'primitive' characteristics is due to him. Yamasaki (1951) has isolated a bile acid (probably C_{27}) from chicken bile salts, the chief constituent of which is, of course, taurochenodeoxycholate. Dr Anderson has found that our specimen of the 'bile salts' of the cassowary is indeed anomalous in its behaviour; he has suggested that it may consist largely of the fat and other materials normally excreted in variable amounts in bile.

(d) It is interesting that ranol sulphate (frog bile, Fig. 5) and the sulphate of scymnol (or a precursor) (dogfish, Fig. 5) both run at almost the same rate as taurocholate; the alcohol sulphate from the carp (Fig. 6) is not very different.

The simple methods of paper chromatography now tested should be of great value for the preliminary examination of bile salts; they have already indicated several lines along which progress may be made.

SUMMARY

1. Simple methods of paper chromatography developed by Sjövall (1952, 1954) have been applied to a search for bile acids conjugated with glycine in the bile salts of seven species of fish and eight species of birds and to the examination of the crude bile salts of a number of vertebrates.

2. No glycine conjugates were found in the fish and birds examined.

3. Some implications of the results have been discussed.

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The Phosphatides of the Latex of *Hevea brasiliensis*

2. PURIFICATION AND ANALYSIS

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Some properties of latex phosphatide were reported in a previous paper (Smith, 1954), but it was found that an extensive analysis could not be usefully carried out without first removing water-soluble impurities which were carried into solution in lipid solvents by the phosphatides. Workers with a variety of phosphatides from both animals and plants have encountered similar difficulties and have used a number of methods for purification.

Thus Channon & Foster (1934), suspended wheat-germ phosphatide in water, and flocculated it by adding acetone and sodium chloride. This method was used by Tristram (1942) for the purification of latex phosphatide. Folch & Van Slyke (1939) and Christensen (1939) washed solutions of blood phosphatides in light petroleum with water. McKibbin & Taylor (1949) found that the large losses of phosphatide incurred by using this method could

be much reduced by using a solution of magnesium chloride for the washing. Folch (1949) and Sinclair (1948) dialysed emulsions of phosphatide, from brain and blood respectively, against distilled water. Water-soluble nitrogenous compounds (urea and choline were identified in certain cases) were usually the main contaminants detected, together with a small amount of water-soluble phosphorus. Folch (1949), however, found gross contamination of brain phosphatide with inorganic phosphates. Some of these methods have been examined with a view to using them for the purification of latex lipid but none was entirely satisfactory. The best results were obtained by using a partition-chromatographic technique which will be described.

Coagulation of latex in ethanol at a temperature below 40° has been previously shown (Smith, 1954) to lead to the enzymic liberation of choline from the phosphatide with an accompanying increase in its acetone solubility. Such a technique followed by acetone precipitation of the extracted lipid was used by most previous workers (Rhodes & Bishop, 1930; Tristram, 1942; Altman, 1946; Resing, 1951) for the preparation of phosphatide from latex. Thus the product examined by these workers was almost certainly an artifact from which, in addition, a fraction may have been lost by solution in acetone. These considerations do not necessarily apply to the method of preparation used by Altman & Kraay (1940) but their product was obtained, in very poor yield, from the silt left after centrifugal clarification of ammoniated latex, and was probably therefore not a representative sample of unchanged phosphatide. Tristram (1942), alone among the above workers, attempted to remove water-soluble impurities from the phosphatide, and this fact must also be borne in mind in assessing the value of analytical results obtained. In particular, the absence of such purification makes figures for total nitrogen valueless.

In the present work, samples of latex lipid, undegraded and degraded by the choline-liberating enzyme, have been prepared and analysed in a variety of ways before and after the removal of water-soluble impurities.

EXPERIMENTAL

Preparation of lipid samples. Seedling latex from the Experiment Station of the Rubber Research Institute of Malaya was extracted essentially according to the scheme outlined previously (Smith, 1954) but on a larger scale. In general, 1 l. batches of latex were coagulated in 3 l. of industrial redistilled methylated spirit (to be referred to as 'alcohol'). For the preparation of lipid in which the phosphatide was undegraded by the choline-liberating enzyme (to be referred to as undegraded lipid), the latex was added to boiling alcohol and the hot serum immediately filtered. When lipid containing phosphatide from which choline had

been liberated enzymically (to be referred to as 'degraded lipid') was required, the latex was added to alcohol at 22° and the mixture stored at this temperature for 24 hr. before boiling for 15 min. and filtering off the hot serum.

Removal of water-soluble impurities from the lipid. Methods of purification involving the addition of metallic ions were rejected as being likely to lead to the formation of artificial metal phosphatides. Water washing of a light petroleum (b.p. 56–70°) solution was unsatisfactory for the purification of undegraded lipid owing to the formation of persistent emulsions which resulted in losses of lipid of the order of 15% despite repeated re-extraction of the water washings with light petroleum. Only a small loss (about 3%) occurred with degraded lipid.

