'glucose + glutamate medium' of the same order (on a dry-weight basis) as the concentration *in vivo*. Presumably this difference is due to the essentially unphysiological conditions of incubation emphasizing the difference from normal brains.

It should be emphasized that the findings in the present work, although reasonably consistent in themselves, are only of a preliminary nature. This is a result of the small numbers of sheep used and of the fact that all these animals were studied in the same stage, the early stages of irreversible coma. Nothing is known of the metabolism of brains from sheep after protracted periods of coma or those showing early symptoms. By analogy, perhaps unwise, from the brains of other animals in early coma (Holmes, 1930; Ashford & Holmes, 1931; Elliott *et al.* 1942; Wortis, 1935; Setchell, 1957), it would seem likely that the metabolism of the brains of sheep in early coma would be normal if glucose were present.

SUMMARY

1. The metabolism has been studied of the brains of sheep in irreversible hypoglycaemic coma after the injection of large doses of insulin.

2. Abnormalities were found in the response of oxygen and glucose uptakes and lactate production to electrical stimulation, in the resynthesis of creatine phosphate, in the reaccumulation of potassium ions *in vitro* and in the oxygen uptake and oxidative phosphorylation by mitochondria.

3. The metabolism of glucose and pyruvate in the absence of electrical stimulation, the hexokinase and cytochrome-cytochrome oxidase activities and the oxygen uptake and oxidative phosphorylation of homogenates were normal.

4. The implications of this 'metabolic lesion' are discussed.

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REFERENCES

- Ashford, C. A. & Holmes, E. G. (1931). *Biochem. J.* **25**, 2028.
 Elliott, K. A. C., Scott, D. B. M. & Libet, B. (1942). *J. biol. Chem.* **146**, 251.
 Fazekas, J. F., Alman, R. W. & Parrish, A. E. (1951). *Amer. J. med. Sci.* **222**, 640.
 Himwich, H. E. (1951). *Brain Metabolism and Cerebral Disorders*. Baltimore: Williams and Wilkins.
 Himwich, H. E., Bowman, K. M. & Fazekas, J. F. (1940). *Arch. Neurol. Psychiat., Chicago*, **44**, 1098.
 Holmes, E. G. (1930). *Biochem. J.* **24**, 915.
 Kerr, S. E. & Ghantus, M. (1936). *J. biol. Chem.* **116**, 9.
 McClymont, G. L. & Setchell, B. P. (1956). *Aust. vet. J.* **32**, 97.
 McIlwain, H. (1955). *Biochemistry and the Central Nervous System*. London: J. and A. Churchill Ltd.
 Olsen, N. S. & Klein, J. R. (1946). *Res. Publ. Ass. nerv. ment. Dis.* **26**, 118.
 Setchell, B. P. (1957). Ph.D. Thesis: University of Cambridge.
 Setchell, B. P. (1959). *Biochem. J.* **72**, 265.
 Setchell, B. P. & McClymont, G. L. (1955a). *Nature, Lond.*, **185**, 998.
 Setchell, B. P. & McClymont, G. L. (1955b). *Aust. Vet. J.* **31**, 204.
 Wortis, S. B. (1935). *Arch. Neurol. Psychiat., Chicago*, **33**, 1022.
 Yannet, H. (1939). *Arch. Neurol. Psychiat., Chicago*, **42**, 237.

Studies on Phospholipids

5. THE SEPARATION OF DINITROPHENYLATED AND METHYLATED PHOSPHOLIPIDS BY COUNTERCURRENT DISTRIBUTION*

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Collins & Wheeldon (1957) have described the preparation of *N*-2:4-dinitrophenylated and methylated phospholipids and, by the use of these derivatives, have demonstrated the presence in

rat liver, ox brain and egg yolk of unknown forms of aminophospholipids in addition to phosphatidylethanolamine and phosphatidylserine and the corresponding plasmalogens. Further study of these hitherto unrecognized forms of phospholipids depended on the development of efficient means of separation and of adequate criteria of purity.

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Adsorption chromatography led to some breakdown but countercurrent distribution was satisfactory. A preliminary account (Collins, 1958) describes the general procedures used, and the isolation from sheep brain of a complex phospholipid which, however, does not occur, except in traces, in liver phospholipids. The term unknown aminophospholipid will be used to describe the three phospholipids with partition coefficients (after dinitrophenylation and methylation) of 1.0, 2.5 and 8.0 in carbon tetrachloride-methanol-water. The first of these corresponds to the complex phospholipid isolated from sheep brain (Collins, 1958). The present paper describes in detail the application of this technique to the phospholipids of rat liver and sheep brain.

