46. The Phosphatides of *Hevea Brasiliensis*

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The presence of phosphatide in *Hevea* latex had long been suspected but it was not until 1931 that Rhodes & Bishop isolated phosphatidic material containing 0.59 % N and 1.72 % P from the serum of latex which had been coagulated with ethanol. The crude material of these authors represented 0.2 % of the latex, and on treatment with acetone yielded an insoluble fraction containing 0.88 % N and 2.56 % P (40 % of the former product). This latter fraction was, according to the accepted analytical composition of phosphatide, still very impure. The work of Rhodes & Bishop was repeated by Altman & Kraay [1940] who isolated in very small amounts a product giving much the same analyses, and extended the findings of Rhodes & Bishop by showing that the crude phosphatide contained both choline and glycerophosphoric acid.

It is very probable that the phosphatide, although present in such small amount, is one of the important non-rubber constituents of latex. Judging from the ease with which phosphatides emulsify in aqueous media, there seems little doubt that the phosphatidic material assists in the maintenance of latex as a stable emulsion. It has been suggested [Bollman, 1926] that crude phosphatide preparations act as the natural antioxidants in vegetable oils. This suggestion has been disputed by Diemair & Fox [1939] and other workers who state that this property is not possessed by pure phosphatide although the complex system from which the phosphatide had been separated was an active antioxidant. Diemair *et al.* [1940] have advanced the opinion that the active antioxidant is a protein-phosphatide complex.

The present work was undertaken as part of an investigation of the non-rubber constituents of latex. The crude phosphatide was prepared by the method of Rhodes & Bishop [1931], the preliminary treatment being carried out at the Rubber Research Laboratories, Ceylon, under the direction of Mr O'Brien to whom the author's thanks are due. The raw material was treated by the method outlined below. The crude phosphatide was fractionated by a method which was essentially that used by Channon & Foster [1934] in their work on the phosphatides of wheat germ. The fractionation resulted in the isolation of a fairly pure form of phosphatidic acid (the possible source of which is discussed later) and an impure form of lecithin. The lecithin fraction contained little or no kephalin (only about 5.5 % of the N in this fraction was amino-N), a finding which was unexpected.

EXPERIMENTAL

Preparation of the crude material

Freshly tapped latex was coagulated by pouring into 3 vol. of 95 % ethanol. The serum was concentrated *in vacuo* at 40° and the concentrate sealed *in vacuo* and shipped to England. Three samples representing 19.5 l. latex were received in good condition. The residues were mixed with anhydrous Na₂SO₄ and extracted with CHCl₃. The extract was concentrated *in vacuo*, dissolved in ether and the phosphatide precipitated by the addition of 6 vol. of acetone. After 15 hr. at 0° the supernatant liquid was decanted and the residue again treated with ether and acetone. This precipitation was repeated three times. 22.95 g. of material containing 1.36 % N and 2.53 % P were isolated.

Fractionation of the phosphatide

Plant phosphatides have been shown by several workers [Smith & Chibnall, 1932, 2; Channon & Foster, 1934; and Jordan & Chibnall, 1933] to contain lecithin and kephalin with varying amounts of phosphatidic acid, the latter compound being present as a salt of Ca, Mg or K (some workers state that the Na salt exists in the plant). It was considered likely that the phosphatide of *Hevea* might be a similar mixture and for this reason the fractionation procedure was essentially that of Channon & Foster [1934]. These workers have described the method in great detail and the accompanying diagram will suffice to illustrate the course of the fractionation.

Diagram 1.

Results

Fraction A 2. This represents a reasonably pure form of lead phosphatidate. Micro-analysis gave C 50.6; H 8.3; P 3.42; ash 30.0 (Smith & Chibnall give C 51.2; H 7.7; P 3.48; ash 33 for lead phosphatidate but the analysis will obviously depend on the nature of the fatty acids present). On hydrolysis it yielded 60.4% fatty acids (i.v. 112; mol. wt. 290.5). A Twitchell lead soap separation of the latter indicated the presence of 21.6%

saturated acids (mol. wt. 280) and 79.4 % liquid acids (i.v. 139; mol. wt. 303). Dihydroxystearic acid m.p. 130° and tetrahydroxystearic acid m.p. 156–157° were obtained on oxidation of the unsaturated fraction by the method of Haworth [1929].