Purification by dialysis was carried out as follows: an aqueous suspension containing approximately 1% (w/v) of the lipid was placed in a cellophane sac and dialysed against distilled water. Nitrogen was slowly bubbled through the suspension and the dialysis was continued for 7 days at 10°, the water being changed twice daily. Analysis of the dialysable materials showed that there was no appreciable removal of nitrogen-containing compounds after this time. The purified emulsion was freeze-dried. This method was tedious to carry out and, as will be seen later, was inefficient. The following chromatographic technique was more satisfactory on both counts and was mostly used in these investigations.

Bevan, Gregory, Malkin & Poole (1951) reported that choline-containing phosphatides could be separated from ethanolamine- and serine-containing phosphatides by partition chromatography on cellulose using as a solvent the water-poor phase from a mixture of chloroform, ethanol and water (80:20:2.5, v/v), referred to as c.e.w. We found that paper chromatography of latex lipid with this solvent gave results similar to those of Bevan *et al.* (1951) in that a ninhydrin-reacting spot near the origin (R_F value about 0.01) and a choline-containing lipid spot at or near the solvent front were obtained. Further examination showed, however, that the former spot was not due to a phosphatide, but to a mixture of water-soluble substances, mainly known amino acids. It appeared therefore that this technique might form the basis of a method of removing water-soluble impurities. Fuller examination, the results of which will be given in a later section, confirmed this view.

Two methods of treatment were used. (a) The lipid (about 30% (w/v) solution in CHCl_3) was spotted 8 cm. from the top of a 30 cm.-long strip of filter paper (Whatman no. 1). This was developed with c.e.w. at 22°, using the ascending technique in an airtight gas jar, until the solvent front had travelled about 15 cm. beyond the starting line. The main lipid band, near the solvent front, was visible to the naked eye in transmitted light after drying at room temperature. A transverse strip containing this material was cut out and extracted with CHCl_3 . The remaining part of the chromatogram was extracted first with water and then with CHCl_3 . The CHCl_3 extractions were carried out in a Soxhlet extractor after cutting the paper into small pieces. The water extraction was made by cutting one end of the strip to a point, then immersing the other end in a trough of water contained in a simple descending type paper chromatographic cabinet (Dent, 1948) and collecting the drippings. (b) Whatman cellulose powder (standard grade, ashless) was slurried with enough c.e.w. solvent to cover it. This, and all subsequent operations, were carried out in a constant-

temperature room at 22°. A wad of glass wool was placed in the bottom of a suitably sized tube fitted with a tap and the slurry added. After the cellulose powder had settled under gravity, the solvent was run out until its level was just above that of the cellulose powder. The liquid level was never allowed to fall below this point. The lipid, dissolved in c.e.w. solvent, was added and the tap opened to allow a dropwise flow (about 1–10 ml./min. depending on the column size). The chromatogram was developed with c.e.w. solvent until all the phosphatide had emerged from the column. Successive washings with CHCl_3 , ethanol and water in that order were then carried out, each fraction being collected separately in an attempt to recover materials held on the column. It was found that, provided the ratio of lipid weight:column volume was less than 1:25, a further passage of the purified lipid through a cellulose-powder column caused no additional change in its composition. Although the c.e.w. solvent of Bevan *et al.* (1951) was used in all the experiments reported, it was subsequently found that CHCl_3 saturated with water could be used equally well.

Total phosphorus, ash phosphorus, choline and total nitrogen determinations. The first three were carried out as described previously (Smith, 1954). Total nitrogen was determined by the micro-Kjeldahl method using a copper–selenium catalyst for the digestion.

Reducing-sugar determination. This was carried out colorimetrically according to Somogyi (1945), the results being calculated as galactose. A lipid sample (about 0.1 g.) was hydrolysed by refluxing for 24 hr. with 20 ml. of 2N- H_2SO_4 . The mixture was cooled and filtered and the filtrate was neutralized with 4N-NaOH. The resultant concentration of Na_2SO_4 was shown to have no influence on the determination. Some sugar destruction probably occurred but higher results were obtained with this procedure than with the milder H_2SO_4 hydrolysis of Scholfield, Dutton & Dimler (1952).

Determination of unsaponifiable matter and total fatty acid. The lipid (about 0.03–0.05 g.) was hydrolysed, and the unsaponifiable matter and fatty acids were extracted in the manner described by Bloor (1943). This extract was evaporated and then dried under vacuum to constant weight. The residue was taken up in neutral ethanol, heated to boiling, and titrated with 0.02N-NaOH using phenolphthalein as indicator. To calculate the respective amounts of fatty acids and unsaponifiable matter in the mixture, we assumed a mean molecular weight of 325 for the fatty acids. This value, which was based on the results of Tristram (1942), may not be correct. It is higher than might be expected, and

although it is assumed that Tristram (1942) separated unsaponifiable matter from his fatty acids before determining their molecular weight, he makes no mention of doing so.

mesoInositol. This was determined microbiologically according to Smith (1951). A lipid sample was hydrolysed by refluxing with 5N-HCl for 48 hr. The cooled mixture was filtered and the filtrate evaporated to dryness. The residue, after removal of most of the HCl under vacuum, was taken up in water and neutralized with NaOH. Inositol was shown to be stable under these conditions, but complete liberation from a combined form might not have occurred. However, higher values were obtained by this method than by 48 hr. hydrolysis with either 2N- H_2SO_4 or saturated methanolic $\text{Ba}(\text{OH})_2$.