EXPERIMENTAL

Solvent mixtures

All solvents used were reagent grade and the following mixtures were used in the countercurrent-distribution experiments. In each case the solvent mixtures were equilibrated and equal volumes of each phase used: Aqueous 85% (v/v) ethanol-light petroleum (b.p. 40–60°), as described by Lovern (1952); aqueous 95% (v/v) ethanol-light petroleum (b.p. 100–120°); methanol-light petroleum (b.p. 100–120°); CCl_4 -methanol-water (62:35:3.15, by vol.), as described by Cole, Lathe & Ruthven (1953); CCl_4 - CHCl_3 -methylene chloride-methanol-water (25:15:10:40:10, by vol.), as described by Cole *et al.* (1953).

Materials

The rat-liver and sheep-brain lipids were prepared as described by Wheeldon & Collins (1957), and were dinitrophenylated and methylated as described by Collins & Wheeldon (1957).

³²P-Labelled rat-liver lipids. The lipids were labelled either *in vivo* by intravenous injection of about 0.1 mc of $\text{NaH}_2^{32}\text{PO}_4$ in 0.2–0.5M-phosphate buffer (pH 7.4) or *in vitro* as described by Collins & Wheeldon (1957). In every case 60 min. elapsed between the introduction of ³²P and the extraction of the lipids.

Dimyristoyl-lecithin. Synthetic material was supplied by La Motte Chemical Products Co.

Methods

The methods for the determination of total P, *N*-2,4-dinitrophenyl (DNP) groups and plasmalogens have been described by Wheeldon & Collins (1957), and that for the quantitative determination of choline by Wheeldon & Collins (1958). The qualitative detection of lecithin was carried out by the method of Huennkens, Hanahan & Uziel (1954). The chromatographic procedure used was described by Collins & Wheeldon (1957).

Radioactivity measurements. The procedure described by Collins & Wheeldon (1957) was used, except for the measurement of the radioactivity in the large number of fractions obtained after chromatography or countercurrent distribution. In these, the solvent was removed from each fraction by distillation *in vacuo*, the residue dissolved in 10 ml. of

light petroleum and the solution counted in a liquid counter.

Fatty acids. The lipids (containing 0.8–8.0 μ moles of fatty acids) were hydrolysed by refluxing with ethanolic *N*-HCl for 3 hr. Ethanol and HCl were removed by heating *in vacuo*; the residue was dissolved in 10 ml. of light petroleum (b.p. 40–60°) and shaken with water to remove soluble materials. The ethanolysis resulted in a quantitative yield of the ethyl esters of the fatty acids, which were determined as described by Stern & Shapiro (1953).

Inositol. The lipid samples were hydrolysed with 6*N*-HCl for 24 hr. in a sealed tube at 110° and the inositol in the aqueous portion of the hydrolysate was determined by a microbiological procedure (Beadle, 1944).

Countercurrent distribution. An all-glass countercurrent-distribution machine (Quickfit) using 25 ml. in each phase and capable of 50 transfers was used. The value of the partition coefficient (*k*) was estimated from the position of the tube with the maximum content of the particular fraction as described by Craig (1944).

RESULTS

Separation of the non-polar and polar fractions

Collins & Wheeldon (1957) have described the separation of dinitrophenylated and methylated phospholipids into a 'non-polar' and a 'polar' fraction by means of a simplified countercurrent distribution with four separating funnels and a solvent system consisting of aqueous 85% (v/v) ethanol-light petroleum. The triglycerides, sterols and the methyl esters of DNP-phosphatidylethanolamine and of DNP-phosphatidylserine were preferentially soluble in the light petroleum-rich phase and constituted the non-polar fraction. The choline- and inositol-containing phospholipids and the unknown aminophospholipids (Collins & Wheeldon, 1957) were preferentially soluble in the ethanol-rich phase and made up the polar fraction.

