2. Chromatography of the washed, deproteinized, lipid extract on alumina with a gradient-elution technique, in which the water content of the solvent was slowly increased, led to the successive elution of cerebroside, sulphatide and aminophospholipid. The cerebroside and sulphatide peaks always overlapped. With one batch of alumina the galactose-containing lipids were completely separated from the aminophospholipid.

3. Gradient-elution chromatography on silicic acid, whereby the methanol content of the solvent was increased, led to the complete separation of the cerebroside from the sulphatide. The former was also separated from the aminophospholipid, whereas there was overlapping between the sulphatide and aminophospholipid.

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The Phospholipids of Butter and their Effect on Blood Coagulation

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The suggestion by Macfarlane (1955) that dietary fats may play an important role in the aetiology of atheroma and that different lipids may have widely different effects on blood thromboplastin formation led us to investigate the effect of a number of dietary fats on blood coagulation *in vitro*. At the start of this work it was generally believed that the lecithin group of phospholipids were strong potentiators of blood coagulation (Macfarlane, Trevan &

Attwood, 1941; Fullerton, Davie & Anastasopoulos, 1953) but later work by a number of workers showed that phosphatidylethanolamine present in the crude lecithin was the active material, and that pure lecithin was completely inactive (Poole, Robinson & Macfarlane, 1955; Lea, Rhodes & Stoll, 1955; Lea, 1956; O'Brien, 1956a, b; Robinson & Poole, 1956). Poole & Robinson (1956) have since demonstrated that synthetic specimens of

phosphatidylethanolamine were as active, in rat-plasma systems, as that isolated from eggs. On the other hand, Biggs & Bidwell (1957) in their experiments on the fractionation of brain phospholipids found the blood-clotting activity variably distributed through the fractions and their phosphatidylethanolamine fraction appeared to be the least active of those tested. They further found that synthetic dimyristoylphosphatidylethanolamine was inactive in human plasma even in the presence of egg lecithin as an emulsifier. The activity of synthetic phosphatidylethanolamine was slight in rat-plasma systems in comparison with human-brain phosphatides.

In the present work, the clotting activity of a large number of fats and lipid fractions had to be tested and hence a rapid blood-clotting technique involving a fat-free thromboplastin was required. The choice of test was therefore limited to the plasma Stypven (Russell viper venom, R.V.V.) method as used by Macfarlane *et al.* (1941), venom being one of the few thromboplastins which is free of fat. A modification of the above method, the 'instantaneous' technique (Billimoria, Lockey & MacLagan, 1957), was preferred, since with this technique the lipolytic action of venom on plasma fats was minimized, and interfering effects of lipids produced by lipolysis were thus reduced.

The effect of a number of the usual dietary fats on this system showed that the clotting effect was almost entirely confined to milk fats and was maximal in butter (MacLagan & Billimoria, 1956). The activity was associated with the lipoprotein in butter, and further separation indicated that the active materials were phospholipids. This paper describes the preliminary separation of the phospholipids from butter and the further fractionation of these lipids by continuous gradient elution from silicic acid columns. The separation was controlled throughout by tests of thromboplastic activity since we were mainly interested in fractions with this action. A preliminary account of some of the work has been published (Billimoria & Curtis, 1959).

METHODS

Total nitrogen. This was determined by nesslerization by the method of King & Wootton (1956) with a modified digestion mixture (C. Long, personal communication) consisting of 100 ml. of H_2SO_4 , 25 g. of K_2SO_4 , 25 mg. of HgO and water to 2 l. Digestion of the lipid containing usually 10–20 μg . of N was carried out with 0.5 ml. of the digestion mixture heated for 20 min. on an electric micro-digestion apparatus (A. Gallenkamp and Co. Ltd., London, E.C. 2).

Amino nitrogen. This was determined on the intact lipids by the procedure of Lea & Rhodes (1954); preliminary hydrolysis with 0.5N-HCl at 100° for 15 min. was employed for the glycosides.