Chromatographic examination of ninhydrin-reacting substances. Lipid hydrolysates were prepared in the same way as those for inositol determination except that, in order to remove traces of HCl, the residues were repeatedly taken up with water, re-evaporated and kept under high vacuum in the presence of KOH. The final residues taken up with water contained about 75% of the non-choline nitrogen in the lipid. Hydrolysis for 48 hr. with 2N- H_2SO_4 and saturated methanolic $\text{Ba}(\text{OH})_2$ gave recoveries of non-choline nitrogen of only 44 and 23%, respectively. The hydrolysates, with and without the addition of known amino acids as reference substances, were submitted to two-dimensional chromatography on Whatman no. 1 filter paper (44.5 × 57 cm.) at a constant temperature (30°), essentially according to Dent (1948).

RESULTS

Purification of latex lipid

Paper chromatographic separation of unpurified undegraded lipid gave the fractions shown in Table 1. Lipid, representing about 94% of the total was recovered from the strip near the solvent front. It contained a smaller percentage of nitrogen than the original lipid but nearly all of the nitrogen lost was recovered in a water-soluble form from the upper part of the paper, and only a trace was soluble in chloroform.

Partition chromatography on a cellulose column permitted separation on a sufficiently large scale for detailed analysis of the fractions (Table 2). Samples of undegraded and degraded unpurified lipid, respectively, were treated, and in both cases it was

Table 1. *Properties of undegraded, impure latex lipid and of fractions obtained therefrom by paper chromatographic separation using c.e.w. solvent*

Four papers (57 × 30 cm.) were used, each bearing 40 × 0.01 ml. spots of an approximately 30% (w/v) light petroleum solution of the lipid. The spots were added at 1 cm. intervals along a line 8 cm. from a longer edge of the paper. Strip B contained only the main lipid band near the solvent front which was sharply visible in transmitted light. Strip A consisted of the rest of the chromatogram.

		Lipid (g.)	Total P (mg.)	Total N (mg.)	Choline N (mg.)	Lipid N/P atomic ratio	Lipid choline N/P atomic ratio
	Unpurified lipid	0.485	9.23	5.34	2.71	1.28	0.65
Strip A	Water-soluble	—	0.51	2.01	0	—	—
	CHCl_3 -soluble	—	0.40	<0.05	0	<0.3	0
Strip B	CHCl_3 -soluble	0.454	8.13	3.40	2.53	0.93	0.69

found that lipid, representing about 95 % of the total by weight, passed through the column with the solvent (in all, 17.48 and 14.33 g., respectively, of undegraded and degraded lipid were treated and 16.46 and 13.77 g., respectively, passed through the column). After each chromatogram had been developed as described in Table 2, it was washed with 400 ml. chloroform, 750 ml. absolute ethanol and 2000 ml. water in that order, the effluents being collected separately. The chloroform effluent contained only about 2 % of the total material retained on the column. The ethanol effluent was evaporated down, after the addition of water, to give an aqueous residue which was added to the water effluent. The recovered materials, which were entirely water-soluble, accounted approximately for the loss in weight on passing the lipid through the column (in all, 1.14 and 0.61 g. water-soluble materials were recovered after treating the undegraded and degraded lipid, respectively). They contained 1.5 and 1.1 % phosphorus, 3.1 and 3.4 % nitrogen (but no choline), 1.6 and 1.6 % inositol, 3.3 and 4.9 % reducing sugar and 49 and 64 % ash (as phosphate) for the undegraded and degraded lipids, respectively.

The metallic impurities, responsible for the ash content of the water solubles, were not soaps, since no free fatty acids could be detected after acidification. Lipid samples from a variety of latices contained impurities in amounts similar to those shown here with the exception that water-soluble nitrogen was found to vary from about 0.15 to 0.5 % depending on the lipid. The nitrogenous impurity never included choline.

The ashes from the purified undegraded and degraded lipids, which were prepared by heating in a muffle at 500° for about 24 hr., contained respectively 22.1 and 23.8 % potassium as K_2O ; 2.7 and 6.0 % magnesium as MgO and 44.2 and 49.0 % phosphorus as P_2O_5 . The remaining constituents were not identified. Sodium and calcium were present in not more than trace amounts but there was an unknown substance which gave a small dark-coloured precipitate with cobaltinitrite during the potassium determination.

