. However, preliminary experiments in which the larvae are fed on a diet containing [$^{32}P$]phosphate have shown that isotopic equilibrium does exist in the emerging adults, and this should provide a convenient method of studying the phospholipids in various tissues of the fly.

Under the conditions used by us for introducing $^{32}P$, the isotope distribution is not the same in the phospholipids as in the acid-soluble compounds. The phospholipid compounds do approach an isotopic equilibrium with the acid-soluble compounds (see Table 5), and therefore the latter compounds may also show uneven labelling among themselves. But as the rate of turnover of phosphorus in the acid-soluble compounds is faster than that in the phospholipids, little error may be involved in assuming that the distribution of $^{32}P$ radioactivity represents the phosphorus distribution in the former compounds in the normal insect under the conditions of labelling. If abnormal conditions are shown to cause rapid turnover or breakdown of phospholipids, then the above assumption may not be justified. These questions have been discussed by Winteringham (1960) in relation to the labelled-pool technique.

A comparison between the above results for the phospholipids of the whole fly and those obtained by other authors for various vertebrate tissues shows some differences that are of interest even when it is remembered that the comparison is being made between a whole insect and various tissues separated from vertebrates. The absence of sphingomyelin and the presence of a sphingolipid containing ethanolamine in houseflies are notable. Sphingomyelin occurs widely in mammals; its presence is reported in larvae of *Aedes aegypti* (Fast & Brown, 1962) but was not found in the blowfly, *Phormia regina*, by Bieber *et al.* (1961). The very low concentration of plasmalogens is also unusual for a whole organism, although similar low concentrations have been reported in trout muscle (Gray & Macfarlane, 1961) and in other vertebrate tissues. Perhaps the most striking difference is the relative abundance of phosphatidylethanolamine compared with phosphatidylcholine; the ratio of these two phospholipids varies between 1:1·5 and 1:2·0 for various mammalian tissues (Dawson, 1960), whereas the ratio is 1:0·25 in the housefly. A similar high concentration of kephalin has been reported in the references to dipteran lipids quoted above and appears to be a characteristic of this Order. The experiments reported above with [$^{14}C$]serine indicate that no labelling of the choline moiety of the phosphatidylcholine occurred in spite of a rapid labelling of the ethanolamine of phosphatidylethanolamine. This suggests that methylation of phosphatidylethanolamine to phosphatidylcholine, as demonstrated in mammalian tissues (Bremer & Greenberg, 1961), cannot occur to any extent in the housefly. The tentative identification of monomethylethanolamine and the failure to detect dimethylethanolamine in hydrolysates of $^{14}C$-

labelled housefly phospholipid indicates that the methylation of the phosphatidylmonomethylethanolamine could be the rate-limiting step in a sequence of methylation reactions between phosphatidylethanolamine and phosphatidylcholine in the housefly.

It seems that a relationship may exist between the low proportion of phosphatidylcholine in houseflies and other Diptera and the dietary requirement for choline shown by many insects (Gilmour, 1961). However, this cannot apply to all insects as we have found (unpublished work) comparatively high amounts of phosphatidylcholine in two species of cockroach (*Periplaneta americana*, 44%, and *Blattella germanica*, 53%, of the total lipid phosphorus). The latter species has a well-established dietary requirement for choline.

## SUMMARY

1. The major components of the phospholipid fraction of the housefly, *Musca domestica*, are phosphatidylethanolamine (65% of the total lipid phosphorus), phosphatidylcholine (17%), phosphatidylserine (3·5%), phosphoinositide (3%), a material believed to be a polyglycerophosphatide (5%), plasmalogens (1·3%), a glycerol ether phosphatide (1%), and a sphingolipid containing phosphorus and ethanolamine (3·5%).

2. Neither sphingomyelin nor sugar-containing lipids could be detected positively in the housefly-lipid extracts.

3. Isotopic equilibrium between the $^{32}P$ introduced and the phosphorus of the housefly lipids was not obtained, even when a long time was allowed to elapse after the introduction of the isotope.

4. The main points of difference between the phospholipids of the housefly and those of mammals appear to be the low plasmalogen content, the absence of sphingomyelin, the occurrence of a sphingolipid containing phosphorus and ethanolamine, and the predominance of phosphatidylethanolamine.

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