Phosphorus. This was determined after removal of the solvent followed by digestion with perchloric acid by a slight modification of the method of Allen (1940) in which the volume used in the colorimetric determination was decreased in order to increase the sensitivity of the method. Perchloric acid (0.5 ml., 60%, w/v) was used for the digestion of lipid containing 2–50 μg . of P. The digest was diluted with 5 ml. of water followed by the addition of 0.25 ml. of 8% (w/v) ammonium molybdate and 0.5 ml. of amidol reagent made up by dissolving 1 g. of amidol in 100 ml. of water containing 2–50 g. of sodium metabisulphite. The blue colour obtained was read after 12 min. in a Unicam SP. 600 spectrophotometer at 690 m μ in a 1 cm. cuvette.

Sugars. These were estimated by the orcinol- H_2SO_4 method cited by Winzler (1955).

Paper chromatography of bases and sugars formed on hydrolysis. For bases, a sample of the combined peak fractions, obtained by chromatography, containing about 5 mg. of the lipids was introduced into a thick-walled Pyrex tube; the solvent was evaporated *in vacuo* and 1 ml. of 6N-HCl added. The tube was sealed and heated overnight at 100°. The tube was cooled and cut open and the HCl evaporated off *in vacuo* (0.1 mm. Hg). Water (0.5–1 ml.) was added and the solution evaporated again to remove traces of HCl. The residue was dissolved in 0.1–0.2 ml. of water, and 5 μl . of the solution was applied to the paper.

For chromatography of sugars, samples containing 5 mg. of sugar were hydrolysed for 2 hr. at 100° with 2 ml. of N- H_2SO_4 . An excess of BaCO_3 was added, and the mixture was heated to boiling and filtered. The precipitate was washed with 5 ml. of water, and the filtrate was evaporated to dryness *in vacuo* (0.1 mm. Hg). The residue was dissolved in 0.5 ml. of water, and 5–10 μl . was applied to paper for chromatography.

For chromatography of phosphate esters, samples containing 5 mg. of phosphate were hydrolysed with 6N-HCl (1 ml.) at 100° for 15 min. After dilution with water (1 ml.), the solution was filtered and the precipitate washed with water (1 ml.). The filtrate and washing were evaporated to dryness at 50° *in vacuo*, and the residue was dissolved in water (0.5 ml.). Portions containing 20 μg . of phosphorus were applied to Whatman no. 54 paper and chromatographed in ethyl acetate–pyridine–water as described by Hanes & Isherwood (1949).

Preparation of silicic acid. Silicic acid (500 g., 100-mesh, Mallinckrodt A.R. grade from Savory and Moore Ltd., Lawrence Rd., London, N. 15) was thoroughly mixed with 2 l. of water and the suspension allowed to settle for 1 hr. in a 2 l. measuring cylinder. The supernatant liquid containing the finer particles was rejected, and the residue was filtered off under suction and dried in an air oven overnight at 105–110°. The material was lightly ground and sieved through a 100-mesh nylon gauze and stored in a tightly stoppered bottle.

Characterization of phospholipids. The various phospholipid fractions, obtained by column chromatography, were identified and characterized by chromatography of the unhydrolysed phospholipids on silicic acid-impregnated paper, by the methods of Marinetti, Erbland & Kochen (1957) and Lea *et al.* (1955). Rhodamine 6G followed by ultraviolet light was used to detect all lipids, the ninhydrin reaction to detect the amino lipids and phosphomolybdic

acid reagent for lipids containing choline. Sphingomyelin was also characterized by its reineckate reaction, its resistance to mild alkaline hydrolysis and the presence of a chloroform-extractable nitrogenous base after prolonged hydrolysis with $\text{Ba}(\text{OH})_2$ solution followed by acidification. Choline was detected by the method of Levine & Chargaff (1951) and by its reineckate reaction (Glick, 1944).

After hydrolysis of the lipids, the bases liberated were characterized by paper chromatography by the methods of Datta, Dent & Harris (1950). Phosphoric esters were detected by the method of Hanes & Isherwood (1949), and glycerophosphoric acid was determined by the method of Burmaster (1946). Sugars were detected by paper chromatography (Jermyn & Isherwood, 1949).