Fraction B. When isolated this fraction contained 0.55 % N and 2.35 % P. Extraction with alcohol, or precipitation with alcohol from ethereal solution, did not increase the purity of the fraction; lead was therefore removed and the free acid emulsified in water, precipitated with acetone and the lead salt regenerated. Severe loss occurred during this procedure but 2.66 g. of material were isolated which contained 0.15 % N; 3.15 % P; ash 31.9 %; 49.15 % C; 7.4 % H. On hydrolysis it yielded 60 % of fatty acids (i.v. 90; mol. wt. 326) of which acids 26 % were solid acids (mol. wt. 326) and 74 % liquid acids (i.v. 123; mol. wt. 327). It thus represented a further amount of fairly pure lead phosphatide. It differed in fatty acid composition from A 2.

Fraction C. This fraction is a crude lecithin and it was impossible to effect any further purification. The difficulties of purifying small samples of phosphatide have been stressed by Smith & Chibnall [1932, 2] and Jordan & Chibnall [1933]. More recently Diemair *et al.* [1937] have isolated phosphatides from oats, barley and wheat which contained only 1.0 % N and 2.2 % P although they were apparently free from sugar. Working & Andrews [1941] state 'It is difficult to imagine what impurities might cling so firmly as not to be removed by the methods used successfully with materials of animal origin'. The results obtained by Rhodes & Bishop [1931] and Altman & Kraay [1940] indicate that they too found difficulty in purifying their products beyond the limits of N 1.0 %, P 2.5 %.

Analysis of fraction C. 3.89 g. were hydrolysed with NH_2SO_4 for 8 hr. 2.40 g. of fatty acids were recovered (62 % yield), i.v. 111; mol. wt. 315. 2.01 g. were fractionated (Twitchell) into 25 % saturated acids (mol. wt. 310) and 75 % unsaturated acids (i.v. 150; mol. wt. 326). Dihydroxystearic (m.p. 125°) and tetrahydroxystearic acid (m.p. 158°) were obtained by oxidation of the unsaturated fraction.

The aqueous fraction was concentrated *in vacuo* and analysed for bases and reducing sugar. Reducing sugar was determined by the method of Maclean [1919] and some 3.3 % was found (calculated as glucose). This figure is a minimal one since appreciable quantities of carbohydrate are decomposed during the acid hydrolysis [cf. Smith & Chibnall, 2]. Even if this is so, a large proportion of the impurity remains uncharacterized [cf. Diemair *et al.* 1937]. The total N of the aqueous fraction was 33.5 mg., some 6.5 mg. (16 %) being lost during hydrolysis. Similar losses (22 %) were reported by Smith & Chibnall [1932, 2] and by Jordan & Chibnall [1933], who found that only 65 % of the original N passed into the aqueous filtrate. It is possible that the loss of N is coupled with the lower amount of reducing sugar (q.v.) in the formation of an insoluble humin. Amino-N as determined by the method of Van Slyke amounted to approximately 1.85 mg., which, if it were all aminoethanol-N, would correspond to 8.15 mg. aminoethanol or to about 98 mg. cephalin. (Thus the cephalin content of the fraction cannot be more than 2.5 % of the crude phosphatide or about 4 % of the calculated phosphatide.)

Isolation of the bases. The aqueous filtrate was freed from H_2SO_4 and analysed for bases. The method has been described in detail by Smith & Chibnall [1932, 2] and depends on the precipitation of primary amines (including amino-acids) by the reagent of Neuberg & Kerb [1912]. Choline is soluble in the reagent but is precipitated almost quantitatively by $HgCl_2$. The following scheme indicates the course of the separation and the distribution of N.