The separation into non-polar and polar fractions has been studied in greater detail by the use of a countercurrent-distribution machine (see Fig. 1). Total lipids rather than the acetone-insoluble lipids were used to ensure that phospholipids with appreciable acetone solubility were not overlooked. The application to dinitrophenylated and methylated rat-liver lipids is shown in Fig. 2, which shows the polar fraction (tubes 1–15) and also the non-polar fraction (tubes 16–50) from which choline was absent. The material with a peak at tube 30 (*k* 1.5) corresponded to a mixture of phosphatidylethanolamine and phosphatidylserine as indicated by the fact that λ_{max} was at 328 m μ and the ratio DNP groups/P was 1.0 (Collins & Wheeldon, 1957). The small peak at tube 47 (*k* 16) lacked DNP groups and was designated fraction X.

When a similar experiment was carried out, with lipids labelled *in vitro* with ³²P, the distribution curve (Fig. 3) was similar in respect to DNP groups

Fig. 1. Rat-liver and sheep-brain lipids (dinitrophenylated and methylated).

Fig. 2. Countercurrent distribution of 200 mg. of rat-liver lipids after dinitrophenylation and methylation. The solvent system was aqueous 85% (v/v) ethanol-light petroleum. Each fraction was analysed for P (—) and DNP groups (---).

Fig. 3. Countercurrent distribution, after dinitrophenylation and methylation, of 323 mg. of rat-liver lipids which had been labelled with ³²P by incubation *in vitro* of a rat-liver homogenate. The solvent system was aqueous 85% (v/v) ethanol-light petroleum. Each fraction was analysed for radioactivity (—) and DNP groups (---).

but the curve obtained by measuring the radioactivity showed two peaks which were not apparent in the first experiment (Fig. 2). The peak at tube 15 (k 0.4) would normally be divided between the polar and the non-polar fractions; that at tube 42 (k 5) was apparent only because it had a high specific radioactivity and will be referred to as fraction Y.

Examination of the non-polar fraction. These results suggested that the non-polar fraction of rat-liver phospholipids contained three phosphorus-containing subfractions. Further separation was obtained either by countercurrent distribution or by chromatography. Chromatographic separation was carried out on Hyflo Super-Cel (Fig. 4) and the eluting solvent was gradually changed from light petroleum, light petroleum-benzene mixtures, benzene and benzene-chloroform mixtures to chloroform. In the first 50 tubes there were no DNP groups but a phosphorus-containing compound was present which on countercurrent distribution proved to be similar to fraction X. The next fraction (tubes 51-65) on countercurrent distribution corresponded to fraction Y. The material in tubes 66-82 had $\lambda_{\max}$ at 410 $m\mu$ and was designated fraction Z. The material in tubes 83-128 had $\lambda_{\max}$ at 328 $m\mu$, and the ratio DNP groups/P was 0.97, and consisted of a mixture of phosphatidylethanolamine and phosphatidylserine. Countercurrent distribution, however, did not separate this material from fraction Z.

Fig. 4. Chromatography, on 50 g. of Hyflo Super-Cel, of 100 mg. of the non-polar fraction of dinitrophenylated and methylated rat-liver phospholipids which had been labelled with ^{32}P by incubation *in vitro*. Gradient elution was used, in which 500 ml. of light petroleum was added to the mixing flask and 500 ml. of benzene was added to the second flask. When the second flask had emptied, 500 ml. of chloroform was added. Each fraction (10 ml.) was analysed for radioactivity (— · —) and the extinction at 400 $m\mu$ (—●—) was measured in 10 ml. of light-petroleum. The numerical values are shown in Table 1.

Additional evidence for the individuality of these fractions is shown in Fig. 5, in which the non-polar fraction of rat-liver lipids, after the removal of most of the phosphatidylethanolamine and phosphatidylserine by chromatography, was distributed in methanol-light petroleum (b.p. 100-120°). The peak at tube 21 (k 0.7) corresponded to a mixture of phosphatidylethanolamine and phosphatidylserine because $\lambda_{\max}$ was at 328 $m\mu$ and the ratio DNP groups/P was 1.0. The peak at tube 30 (k 1.5) had no $\lambda_{\max}$, but a high radioactivity, and its identity with fraction Y was confirmed by measurement of k in methanol-light petroleum and in aqueous 85% (v/v) ethanol-light petroleum. The material with a peak at tube 39 (k 3.5) also showed no $\lambda_{\max}$, and corresponded with fraction X, as shown by countercurrent distribution.