Fractions containing sugar. The sugar was shown to be present in chemical combination as follows. Emulsification of the individual fraction with water in a sonic vibrator followed by Seitz filtration gave a filtrate free of sugar, and fractionation between water and chloroform gave an aqueous layer free of sugar. The sugars were, however, readily liberated by acid hydrolysis as indicated above.

Butter. Numerous samples of butter were used in this work, all obtained by retail purchase. No obvious differences were noted between different brands bought at various seasons of the year.

Phospholipids. Synthetic L- α -dimyristoylphosphatidylethanolamine and L- α -dimyristoyllecithin were kindly supplied by Dr Erich Baer (University of Toronto, Canada). A sample of sphingomyelin was kindly supplied by Dr Elizabeth Smith (Courtauld Institute of Biochemistry, Middlesex Hospital Medical School, London, W. 1) and others were purchased from L. Light and Co. Ltd., Colnbrook, Bucks, England.

Thromboplastic activity. The blood-clotting activity of the lipids was tested on normal fasting-human plasma by the Stypven method and by plasma and whole-blood recalcification techniques as described earlier (MacLagan & Billimoria, 1956; Billimoria *et al.* 1957). The thrombin- and thromboplastin-generation tests used were performed as described by Biggs & Macfarlane (1957). The weight of lipid added was obtained by multiplying its P content by 25.

Apparatus. A graduated cylindrical donor vessel diam. 60.6 mm. containing methanol (1.5 l.) was connected at the base by means of a stopcock (S_1) to a similar receiver vessel (R) diam. 83.5 mm. containing CHCl_3 (1.5 l.). The column containing silicic acid was connected by means of a stopcock (S_2) attached to a second outlet in (R) and the fractions were collected in a fraction collector. Vessel (R) carried a small iron nail covered in a Fluon sleeve which, in conjunction with a rotating magnet held under the receiver, acted as a stirrer.

The diameters of the vessels were such that when, with the stirrer in motion, the stopcock (S_2) was closed and (S_1) opened, there was no solvent flow from one cylinder to the other. The gradient elution was started by opening the stopcock (S_2).

The gradient obtained was checked by introducing a standard dye solution of Sudan Black in methanol in the donor vessel and reading the colour of the resulting mixture of CHCl_3 -methanol-dye issuing from the receiver vessel and collecting in the fraction cutter tubes.

The gradient could also be obtained by a simple calculation without performing the above experiment. If H_1 is the height of the methanol and H_2 the height of the mixture

after a volume V has been collected, then $H_1/H_2 = \rho_1/\rho_2$, where ρ_1 and ρ_2 are the densities of methanol and mixture respectively. The density of the mixture (ρ_2) can be found after a fixed volume V leaves the cylinder (R) from measurements of H_1 and H_2 . Since the change in volume due to the mixing of CHCl_3 and methanol was found to be negligible, the percentage composition of the two solvents may be read off directly from a reference line drawn joining the two points corresponding to the density of pure CHCl_3 and pure methanol.

RESULTS

Dietary fats and Stypven times

The Stypven clotting times of plasma were substantially decreased by adding certain fats to plasma. Thus Fig. 1 shows the effect of two typical fats, indicating that butter is much more effective than margarine at all concentrations tested. The maximum effect was produced by 10 mg. of butter/system, which produced a 78% drop in clotting time.

Clotting unit. Since butter was the most active natural fat tested, the activity of the least amount of butter (10 mg.) which produced the maximum shortening of clotting time was chosen as one clotting unit. The other fats could then be assigned units/mg. With feebly active fats or certain lipid fractions available only in limited quantity, it was impossible to produce minimum clotting times and the units were read from the calibration curve based on a serial dilution of butter. Thus if 0.01 mg. of a substance gave a clotting time of 11 sec. with the plasma used in Fig. 1, corresponding to 1 mg. of butter (0.1 unit), this substance would have an activity of 10 units/mg.

Fig. 1. Effect of adding butter or margarine on the Stypven clotting times of normal fasting plasma. ●, Butter; ○, margarine; ×, control.