When the purified lipids were hydrolysed by refluxing with 0.1N alcoholic NaOH for 1 hr. about 97 % of the phosphorus was recovered in a water-soluble organic form. None was liberated in an inorganic form on heating a 5 ml. sample of this solution, containing about 0.03 mg. phosphorus, with 1 ml. 10N- H_2SO_4 in a boiling water bath for 1 hr. Similar treatment with the addition of 1 ml. 0.05M periodic acid (Burmaster, 1946) gave recovery of 89–91 % of the phosphorus in an inorganic form for both the undegraded and degraded lipins. Glycerophosphate is broken down quantitatively under these conditions so that part of the lipid

Table 2. *Effect of purification by dialysis and cellulose column partition chromatography respectively on the properties of undegraded and degraded latex lipid*

For the first method approximately 100 ml. batches of 1 % (w/v) aqueous suspensions of the lipids were dialysed against distilled water. For the second method 7–8 g. batches of the lipids, dissolved in 50 ml. c.e.w. were added to cellulose-powder columns (4 cm. diam. $\times$ 25 cm. length) and washed through by a further 750 ml. c.e.w. The last 200 ml. of effluent contained negligible amounts of phosphorus (about 0.25 % of the total). For further details of both procedures see text.

	Total P (%)	Total N (%)	Ash P (%)	Choline N (%)	Non-choline N (%)	Choline N/P (atomic ratio)	Ash-P/total-P (ratio)	N/P + ash-P/total-P	Total ash (%)	Inositol (%)	Reducing sugar (%)	Total fatty acid (%)	Unsaponifiables (%)
Undegraded lipid													
Unpurified	1.90	0.91	0.78	0.57	0.34	1.06	0.67	1.47	4.14	1.34	8.94	53	16
Purified (dialysis)	1.94	0.81	0.52	0.59	0.22	0.93	0.68	1.20	2.93	—	—	—	—
Purified (chromatography)	1.82	0.72	0.33	0.59	0.13	0.88	0.72	1.06	1.71	1.30	8.60	55	14
Degraded lipid													
Unpurified	1.91	0.42	1.67	0.07	0.35	0.49	0.08	1.37	7.91	0.80	8.14	54	18
Purified (dialysis)	1.95	0.28	1.20	0.06	0.22	0.32	0.07	0.94	6.05	—	—	—	—
Purified (chromatography)	1.79	0.21	1.32	0.06	0.15	0.26	0.08	1.00	6.15	0.72	7.84	56	15

phosphorus does not appear to be present as glycerophosphate. It is not clear in what form it occurs. It would be expected that inositol monophosphate, if present, would react in the same way as glycerophosphate.

Analytical results for lipid samples after dialysis in aqueous suspension against distilled water are also given in Table 2. In all, 15.98 and 15.28 g., respectively, of the undegraded and degraded lipid were treated and 15.39 and 14.13 g., respectively, were recovered. Part, at least, of the lipid loss was mechanical. The procedure did not remove any appreciable amount of water-soluble phosphorus and the amounts of nitrogen removed were less than by chromatographic purification, indicating a less efficient purification by dialysis. The ash content of the undegraded lipid also indicated that the chromatographic purification was the more efficient but the ash content of the degraded lipid was reduced to a greater extent by dialysis. It may be that certain of the phosphatidic acid salts, present in the latter lipid as degradation products of the choline containing phosphatide, are somewhat soluble in water (Witcoff, 1951). If this were so the setting up of successive Donnan equilibria for each change of water could result in the loss of metallic ions therefrom and the consequent artificial formation of free phosphatidic acid.

It has been shown (Smith, 1954) that the coagulation in alcohol of amounts of latex greater than about 40 ml. may lead to inefficient lipid extraction owing to the lipid being trapped within the bulky rubber coagulum. This is particularly apparent in the preparation of degraded lipid when the latex is coagulated in the cold and the mixture only subsequently heated to boiling and filtered. In the large-scale extractions used for the preparation of lipids, for which analysis figures are given in Table 2, the calculated yields on the latex (allowing for serum not recovered from the coagulum) were 0.73 and 0.60% for the undegraded and degraded unpurified lipids, respectively. The difference may well be due to less complete removal of phosphatide, insoluble in cold ethanol, in the latter case. We believe that this explains the differences in inositol and reducing-sugar contents between the undegraded and degraded lipids. This belief was supported by a further experiment in which undegraded and degraded lipid samples were prepared from 40 ml. lots of latex in the manner described previously (Smith, 1954). In this way, calculated lipid yields of 0.71 and 0.73% were obtained for the undegraded and degraded unpurified lipids, respectively. These lipids contained 1.89 and 1.91% phosphorus, 0.55 and 0.10% choline nitrogen, 1.31 and 1.34% inositol and 9.8 and 10.1% reducing sugar for the former and latter, respectively.