The distribution of both fractions X and Y was further investigated and the results with fraction X (Fig. 6) demonstrate that within the experimental error the radioactivity, weight and extinction at 400 $m\mu$ all follow the theoretical curve for k 3.64. The lack of selective absorption in the region 300-400 $m\mu$ confirmed the absence of DNP groups, but the fact that the extinction was proportional to P content suggested end-absorption due to a peak below 300 $m\mu$. Although the absorption was high in this region no well-defined band was present.

A similar result (Fig. 7) was obtained with fraction Y, in that there was close agreement between the distribution of radioactivity and the theoretical curve for k 1.75, indicating the presence of a single ^{32}P -containing compound. Analysis of this material, of which 60 mg. was obtained, showed the presence of 11 μmoles of P, 43 μmoles of fatty acids and 4 μmoles of fatty aldehydes, giving a ratio of fatty acid and fatty aldehyde/P of 4.2.

Fig. 5. Countercurrent distribution of 228 mg. of the non-polar fraction of dinitrophenylated and methylated rat-liver lipids after separation from most of the DNP-phosphatidyl-ethanolamine and -serine by chromatography. Radioactivity (— · —) and the extinction at 400 $m\mu$ in 10 ml. of light petroleum (—●—) were determined on each fraction.

Hydrolysis by refluxing with ethanolic 2N-KOH for 2 hr. yielded 31 mg. of unsaponifiable material, which gave a positive Liebermann-Burchard reaction, probably due to contaminating sterols because total lipids rather than the acetone-insoluble lipids had been taken.

Analysis of rat-liver lipids showed the presence of 2-3 % of the minor phospholipids (fractions X, Y and Z), the properties of which were summarized by Collins (1958).

Experiments with ^{32}P *in vivo* showed that fractions Y and Z were labelled to approximately the same degree as phosphatidylethanolamine and that fraction X was somewhat less radioactive (Table 1). Labelling *in vitro*, however, resulted in a change in this relationship; thus whereas fraction Z was about twice that of phosphatidylethanolamine, both fractions X and Y, and particularly the latter, were more than ten times as radioactive.

Examination of the polar fraction. Chromatography on Hyflo Super-Cel (1 g./mg. of lipid) produced breakdown of the lipids, as indicated by the increased number of fractions obtained on re-chromatography and by pronounced changes in absorption spectra. For these reasons countercurrent distribution was used and in rat-liver and sheep-brain lipids showed the presence of three fractions, containing both P and DNP groups, with characteristic absorption maxima and partition coefficients.

Rat-liver lipids (170 mg.) yielded 93 mg. of a polar fraction, with amino N/P 0.17, which was distributed in $\text{CCl}_4\text{-CHCl}_3\text{-methylene chloride-methanol-water}$ (100 transfers). Two peaks in the distribution of DNP groups were obtained which corresponded to material with partition coefficients of 0.5 and 4. The tubes containing the material with k 0.5 were combined (giving 26 mg. with amino N/P 0.30) and redistributed in $\text{CCl}_4\text{-methanol-water}$ (50 transfers). Two phosphorus-containing fractions were obtained (Fig. 8; k 0.9 and 2.5) but only one with DNP groups (k 2.5). The first fraction contained combined choline and was largely lecithin; a synthetic dimyristoyl-lecithin had

Fig. 6. Countercurrent distribution of 146 mg. of fraction X in methanol-light petroleum (b.p. 100-120°). Curve A is the theoretical curve calculated for k 3.64; curve B represents the extinction at 400 μ in 10 ml. of light petroleum (one division = 0.1); curve C represents the weight of each fraction (one division = 5 mg.); curve D represents the radioactivity (one division = 10 counts/min.).

Fig. 7. Countercurrent distribution of 63 mg. of fraction Y in aqueous 95% (v/v) ethanol-light petroleum (b.p. 100-120°). The solid line has been calculated for k 1.75; the circles represent the total radioactivity in each fraction.

Table 1. *Minor phospholipids of rat liver*

Lipids were labelled either *in vivo* or *in vitro* with ^{32}P and the specific radioactivity of the total lipid was put equal to 1.0.