Other clotting systems

The effect of various concentrations of butter and margarine on the plasma recalcification system were also studied; Fig. 2 shows that again butter was far more active than margarine. Maximum effects were obtained from 5 mg. of butter and a much smaller maximum from 1 mg. of margarine. The inhibitory effects obtained with larger quantities of the fats were quite pronounced with this clotting system, especially with margarine, which gave a clotting time longer than the control at a concentration of 10 mg./system. This latter effect is not clearly understood but may be due to mechanical interference of neutral fat with the clotting system.

Butter was also tested in the whole-blood coagulation system, and the thrombin- and thromboplastin-generation tests; it was active in all three systems.

Fractionation of butter

A typical preliminary separation of active material from butter is shown in Fig. 3, where the progress of fractionation was followed by estimation of clotting activity at various stages. Butter (1) was melted overnight at 40° and centrifuged (2000 *g* for 0.5 hr.), when four visible layers were formed. The top, clear, neutral-fat layer (2) was almost inactive; this was followed by three aqueous lipoprotein layers which contained the major proportion of the activity. These aqueous layers (3) were combined and extracted with ether at 40° to remove most of the residual neutral fat. In this process only 10% of the activity was found in the

extract (4), even when chloroform was used instead of ether, suggesting that the active lipid is present mostly in the form of lipoprotein at this stage. In confirmation of this, when the ether-insoluble residue (5) was treated with boiling acetone and the mixture filtered after cooling, the activity of the dry, acetone-insoluble precipitate (7) was almost quantitatively extracted by chloroform (Soxhlet extraction). The chloroform extract (9) was evaporated to a small bulk, and the phospholipids (10), precipitated at 0° with a large volume of acetone, had an activity of approx. 40 units/mg. This represented a 400-fold concentration and an overall recovery of 74% of the total original activity. The phospholipids, dissolved in a minimum of chloroform, were adsorbed on a short silicic acid column, and the column was washed with chloroform to remove non-phospholipid fractions. The crude phospholipids (12) were eluted with ethanol-chloroform-water (6:3:1, by vol.) and, after evaporation of the solvent, had an activity of approx. 60 units/mg. The acetone-soluble fractions (6) and (11) and the residue insoluble in chloroform (8) were almost inactive.

Having once established that the activity from butter was due to its phospholipid content, for further work, the chloroform extracts from stage (9) were directly adsorbed on a column of silicic acid and concentrated as indicated at stage (12). Thus, in large-scale work, extracts of as much as 15 g. of total lipid containing about 1 g. of phospholipid could be directly adsorbed on large-diameter columns (2½ in.) containing silicic acid (125 g.), and the lipids other than phospholipids eluted off the column with chloroform (usually about 2 l.). The phospholipids were then eluted off the columns with the mixture of ethanol-chloroform-water. After evaporation of this solvent, the lipid, dissolved in a small volume of dry chloroform, was adsorbed on to a second column for stepwise or gradient elutions, as described below.

Stepwise elution of butter phospholipids from a silicic acid column

The above phospholipids, dissolved in a small volume of dry chloroform, were readsorbed on a column of silicic acid (125 g.) and eluted with graded amounts of methanol, 1, 2, 4, 8, 16 and 32% (v/v) in chloroform and finally with a mixture of ethanol-chloroform-water (6:3:1, by vol.). The separation was followed by weighing the lipids eluted and determining their clotting activity. At each change of methanol concentration a sharp peak occurred, but the analytical data on the eluates showed that most of the substances obtained were still mixtures, and consistent results were unobtainable from repeated experiments.

Fig. 2. Effect of adding butter or margarine on the recalcification times of normal fasting plasma. ●, Butter; ○, margarine; ×, control.

Fig. 3. Preliminary separation of phospholipids from butter. u, Stypven clotting units; t.u., total units of clotting activity.