The choline aurichloride was isolated in two crops: (1) m.p. 268–270°; Au 44.14; C 13.65; H 3.2; (2) m.p. 265°; Au 44.5; C 13.6; H 3.2. (The analysis in crop 2 was corrected for a small amount of free gold which was found to be contaminating the sample.) $C_5H_{14}ON.AuCl_4$ has m.p. 269–270° and requires Au 44.5; C 13.5; H 3.2 %.

Diagram 2.

the presence of phosphatidic acid after this stage of the fractionation suggests that it is present in salt form in P 1. Unfortunately it was not possible to analyse P 1 for the alkali metals (Na and K) since NaCl had been used to assist in the flocculation of P 1 with acetone. In order to check this point, and the apparent absence of kephalin, a sample of phosphatide was prepared from dried latex (a product in which the breakdown should have been small).

1.48 g. of crude phosphatide were obtained (N 1.2 %, P 2.2 %) from 850 g. dried latex. The crude material after three precipitations with acetone was dissolved in ether and the solution shaken with 0.05 *N* HCl in 40 % acetone-water. The aqueous extract was analysed for Na, K, Ca and Mg. Na and Mg were absent. The extract was found to contain the equivalent of 0.48 % Ca (estimated by the method of Wiley [1931]) and 2.6 % K (estimated by the method of Hamid [1926]).

If these elements were in combination with phosphatidic acid then the crude phosphatide would contain 0.14 g. (9.4 %) calcium phosphatide and 0.41 g. (27.6 %) dipotassium phosphatide. Thus fraction P 1 (diagram 1) should contain 5.9 g. phosphatidic acid (0.03 % latex). Only 4.2 g. (0.02 % latex) were isolated, since severe losses occurred during the final purification of the phosphatidic acid fractions A 2 and B. It is considered therefore that the total amount of phosphatidic acid present in fraction P 1 may well be 5.9 g. (0.03 % latex) and that it is probably present as both the calcium and potassium salts in the ratio of 1 : 3.

No amino-nitrogen, in amounts greater than those suggested above, could be detected in the phosphatide and it must be concluded that the phosphatide of *Hevea Brasiliensis* does not contain appreciable amounts of kephalin. This finding is in keeping with that of Altman & Kraay [1940].

The fatty acids of the fractions A, B and C had i.v. 90–112 and contained 21–26 % saturated acids, whilst the fatty acids from the glycerides [Tristram, 1942] had i.v. 135–148 and contained 24–26 % saturated acids. Thus the glyceride fatty acids are slightly more unsaturated than the phosphatide fatty acids. This finding is similar to those of Smith & Chibnall [1932, 1, 2] who found that the glyceride and phosphatide fatty acids of cocksfoot grass (*Dactylis glomerata*) had i.v. 170 and 128–144 respectively.

In a recent paper [Baker, 1941] it was stated that on purifying latex by creaming or centrifuging the nitrogen content of the rubber was reduced to a lower limit of 0.03 %. This N was said to be due to the 'phospholipin-sterol complex which is also thought to be associated with the rubber phase'. Now if the pure phosphatide of *Hevea Brasiliensis* is assumed to be lecithin only, the residual N referred to by Baker corresponds to the presence in the crude rubber of 1.7 % of lecithin. If there is any phosphatidic acid present the amount of phosphatide would be even higher (1.7–3.4 %). Now the maximum amount of phosphatide (even in the crude condition) which has been isolated is 0.2 % (N 0.59; P 1.72) [Rhodes & Bishop, 1931] and there is every reason to suppose that some of the N in this product is non-phosphatide-N. The assumption of Baker could be tested by a determination of the phosphorus content (0.06 if the phosphatide is lecithin only). The statement of Baker infers that the phosphatide is only removed from the rubber globule with great difficulty. It must be pointed out however that the lipin was isolated from the serum after coagulation of the latex with alcohol. The fact that crepe rubber contains little or no phosphatide may indicate that the reverse is the case, namely that the phosphatide, whilst assisting in the maintenance of the latex in equilibrium, will appear in the aqueous fraction when that equilibrium is upset.

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