Fractionation of the lipids according to acetone and ethanol solubility

Latex phosphatide, particularly when degraded by the choline-liberating enzyme, has been shown to be soluble in acetone (Smith, 1954). Consequently although it was impossible to obtain the total phosphatides free of non-phosphatide lipid by this means, we considered that useful data concerning the components of the phosphatide mixtures might be obtained by fractionating with acetone. Further information was sought by fractionating the acetone insolubles with ethanol. Complete separation of phosphatide mixtures by making use of their solubility differences is very difficult even using a counter-current extraction technique (e.g. Lovern, 1952). Consequently it was thought that more useful information would be obtained in the first instance by carrying out a simple separation so that quantitative recovery of the lipid could be easily effected, rather than by attempting to isolate pure components. Table 3 shows the properties of fractions from the undegraded and degraded latex lipid purified by partition chromatography and described in Table 2. The fractions were prepared by adding 6 vol. acetone to 30 ml. of a 15% (w/v) solution of the lipid in light petroleum (b.p. 56–70°), keeping at 10° overnight and pouring off the supernatant liquid after centrifuging. Absolute ethanol (20 vol.) was rapidly added to the washed residue (the dry residue discoloured and became partially insoluble in light petroleum after only a few minutes exposure to the air). The mixture was heated to boiling under nitrogen with constant shaking, cooled to 10° and kept overnight at this temperature. The supernatant liquid was decanted off after centrifuging, and the washed residue was dissolved in light petroleum. As was to be expected, there was no clear-cut separation of the different components of the mixture.

A small amount of undegraded phosphatide and a larger amount of degraded phosphatide were present, together with the bulk of the non-phosphatide lipids, in acetone solution. In another experiment, 0.3 g. samples of the total lipids dissolved in 1 ml. light petroleum were precipitated with 6 ml. acetone and 3 drops of a saturated ethanolic solution of magnesium chloride (Bloor, 1929). The solutions were evaporated to 1 ml. and the precipitation repeated. Under these conditions 18.3% of the undegraded lipid was soluble in acetone and this fraction contained only 0.012% phosphorus (<0.2% of the total lipid phosphorus) but 0.125% nitrogen. Almost all of the latter must therefore be non-phosphatide. The degraded lipid gave 37.7% acetone-solubles containing 0.23% phosphorus and 0.089% nitrogen. Although all the phosphatide was not precipitated in this instance it

Table 3. Properties of fractions obtained from chromatographically purified lipids according to their acetone and ethanol solubility

The properties of the total lipids are given in Table 2. Fractions were obtained by single precipitations and quantitative recovery of the lipids. For details see text.

	Percent of total lipid	Total P (%)	Total N (%)	Ash P (%)	Choline N (%)	Non-choline N (%)	N/P (atomic ratio)	Choline N/P (atomic ratio)	Ash-P/total-P (ratio)	N/P + ash-P/total-P	Inositol (%)	Reducing sugar (%)	Total fatty acid (%)	Unsaponifiables (%)
Undegraded lipid														
Acetone-soluble	25.2	0.40	0.27	0.03	0.17	0.10	1.50	0.92	0.07	1.57	0.07	4.4	34	51
Acetone- (Ethanol-soluble	62.0	2.20	0.91	0.15	0.88	0.03	0.92	0.89	0.07	0.99	0.54	12.2	64	1
insoluble { Ethanol-insoluble	12.8	2.48	0.43	1.62	0.22	0.21	0.38	0.20	0.65	1.03	8.17	11.2	56	10
Degraded lipid														
Acetone-soluble	45.0	0.81	0.17	0.29	0.04	0.13	0.48	0.11	0.36	0.84	0.04	4.2	51	32
Acetone- (Ethanol-soluble	39.5	2.45	0.20	1.87	0.12	0.08	0.18	0.11	0.76	0.94	0.13	12.9	65	0
insoluble { Ethanol-insoluble	15.5	3.10	0.19	2.65	0	0.19	0.14	0	0.86	1.00	4.06	12.0	67	8

can be justifiably assumed that virtually all nitrogenous phosphatide was removed so that the nitrogen in this fraction must also be non-phosphatide. Titration of an ethanolic solution of the acetone-soluble material from the undegraded phosphatide, after water washing to remove traces of magnesium chloride, showed no free fatty acids to be present. This determination was not possible with the degraded lipid owing to the presence of phosphatides in the acetone-solubles.

Chromatographic examination of ninhydrin-reacting substances

Fig. 1a shows that the impure undegraded lipid, for which analysis figures are given in Table 2, contains a number of ninhydrin-reacting substances including some known amino acids and ethanolamine. Among the former, only serine has been previously reported as a phosphatide constituent (e.g. Hutt, Malkin, Poole & Watt, 1950). Closely similar chromatograms were obtained with the hydrolysates of a number of unpurified latex lipid samples examined. Although dialysis failed to remove most of the ninhydrin-reacting materials from the lipids (Fig. 1b) purification on the cellulose column removed all but traces of all of them except ethanolamine (Fig. 1c). These substances, again with the sole exception of ethanolamine, were detected in a free form in the water-soluble impurities recovered from the column (Fig. 1d). Results obtained using the degraded lipid were apparently identical with those for the undegraded material and the chromatograms obtained are consequently not illustrated. It is possible that there were undetected quantitative differences. Examination of the fractions obtained by the paper chromatographic purification of undegraded lipid (Table 1) gave similar results.