Elementary chemical analysis of the lipids and paper chromatography of intact lipids and their hydrolysis products show that the 8% fraction contained mainly serine, glycerophosphoric acid and galactose; ethanolamine was also present to a lesser extent in the hydrolysate. The fraction had a clotting activity of approx. 200 units/mg. The analysis (Found: C, 61.6; H, 10.1; N, 1.5; P, 3.7%) was in close agreement with that of a phosphatidylserine galactoside containing C_{18} and C_{20} acids, with an empirical composition $C_{49}H_{95}O_{14}NP$ (requires C, 61.8; H, 10.0; N, 1.5; P, 3.4%).

The 32% fraction was of the lecithin type since it gave no ninhydrin reaction even after hydrolysis and contained choline, glycerophosphate, glucose and galactose. The P:N ratio was 1:1.14 and the low carbon value was due to the glycoside structure (Found: C, 57.1; H, 10.1; N, 1.9; P, 3.7%). The fraction was inactive in the clotting test.

The ethanol-chloroform-water fraction contained mainly sphingomyelin as shown by paper chromatography, precipitation reaction with Reinecke's salt, and presence of choline and absence of glycerophosphate in the hydrolysate. The P:N ratio was 1:2.14 (Found: C, 57.9; H, 10.3; N, 2.9; P, 3.0%). The 1, 2, 4 and 16% fractions appeared to be mixtures and were not further investigated.

Gradient elution chromatography of phospholipids

Further information was obtained by elution of the lipids from a silicic acid column by a linear gradient elution technique; the crude phospholipids, after removal of neutral fat, were dissolved in dry chloroform and adsorbed on a column of silicic acid. Fig. 4 shows the separation of the lipids on elution with a continuous linearly increasing concentration of methanol in chloroform so adjusted that after 108 fractions of 25 ml. had been collected, the solvent in the mixing chamber was approx. 90% of methanol and 10% of chloroform. Each fraction of the eluate was analysed for phosphorus, total nitrogen and sugar; these are plotted as $\mu\text{g. atoms}$ of P and N, and μmoles of sugar per fraction. The clotting activities were also determined.

The earlier fractions 17–49 which constitute the first five peaks were poorly separated and after acid hydrolysis were found to contain over 90% of the total nitrogen as amino nitrogen; these fractions were further investigated after rechromatography. Fractions 50–108 contain choline as the base and were free from amino nitrogen. The results of rechromatography of these fractions is shown below.

Fig. 4. Chromatography of butter phospholipids by a continuous linear gradient elution with methanol- CHCl_3 from a column of silicic acid. ●, Phosphorus; ○, nitrogen; ×, sugar; —, slope of the solvent gradient. Peak numbers are in parentheses.

Peak 6 (fractions 50–59, Fig. 4), lecithin galactoside. The intact lipid was detected on a silicic acid paper chromatogram with Rhodamine 6G and the phosphomolybdic acid reagent; its R_F was 0.44. The P:N:sugar ratios were approx. 1:1:1, and after hydrolysis the products identified were glycerophosphoric acid, choline and galactose. The lipid had no clotting activity.

Peak 7 (fractions 70–79, Fig. 4), a sugar-containing phosphatidylcholine. The lipid was detected by silicic acid paper chromatography with Rhodamine 6G and the phosphomolybdic acid reagent. The P:N ratio after reprecipitation (1 vol. of chloroform plus 10 vol. of acetone) was 1:1.2. After hydrolysis, galactose and glucose were detected, and the lipid had a clotting activity of 50 units/mg.

Peak 8 (fractions 82–89, Fig. 4), lecithin. The intact lipid on silicic acid paper chromatography was detected with Rhodamine 6G and phosphomolybdic acid; its R_F , 0.71, was identical with that of synthetic lecithin run alongside and in admixture. The P:N ratio, after reprecipitation as above, was 1:1.08 (theory requires 1:1) and acid-hydrolysis products were choline and glycerophosphate. The lipid had no clotting activity.

Peak 9 (fractions 100–108, Fig. 4), sphingomyelin. The intact lipid on silicic acid paper chromatography was detected with the Rhodamine 6G and phosphomolybdic acid reagents, R_F 0.61. It readily gave a reneckate and had a P:N ratio of 1:2.18 (theory 1:2). On acid hydrolysis followed by chromatography (as described for phosphate esters) the phosphate ester had R_F 0.8 (with reference to glycerophosphate R_F 1). Choline was liberated on prolonged acid hydrolysis, as described under hydrolysis for bases. The lipid had no clotting activity.