Fraction	X	Y	Z	Phosphatidyl- ethanolamine and -serine
Lipids labelled <i>in vivo</i>				
P as % of total lipid P	0.78	0.33	1.15	9.40
Relative specific radioactivity	0.64	2.06	1.75	1.92
Lipids labelled <i>in vitro</i> (Fig. 3)				
P as % of total lipid P	0.48	0.89	1.54	13.3
Relative specific radioactivity	2.2	11.0	0.54	0.20

k 0.9 in CCl_4 -methanol-water. The second fraction (k 2.5) contained amino N and P in a ratio of 0.46 but also some lecithin, which was removed by a further 50 transfers. The overall yield of material amounted to 19% of the DNP groups of the polar fraction and had an amino N/P 0.51. The rat-liver phospholipids had been labelled *in vitro* with ^{32}P and Fig. 8 shows that the specific radioactivity of the aminophospholipid was nearly ten times that of the lecithin.

Fig. 8. Countercurrent distribution in CCl_4 -methanol-water of 26 mg. of the polar fraction of rat-liver lipids after the removal of some of the lecithin in a previous countercurrent distribution in CCl_4 - CHCl_3 -methylene chloride-methanol-water (see text). Each fraction was analysed for P (—), DNP groups (—○—) and radioactivity (---).

Fig. 9. Countercurrent distribution of 119 mg. of the polar fraction of sheep-brain lipids after dinitrophenylation and methylation. The solvent system was CCl_4 -methanol-water and the curve represents the extinction at 335 $\text{m}\mu$ of each fraction in 10 ml. of light petroleum.

When the polar fraction (119 mg.) from sheep-brain lipids (296 mg.) was distributed in CCl_4 -methanol-water two peaks in the distribution of DNP groups were obtained (Fig. 9), corresponding to partition coefficients of 1.0 and 8.0. The material with k 1.0 was free from inositol-containing phospholipids but had not been separated from the choline-containing phospholipids. Such a separation is, however, impossible to achieve in this system because of the closeness of the partition coefficient to that of lecithin (k 0.9). Separation was achieved by the use of the solubility of the material in acetone at 0° , under which conditions lecithin is insoluble. The purified material was then distributed in CCl_4 -methanol-water (50 transfers), and the product (38 mg.) had a ratio of DNP groups/P equal to 0.50 and a ratio of choline/P of 0.48. Collins (1958) has given a preliminary account of further work on this material.

The material with k 8, which was present in both rat-liver and sheep-brain phospholipids, was purified by countercurrent distribution in CCl_4 -methanol-water and in four distinct preparations yielded products with ratios DNP groups/P 0.70, 0.64, 0.65 and 0.66.

Amounts of up to 50 mg. of each of these fractions (k 1.0, 2.5 and 8.0) were isolated and certain of their properties determined (Collins, 1958). The ratio fatty acids/P was 0.96, 0.87 and 1.07 respectively whereas the ratio aldehyde/P was 0, 0.05 and 0 respectively.

DISCUSSION

Lovern (1952) and Cole *et al.* (1953) have applied countercurrent methods to the fractionation of lipid material from brain without obtaining any marked separation of the component phospholipids. However, the solvent systems used by these authors can be successfully applied to the fractionation of phospholipids after dinitrophenylation and methylation, which results in a reduction of the number of ionizable groups (i.e. free amino, carboxyl and phosphate groups) and in an alteration of the partition coefficients. Little or no emulsification has been experienced in the use of these solvent systems with DNP-lipids.

The application of these techniques to rat-liver and sheep-brain lipids involves a preliminary separation into polar and non-polar fractions followed by further fractionation which has resulted in the isolation of three minor phospholipids and three unknown aminophospholipids. That from sheep brain has fractions with k values 1 and 8, whilst that from rat liver has fractions with k values 2.5 and 8.0 in carbon tetrachloride-methanol-water. At present it is not possible to indicate their chemical nature but that fraction having k 8 and

isolated from rat liver would seem identical with that isolated from sheep brain, for similar values of $\lambda_{\max}$, and of the ratio DNP groups/P have been obtained.

Lecithin and the inositol-containing phospholipids have partition coefficients of 0.9 and approximately 4 respectively in carbon tetrachloride-methanol-water, and the similarity between these values and those of the aminophospholipids renders it difficult to isolate pure materials. However, small amounts have been obtained and those with k 1.0 and 2.5 had a ratio of DNP groups/P of 0.5, whereas those with k 8 had the same ratio, equal to 0.67. It may therefore be concluded that these compounds have more than one atom of P/molecule. All three compounds had a fatty acid/P ratio of 1.0.