Hydrolysates of the acetone/ethanol lipid fractions, properties of which are given in Table 3, all gave a strongly marked ethanolamine spot together with trace spots of all or some of the amino acids. Qualitative results were identical for the undegraded and degraded lipids.

DISCUSSION

The statement of Bevan *et al.* (1951) that a separation of choline-containing phosphatides from other nitrogen-containing phosphatides could be achieved by partition chromatography on cellulose using a chloroform/ethanol/water solvent has not been confirmed. Thus although a considerable amount of nitrogenous material, including certain known amino acids and other ninhydrin-reacting substances, was retained by the stationary phase on treating latex lipid in this way (using either paper or a cellulose-powder column) this nitrogenous

Fig. 1. Ninhydrin-reacting substances on two-dimensional chromatograms prepared from (a) hydrolysate of 20 mg. unpurified, undegraded lipid; (b) hydrolysate of 20 mg. dialysis-purified, undegraded lipid; (c) hydrolysate of 20 mg. chromatographically purified, undegraded lipid; (d) water-soluble impurities removed on the chromatograph column during the purification of 20 mg. undegraded lipid. The spots with dotted outlines were very faint. (1) unknown substance—blue coloration, (2) unknown substance—purple coloration, (3) glycine, (4) serine, (5) alanine, (6) ethanolamine, (7) valine, (8) leucine (or isoleucine).

material was almost completely recoverable in a water-soluble form together with a number of other water-soluble substances. The lipid not retained on the paper or column contained, as well as choline, ethanolamine in roughly the same amount as in the untreated material. The technique appears to provide an efficient method of removing water-soluble impurities of a varied nature from the lipid. Unless egg phosphatides behave differently from latex phosphatides, it seems that Bevan *et al.* (1951) may have mistaken ninhydrin-reacting impurities for ninhydrin-reacting phosphatides. Since completing this work, a paper by Lea & Rhodes (1953) has come to our notice. These workers repeated the experiments of Bevan *et al.* (1951) and found evidence that the slowly moving ninhydrin-reacting substances in egg-yolk phosphatides were due to water-soluble impurities including known amino acids. They also found an unknown substance giving a blue coloration with ninhydrin (cf. Fig. 1a and d).

An examination of lipid samples, purified by this method, has confirmed the observation of Tristram (1942) that much of the reducing sugar associated

with the phosphatide is not easily removed in a water-soluble form. Inositol, detected by Resing (1951) in latex phosphatide from which water-soluble impurities were not removed, has been found in the purified material; but serine, also reported to be present by Resing (1951), appears to be a water-soluble impurity.

The undegraded and degraded purified lipids show marked differences apart from the loss of choline. Variations in inositol and reducing sugar appear, however, to be due to incomplete extraction from the latex rather than to intrinsic differences in the lipid. The only other obvious change on degradation is an increase in ash content. This was indicated previously (Smith, 1954), but the presence of impurities rendered the results of doubtful value. Further information can be derived from a study of the nitrogen/phosphorus and ash phosphorus/total phosphorus ratios of the purified lipids and their acetone/alcohol fractions. The phosphatides likely to be present can be put into one of three classes, irrespective of the main structure of the molecule, according to the substituent groups combined with the phosphate radical. These are (i) nitrogenous

phosphatides containing equimolecular amounts of nitrogen and phosphorus, (ii) metal phosphatidates and (iii) free phosphatidic acids. Ashing of a phosphatide mixture, from which complete removal of contaminating metal salts had been achieved, would result in the complete loss, by volatilization, of the phosphorus in any wholly organic phosphatide. The phosphorus in any metal phosphatidate present, on the other hand, would be quantitatively retained in the ash. Thus the ash phosphorus/total phosphorus ratio of the mixture would give the proportion of phosphatide phosphorus present as metal phosphatidate. Similarly, in a phosphatide mixture free of nitrogenous impurities, the nitrogen/phosphorus atomic ratio would give the proportion of phosphatide phosphorus present as nitrogenous phosphatide. The sum of these two ratios, which will be subsequently referred to as the $(N/P + \text{ash-P}/P)$ sum, should be unity if free phosphatidic acids are absent and less than unity if they are present. The extent to which this sum is less than unity would give the proportion of phosphatide phosphorus present as free phosphatidic acids. The total undegraded and degraded purified lipids are not completely free of non-phosphatide nitrogen. They contain traces of amino acids, although the amounts must be very small and are probably negligible; more important they contain acetone-soluble non-phosphatide nitrogen. The undegraded and degraded total lipids possess $(N/P + \text{ash-P}/P)$ sums of 1.03 and 0.96, respectively, after correction for this material. This indicates the possible presence of free phosphatidic acid in the degraded lipid although the effect is small enough to be accountable as experimental error. The finding is confirmed, however, by the fact that the acetone-soluble fraction from the degraded lipid possesses a $(N/P + \text{ash-P}/P)$ sum of considerably less than unity even before correction for known non-phosphatide nitrogen. When this correction is made, a sum of only 0.61 is obtained, indicating the presence of 39% phosphatide phosphorus as free phosphatidic acid. It is presumably this substance which is largely responsible for the high acetone solubility of the degraded phosphatide. The corresponding fraction from the undegraded lipid has a $(N/P + \text{ash-P}/P)$ sum of 1.09 after correcting for known non-phosphatide nitrogen. All the acetone-insoluble fractions obtained, with the exception of the ethanol-soluble part from the degraded lipid which appears to contain a small amount of free phosphatidic acid, possess $(N/P + \text{ash-P}/P)$ sums close to unity. It seems probable therefore that the phosphatides in these fractions are made up almost entirely of nitrogenous phosphatides and metal phosphatidates and that no appreciable nitrogenous or metallic impurities are present. Traces of amino acid impurities were detected in all