Rechromatography of peaks 1–5

Fractions 17–50 (Fig. 4), after removal of solvent, were dissolved in a small volume of chloroform, rechromatographed on a similar silicic acid column and eluted with a linear gradient of methanol in chloroform, so adjusted that after 60 fractions of 25 ml. a concentration of 30% (v/v) of methanol in chloroform was reached in the mixing vessel. The separation of the early peaks (Fig. 5) was considerably improved, especially with respect to the sugar peak, and the nitrogen and sugar values of the fractions ran closely parallel with the phosphorus values. The peak fraction after reprecipitation as above gave the following results.

Peak 1 (Fig. 5), a complex phosphatidic acid. On silicic acid paper chromatography this gave no ninhydrin reaction and was detected with Rhodamine 6G and phosphomolybdate reagents, R_F 0.95 (considerable 'tailing'). The N content was negligible, and on hydrolysis no base was detectable and glycerophosphoric acid was obtained (tailing on chromatogram). The lipid had a weak clotting activity of 5 units/mg.

Peaks 2, 3 and 4 (Fig. 5), phosphatidylserine and phosphatidylserine galactoside. On silicic acid paper chromatography these peaks gave ninhydrin-positive spots which were also detectable with Rhodamine 6G (violet fluorescence under u.v. light) at R_F 0.73, 0.72, 0.72 respectively. The P:N ratio of each of the compounds was 1:0.87, 1:1.1 and 1:0.88 respectively, whereas peak 4 contained galactose (P:galactose ratio, 1:4.5). Hydrolysis of peaks 2 and 3 gave glycerophosphoric acid and serine whereas hydrolysis of peak 4 gave glycerophosphoric acid, serine and galactose. The clotting

Fig. 5. Rechromatography of peaks 1–5 from Fig. 4 with gradient of methanol increasing to 34% after 70 fractions. ●, Phosphorus; ○, nitrogen; ×, sugar; —, slope of gradient. Receiver diam. 83.5 mm. and donor 73 mm. Concentration of methanol in CHCl_3 in donor vessel, 57% (v/v).

activities of peaks, 2, 3 and 4 were 85, 60 and 30 units/mg. respectively.

Phosphatidylserine appears as two distinct fractions (peaks 2 and 3) when run with a neutral solvent system whereas both compounds appear together when chromatographed on silicic acid paper with acid solvents.

Peak 5 (Fig. 5), phosphatidylethanolamine, phosphatidylserine traces, compound X. The lipid in this fraction appears to be the least homogeneous and on silicic acid paper chromatography gave three distinct components. (1) Phosphatidylethanolamine, R_F 0.76, which gave a positive ninhydrin reaction and with Rhodamine 6G a yellow fluorescent spot when viewed under u.v. light. Authentic L- α -dimyristoylphosphatidylethanolamine gave identical reactions and had R_F 0.74–76. (2) Phosphatidylserine, R_F 0.72, gave a ninhydrin-positive reaction and blue fluorescence with the Rhodamine 6G reagent when viewed under u.v. light. (3) Compound X, with R_F 0.54, gave a negative reaction with ninhydrin and phosphomolybdic acid reagent, but it appeared as a blue fluorescent spot with the Rhodamine 6G reagent under u.v. light. The mixed lipids showed a clotting activity of 500 units/mg. The mixture on hydrolysis showed the presence of ethanolamine and serine.

The mixture of phosphatidylethanolamine and phosphatidylserine was separated from compound X by cutting the paper. After elution of X, its clotting activity/mg. of P was 100 times as great as that of the mixed phosphatidylethanolamine and phosphatidylserine fraction.

Summary of phospholipid fractions and their blood-clotting activities

A list of the phospholipids separated from butter is shown in Table 1, together with their clotting activities. It will be seen that the strongest clotting activity was in fraction 5 and, as shown above, the most active in this group is compound X. The synthetic L- α -dimyristoylphosphatidylethanolamine which was tested at the same time on the same plasma specimen exhibited only a weak activity.