Kennedy (1953, 1956) and Marinetti, Erbland, Albrecht & Stotz (1957*a, b*) have shown that, *in vitro*, [32 P]orthophosphate was mainly incorporated into phospholipid fractions which resembled phosphatidic acids. Marinetti *et al.* (1957*b*) have shown that there are two phosphatidic acid fractions which constituted 7% of the rat-liver lipid P and that one of these had an ester/P ratio of 6.04. In the present work fraction Y is a phosphatidic acid with an ester/P ratio of 4.2 and has a high specific radioactivity after incorporation of [32 P]orthophosphate *in vitro* and may correspond to one of the two fractions described by Marinetti *et al.* (1957*b*). The other fraction which has a high specific radioactivity after incorporation of [32 P]-orthophosphate *in vitro* is the unknown aminophospholipid, with k 2.5 (carbon tetrachloride-methanol-water), shown to be present in rat liver. This is not a phosphatidic acid, but because the ratio DNP groups/P is 0.5 there must be two P atoms per molecule, and it may be a labile compound of a phosphatidic acid and an amino N-containing phospholipid. If this were so then the phosphatidic acid component may correspond to the second fraction described by Marinetti *et al.* (1957*b*). The instability of the unknown aminophospholipids on chromatography on even such a weak adsorbent as Hyflo Super-Cel is consistent with this hypothesis.

The precise nature of fraction X remains obscure but sufficient of fraction Y was obtained to show that the ratio fatty acids/P was 4.2, which may indicate that this fraction is a bis-phosphatidic acid (see Baer, 1952; Garcia, Lovern & Olley, 1956; Olley, 1956 and Benson & Maruo, 1958).

SUMMARY

1. Countercurrent methods have been applied to the separation of dinitrophenylated and methylated phospholipids from rat liver and sheep brain.

2. Three minor phospholipids have been characterized from rat-liver lipids, two of which resemble phosphatidic acids.

3. Three different forms of unknown aminophospholipid have been distinguished by their partition coefficients (k) in carbon tetrachloride-methanol-water. Rat-liver lipids contain two with k 2.5 and 8.0 and the ratio dinitrophenyl groups/P equal to 0.5 and 0.67 respectively. Sheep-brain lipids contain phospholipids with k 1.0 and 8.0 and the ratio dinitrophenyl groups/P equal to 0.5 and 0.67 respectively, and the material with k 1.0 also contains choline (choline/P 0.48).

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REFERENCES

- Baer, E. (1952). *J. biol. Chem.* **198**, 853.
- Beadle, G. W. (1944). *J. biol. Chem.* **156**, 683.
- Benson, A. A. & Maruo, B. (1958). *Biochim. biophys. Acta*, **27**, 189.
- Cole, P. G., Lathe, G. H. & Ruthven, C. R. J. (1953). *Biochem. J.* **54**, 449.
- Collins, F. D. (1958). *Nature, Lond.*, **182**, 865.
- Collins, F. D. & Wheeldon, L. W. (1957). *Biochem. J.* **66**, 441.
- Collins, F. D. & Wheeldon, L. W. (1958). *Biochem. J.* **70**, 46.
- Craig, L. C. (1944). *J. biol. Chem.* **155**, 519.
- Garcia, M. D., Lovern, J. A. & Olley, J. (1956). *Biochem. J.* **62**, 99.
- Huennekens, F. M., Hanahan, D. J. & Uziel, M. (1954). *J. biol. Chem.* **206**, 443.
- Kennedy, E. P. (1953). *J. biol. Chem.* **201**, 339.
- Kennedy, E. P. (1956). *Canad. J. Biochem. Physiol.* **34**, 334.
- Lovern, J. A. (1952). *Biochem. J.* **51**, 464.
- Marinetti, G. V., Erbland, J., Albrecht, M. & Stotz, E. (1957*a*). *Biochim. biophys. Acta*, **25**, 585.
- Marinetti, G. V., Erbland, J., Albrecht, M. & Stotz, E. (1957*b*). *Biochim. biophys. Acta*, **26**, 130.
- Olley, J. (1956). *Biochem. J.* **62**, 107.
- Stern, I. & Shapiro, B. (1953). *J. clin. Path.* **6**, 158.
- Wheeldon, L. W. & Collins, F. D. (1957). *Biochem. J.* **66**, 435.
- Wheeldon, L. W. & Collins, F. D. (1958). *Biochem. J.* **70**, 43.