the fractions but the amounts are apparently negligible.

It appears probable from the manner in which the various phosphatide constituents distributed themselves in the acetone/ethanol fractions that the phosphatides in the undegraded lipid consist of (i) an essentially ethanol-soluble phosphatide containing choline (presumably lecithin) and associated with reducing sugar, (ii) an essentially ethanol-insoluble metal phosphatidate containing inositol also associated with reducing sugar, and (iii) a non-choline nitrogenous phosphatide which from the chromatographic results appears to be a phosphatidyl ethanolamine. It is possible that the ethanolamine occurs in combination with the inositol phosphatide as, according to Woolley (1943), is the case in 'lipositol' from soya beans. The non-choline nitrogen figures are quite unreliable as a means of estimating the amount of ethanolamine—they are measured as small differences between two large values and residual amounts of nitrogenous impurity are almost certainly present, which although small in relation to the total lipid may be large in comparison with the small amount of ethanolamine. However, since chromatographic examination showed strong ethanolamine spots in fractions with very little inositol, the suggestion of a separate phosphatidyl ethanolamine is preferred. The phosphatides in the degraded lipid appear also to contain these three components although with the first in much reduced amount. It has previously been shown that some free phosphatidic acid is present in this lipid. In addition, the amount of ash phosphorus is much more than can reasonably be accounted for as an inositol-containing metal phosphatidate, particularly in the acetone-insoluble, ethanol-soluble fraction. It seems that a further metal phosphatidate, not containing inositol, must be present. These last two products are presumably formed from the lecithin as a result of the action of the choline-liberating enzyme. As well as the phosphatides, the lipids contain unsaponifiable material and, since in the acetone-soluble fractions there was far more fatty acid than could reasonably be attributed to the phosphatide they must also contain other lipids, e.g. triglycerides or free fatty acids. There were no free fatty acids in the undegraded lipid and probably not in the degraded lipid although they were not determined in the latter. It is possible that part of the extra fatty acid was present, not as triglyceride, but in combination with unsaponifiable matter; the nature of the unsaponifiable matter has not, however, been ascertained.

Our results show that latex lipid is a mixture containing a number of more or less ill-defined components. With the present data it is not possible to derive quantitative information concerning the composition of this mixture without making

numerous assumptions of varying degrees of reliability. It must be stressed, therefore, that the following deductions are tentative, although we consider it likely that, with the probable exception of the figures for phosphatidyl ethanolamine, they are substantially correct. The assumptions made are as follows: (i) All the non-choline nitrogen present (excepting the acetone-soluble non-phosphatide nitrogen) is due to ethanolamine which is present in a phosphatidyl ethanolamine of normal structure. As previously stated the ethanolamine may not occur in this form and even if it does it is unlikely that an accurate estimate is obtained on this basis. The amount is so small, however, that an error would not greatly affect the calculation of the remaining constituents. (ii) All the phosphatides present possess fatty acid/phosphorus molecular ratios of 2/1. This is theoretically true for lecithin and phosphatidyl ethanolamine and was also found by Woolley (1943) to be approximately true for the inositol-containing phosphatide from soya beans. The experimental results show that this ratio, even for the acetone insoluble fractions, is rather more than 2/1. The excess of fatty acid is regarded as being present as non-phosphatide lipid (e.g. triglyceride), precipitated from acetone solution with the phosphatides. (iii) The fatty acids have an average molecular weight of 325 (Tristram, 1942). (iv) The nitrogenous phosphatides possess a nitrogen/phosphorus ratio of 1/1. (v) The inositol-containing metal phosphatide has an ash phosphorus/total phosphorus ratio of 1/1. The unsaponifiable contents of the fractions are known and the non-phosphatide fatty acid and phosphatidyl ethanolamine contents can be calculated by making the above assumptions. Calculation by difference shows that the remaining phosphatide mixtures in the acetone-insoluble fractions, both ethanol-soluble and ethanol-insoluble, contain about 2.8% phosphorus. This, despite the fact that in one fraction the majority is lecithin and in the other the majority is metal phosphatide containing inositol. It appears therefore that both the lecithin and the inositol phosphatide contain nearly the same phosphorus percentage (about 2.8%) and their relative amounts in the fractions can consequently be calculated from the choline nitrogen/phosphorus and the ash phosphorus/total phosphorus ratios. By accepting these values the approximate inositol and reducing-sugar contents of the individual phosphatides can be deduced.