DISCUSSION

Although much has been published on the analysis of butter and dairy fats (reviewed by Hilditch, 1949; Davis & Macdonald, 1953), little attention has been directed to the butter phospholipids which are described here. We have found that the whole of the strong thromboplastic activity of butter resides in a number of the phospholipid fractions and these appear to be present as lipoproteins in the original butter. Ten phospholipids have been separated and six of these have thromboplastic activity. The most active, component X, which has been recognized only by silicic acid paper chromatography, remains unidentified; it was approximately 100 times as active as phosphatidylethanolamine and phosphatidylserine prepared from butter and far more active than synthetic phosphatidylethanolamine.

During the progress of this work Poole & Robinson (1956) and O'Brien (1956*a*) reported blood-thromboplastic activity from phospholipid fractions of egg and, when Lea *et al.* (1955) isolated phosphatidylethanolamine from the same source, the activity of egg yolk was attributed to its phosphatidylethanolamine content. In our experience egg yolk was only weakly active compared with butter, and as butter contains only traces of phosphatidylethanolamine its activity is unlikely to be due to this substance. It appears from the evidence presented here that phospholipids other than phosphatidylethanolamine are strong potentiators of blood coagulation. The difficulty of assessing the activity of thromboplastic agents has been overcome by the use of an arbitrary system of blood-clotting units where butter has been chosen as a standard of comparison, not only because it was the most active fat, but also because a large number of samples of butter tested had approximately the same thromboplastic activity.

Silicic acid chromatography has long been recognized as a powerful tool in the separation of phospholipids and although various techniques have been reported with this absorbent, it appears that the choice of the method of elution is dependent on the composition of the lipids requiring

Table 1. *Thromboplastic activity of butter phospholipids*

Peaks 1–5 refer to Fig. 5 and 6–9 to Fig. 4. Compound X [5 (c)] was approx. 100 times as active (per mg. of P) as the mixture of 5 (a) and 5 (b).

Peak no.	Phospholipid	Clotting activity (units/mg.)
1	A complex phosphatidic acid	5
2	Phosphatidylserine	85
3	Phosphatidylserine	60
4	Phosphatidylserine galactoside	30
5	Mixture:	
	(a) Phosphatidylethanolamine	500
	(b) Phosphatidylserine	
	(c) Unknown compound X	
6	Phosphatidylcholine galactoside	0
7	A choline lipid containing glucose and galactose	50
8	Lecithin	0
9	Spingomyelin	0
	Synthetic L- α -dimyristoylphosphatidylethanolamine	7

separation (Lea *et al.* 1955); Hanahan, Dittmer & Warashina, 1957; Marinetti, Scaramuzzino & Stotz, 1957). With the Lea *et al.* method the butter phospholipids were eluted as a single peak, and further experience indicated the need for a gradient elution system.

When a modified exponential system of elution, as used by Alm, Williams & Tiselius (1952) for the separation of fatty acid mixtures, was used with the methanol-chloroform solvent system, the concentration of the polar solvent increased too rapidly in the early stages of chromatography. The linear gradient chromatography eventually used in this work is a modification of the systems of Kellie & Wade (1957) and Edwards (1958). The large differences in the densities of methanol and chloroform necessitated the use of donor and receiver vessels of different cross-sectional areas. With such a gradient elution system, highly reproducible chromatographic separation was obtained and preliminary experiments on the separation of phospholipids from egg yolk and blood plasma suggest that the method may be of general use in the fractionation of phospholipids.

The fatty acid composition of the phospholipids has not been investigated and the nature of the sugar linkage in the glycosides is uncertain, although it has been established that the sugar is not present in a free state and is probably linked through the amino nitrogen in the 'kephalin' group of lipids. Phosphatidylserine appears as two distinct peaks (peaks 2 and 3, Fig. 5) and it is unlikely that separation within this group is obtained through a difference in fatty acid compositions, for on silicic acid paper chromatography in acid media the two components run together. This observation is in agreement with that of Marinetti, Erbland & Stotz (1958), who found two ionic forms of phosphatidylserine in brain phospholipids.

The physiological importance of the butter phospholipids described is uncertain since there is evidence that such compounds are probably modified during intestinal absorption (Ahrens & Borstrom, 1956; O'Brien, 1957; MacLagan, Billimoria & Curtis, 1958). Nevertheless, the possible influence of dietary phospholipids on blood phospholipids is of some interest and is under further investigation.

SUMMARY

1. The addition of butter to plasma shortened the Stypven clotting time of human plasma by over 75% and butter was more active than any other fat tested.

2. A preliminary separation of butter lipids indicated that the thromboplastic activity was confined to the phospholipids originally bound to the proteins of butter.

3. Butter phospholipids were separated by chromatography on silicic acid columns by stepwise and continuous gradient elution techniques, with methanol-chloroform mixtures. A simple method of obtaining an approximately linear gradient is described which may be of general use in the separation of phospholipid mixtures.

4. Ten major phospholipids have been detected in butter and most of these have been identified by paper chromatography and chemical analysis of the intact lipids and their hydrolysis products.

5. A comparison of the Stypven clotting activities of the butter-phospholipid fractions with that of synthetic L- α -dimyristoylphosphatidylethanolamine has shown that a number of these fractions are far more active than the synthetic compound. The most active butter phospholipid (compound X), has not yet been identified.

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On the Mechanism of α -Oxoglutarate Oxidation in *Escherichia coli*

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The enzymic formation of succinyl-coenzyme A from α -oxoglutarate is known to require diphosphopyridine nucleotide (DPN⁺), thiamine pyrophosphate (TPP), lipoic acid (lip-S_{ox} or lip-S_{red}) and coenzyme A (CoA). It has been postulated (Gunsalus, 1953; Kaufman, 1955; Sanadi, Langley & White, 1959) that the sequence of reactions involved in the overall reaction is:

- (1) α -oxoglutarate + TPP $\rightleftharpoons$ [succinic semialdehyde]-TPP + CO₂;
- (2) [succinic semialdehyde]-TPP + lip-S_{ox} $\rightleftharpoons$ succinyl-S-lip + TPP;
- (3) succinyl-S-lip + CoA-SH $\rightleftharpoons$ succinyl-S-CoA + lip-S_{red};
- (4) lip-S_{red} + DPN⁺ $\rightleftharpoons$ lip-S_{ox} + reduced DPN + H⁺.

Sum:

Attempts to isolate the enzymes catalysing these individual steps have been largely unsuccessful, although lipoic dehydrogenase (reaction 4) has been separated and shown to be a component of the α -oxoglutarate-dehydrogenase system of *Escherichia coli* (Hager & Gunsalus, 1953). More recently the mammalian α -oxoglutarate-dehydrogenase system has also been resolved with respect to lipoic dehydrogenase (Massey, 1960) and it has been shown to be a flavoprotein (Massey, 1958; Searls & Sanadi,

1959) identical with Straub's (1939) diaphorase (Massey, 1960).

It is the purpose of this paper to characterize the biochemical lesion in a succinate-requiring mutant of *E. coli*. The experiments reported indicate that the mutant lacks the enzyme catalysing reaction 1, α -oxoglutarate carboxylase, or reactions 1 and 2. It therefore provides a source for the α -oxoglutar-

ate-dehydrogenase complex which is deficient in this enzyme or these enzymes.

METHODS AND MATERIALS

Maintenance of stock culture. *E. coli* W (wild type) and the succinate-requiring mutant (*E. coli* 309-1 R) were gifts from Professor B. D. Davis (Harvard University Medical School, Boston, Mass., U.S.A.). The organisms were maintained in stock culture on agar slopes containing (per 100 ml. of medium) 1 g. of tryptone, 1 g. of Difco yeast extract, 0.5 g. of dipotassium hydrogen phosphate, 0.3 g. of glucose, 0.2 g. of sodium succinate and 2 g. of agar agar (Hopkin and Williams Ltd., Chadwell Heath, Essex).

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