From the derived properties of the lecithin it is possible to calculate approximate properties for the free phosphatidic acid and its metallic salts (as potassium phosphatide) which are apparently formed in the degraded lipid as a result of simple loss of choline from the lecithin. Accepting these properties and making the same assumptions as

Table 4. *Estimated composition of the lipids and their acetone/ethanol fractions*

The estimation is based on the results in Tables 2 and 3 making the assumptions described in the text (on this page). Values are expressed to the nearest 0.5%. The difference between the amounts of inositol phosphatide in the undegraded and degraded lipids is believed to be due to inefficient extraction of the latter from the latex rather than to any intrinsic difference in the lipids.

		Percentage (w/w) of total lipid							
		Total fraction	Lecithin	Phosphatidyl ethanolamine	Inositol phosphatide	Free phosphatidic acid*	Metal phosphatide*	Tri-glycerides†	Unsaponifiables
Undegraded lipid	Acetone-soluble	25	3.5	0.5	0	0	0	7.5	13.5
	Acetone-insoluble	62	45.5	1.0	3.0	0	0	12.0	0.5
		13	2.0	1.5	7.5	0	0	0.5	1.5
	Total	—	51.0	3.0	10.5	0	0	20.0	15.5
Degraded lipid	Acetone-soluble	45	1.5	1.5	0	5.0	5.0	17.0	15.0
	Acetone-insoluble	39.5	4.0	2.0	0.5	2.0	25.0	6.0	0
		15.5	0	1.5	4.5	0	8.0	0.5	1.0
	Total	—	5.5	5.0	5.0	7.0	38.0	23.5	16.0

* Breakdown products from lecithin.

† Representing fatty acid in excess of that attributable to phosphatide.

The estimation is based on the results in Tables 2 and 3 making the assumptions described in the text (on this page). Values are expressed to the nearest 0.5%. The difference between the amounts of inositol phosphatide in the undegraded and degraded lipids is believed to be due to inefficient extraction of the latter from the latex rather than to any intrinsic difference in the lipids.

before, a quantitative estimation of the components in the degraded lipid can be made.

Calculated values for the compositions of the undegraded and degraded lipids and their acetone/ethanol fractions are given in Table 4.

If these values are correct it can be calculated that the lecithin component contains reducing sugar (calculated as hexose) and phosphorus in a roughly equimolecular ratio. The inositol-containing metal phosphatide has been assumed to possess a fatty acid/phosphorus molecular ratio of 2/1 (an assumption which is supported by the fact that the experimentally determined fatty acid/phosphorus ratio of the ethanol-insoluble fraction of the undegraded lipid which appears to consist largely of this phosphatide is 2.16/1). Calculations indicate that it contains both inositol and reducing sugar in roughly equimolecular proportions to phosphorus. These findings are in close agreement with those for the soya bean 'lipositol' of Woolley (1943).

Comparison of our results, with the results of previous workers, gives further evidence that the phosphatides which they examined were partially degraded by the choline-liberating enzyme. Thus Tristram (1942) found about 35 % lecithin and 35 % phosphatidic acid (or its salts) in his acetone-insoluble lipid. Corresponding estimated figures for our undegraded and degraded products, respectively, were 63 and 7 % lecithin and 14 and 73 % phosphatidic acid (or its salts—including the inositol containing phosphatide). Altman (1946), fractionating total lipid, found 45 % acetone-soluble, 33 % acetone-insoluble, ethanol-soluble and 22 % acetone-insoluble, ethanol-insoluble. These figures are close to our findings for degraded lipid.

SUMMARY

1. The statement of Bevan *et al.* (1951) that choline-containing phosphatides are separated from non-choline nitrogenous phosphatides by partition chromatography on cellulose could not be confirmed. Instead it was found that the removal of water-soluble impurities from latex lipid, which is usually difficult to achieve was efficiently accomplished by this technique.

2. Inositol, reducing sugar and substances containing nitrogen, phosphorus and metals, were shown to be present partly as water-soluble impurities in unpurified latex lipid preparations. The nitrogenous impurities contained certain known amino acids but did not contain ethanolamine or choline.

3. Evidence is presented which tentatively suggests that purified undegraded latex lipid consists of (i) lecithin containing reducing sugar (51 %); (ii) metal phosphatide containing inositol and reducing sugar (10.5 %); (iii) phosphatidyl ethanolamine (3 %); (iv) triglyceride (representing fatty acid in excess of that attributable to phosphatide) (20 %); and (v) unsaponifiables (15.5 %).

4. The enzymic liberation of choline from the lecithin fraction leads to the formation of free phosphatidic acid and its metallic salts. No other marked changes are apparent.

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