The Phosphatides of the Latex of *Hevea brasiliensis*

1. BIOCHEMICAL CHANGES

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(Received 28 July 1953)

The presence of phosphatide material in the latex of *Hevea brasiliensis* was first definitely demonstrated by Rhodes & Bishop (1930). These and later workers all separated the phosphatides from the total lipids by precipitation with acetone. It was found that the acetone precipitate could not be separated from certain carbohydrate substances, and contained only about 2.5% phosphorus. Altman & Kraay (1940) showed that choline and glycerophosphate were present, and identified a number of fatty acids. Tristram (1942) found that, after purification, the phosphatides possessed a nitrogen/phosphorus ratio of less than one, and succeeded in separating a sample into a choline-containing fraction and a fraction which he considered to be derived from phosphatidic acid salts. Resing (1951) reported the identification in phosphatide hydrolysates, of glucose, galactose, inositol, a polysaccharide, and serine, by paper chromatography.

Hevea latex in the tree is a complex colloidal system in which the rubber particles are considered by some authorities (Cockbain, 1948) to be stabilized by an interfacial coating comprising an inner layer of lipid material and an outer layer of protein. In any case, it is to be expected that the phosphatides, in view of their known surface activity, would play an important part in the stabilization. When latex is tapped, changes occur which may be partially enzymic and partially bacterial, and which lead, in the course of some hours, to coagulation of the latex. This coagulation is presumably brought about by physical or chemical modification of the layers stabilizing the rubber particles. Thus, any chemical changes which the phosphatides might undergo during the period between tapping and spontaneous coagulation would probably be concerned with the loss of stability. The present work was undertaken to examine such changes in the phosphatides, with a view to obtaining information of value in explaining the spontaneous coagulation of latex.

A quantitative method was developed for the extraction and estimation of phosphatide in latex which was based on the coagulation of latex in alcohol, followed by the separation of the serum which formed. When, following previous workers, we used alcohol at room temperature (about 30°) for the coagulation, we found marked changes in the

properties of the phosphatides extracted, according to the time that the latex had been stored after tapping. The first change observed was a decrease in the acetone solubility of the phosphatide phosphorus with increased storage time. This was subsequently shown to be associated with an increase in the choline nitrogen/phosphorus atomic ratio (choline N/P ratio) of the lipid. Thus, in one experiment, the lipid samples extracted had choline N/P ratios of 0.19, 0.38 and 0.51 after 0, 4 and 8 hr. storage respectively. This finding, which was quite unexpected, appeared to show that after tapping the latex, a progressive synthesis of lecithin occurred. It later became clear that this interpretation was incorrect, and evidence is presented here to show that there is an enzyme present in latex which can liberate choline from latex phosphatide. This enzyme is inactive in fresh latex and is progressively destroyed as the latex is stored. It is, however, active after alcohol coagulation, in spite of the high alcohol content of the medium. These facts were responsible for the earlier misconception.

Hanahan & Chaikoff (1947*a, b*, 1948) and Acker, Diemair & Jager (1952) have reported the presence of an enzyme causing a similar liberation of choline from phosphatide, the former workers finding it in carrots and in cabbage leaves, and the latter in carrots and in germinated wheat.

EXPERIMENTAL

Total phosphorus determination. Total phosphorus (total P) was estimated by a modification of the method of van der Bie (1948). The method was developed for the determination of phosphorus in rubber, but was found to be quite suitable for lipid analyses. The ashing procedure employed was as described by van der Bie (1948) except that platinum, not porcelain, basins were used. A sample of the final phosphate solution (containing about 0.04 mg. P) was placed in a 50 ml. volumetric flask and 5 ml. 10*N*-H₂SO₄ and 2.5 ml. 0.04*M* ammonium molybdate were added. This was shaken and then 2 ml. SnCl₂ solution (prepared by dissolving 5.26 g. A.R. tin foil in 25 ml. 11*N*-HCl and, just before use, diluting 0.1 ml. in 20 ml. water) were added and the flask contents made up to the same volume and well mixed. The solution was allowed to stand for 20 min., to allow for maximum colour development, and then compared with standard solutions, prepared similarly, using a Hilger Biochem Absorptiometer with a red O.R. 2 glass filter.

Ash phosphorus determination. In the ashing procedure used for the determination of total phosphorus, Na_2CO_3 is added to the lipid in order to prevent volatilization of organically bound phosphorus. If this is omitted, and the lipid is ashed alone, then the amount of phosphorus which is retained depends upon the amount of metallic constituents present in the lipid. Phosphorus which is determined in this way is referred to as ash phosphorus (ash P). In the absence of non-phosphatide metals, the ratio ash P/total P should give a measure of the phosphatidic-acid metal salts present in the phosphatide mixture.

Choline determination. This was carried out essentially according to the method of Glick (1944), but different methods of extraction and hydrolysis were employed. A lipid sample, extracted according to the technique to be described later, and containing about 2–8 mg. choline, was hydrolysed by refluxing with 5N-HCl. This technique was shown not to lead to destruction of added choline, even after 48 hr. refluxing, and since there was no change in the determined choline content of the lipids whether refluxing was continued for 6, 18 or 48 hr., it appeared that complete liberation from the lipid occurred during the first 6 hr. In practice, a hydrolysis time of 18 hr. was used.

The hydrolysis mixture was cooled in the refrigerator, filtered, and the filtrate was evaporated to dryness on a steam bath. The residue was taken up with water, again filtered, and the filtrate made slightly alkaline to thymolphthalein with N-NaOH. The blue colour was then just discharged by the dropwise addition of 0.15N acetic acid (pH now approx. 9). Choline was precipitated and determined colorimetrically as the reineckate according to Glick (1944) using a Spekker absorptiometer with Ilford 604 Spectrum Green filter. The amounts of choline are given as choline nitrogen (choline N).

To determine free choline in aqueous extracts, the pH of the solution was again adjusted to approximately 9 and the reineckate precipitated and estimated as before.

Acetone solubility of latex phosphatide. This was found to be a variable property depending upon the choline content of the phosphatide. The solubility, as measured by one precipitation, also depended upon the concentration of the phosphatide and consequently, in order to make a simple comparison of the degree of acetone solubility of different samples, an arbitrarily standardized technique was used. A sample of a light petroleum (b.p. 56–70°) solution of the lipid containing 1 mg. phosphorus was added to a 15 ml. centrifuge tube. The volume was adjusted to 1 ml., and 6 ml. of acetone were added. The tube was allowed to stand overnight at 22°, when it was centrifuged, and the supernatant liquid and precipitate were separately analysed for phosphorus.

Collection of latex samples. Seedling latex, obtained from the Experiment Station of the Rubber Research Institute of Malaya was used throughout the work. Latex was, except where stated otherwise, collected straight from the tree into a bottle surrounded by freezing mixture in order to avoid changes during the time of tapping (about 2 hr.). It was thawed out immediately before starting an experiment. Such latex was regarded as being in the same state as it is in the tree and was examined either immediately (fresh latex, in this paper), or at different storage times after thawing. No special precautions against bacterial contamination were taken, although the conditions of collection were cleaner than is usual when commercial tapping into open cups is carried out.

Extraction and estimation of free choline and phosphatide in latex. The quantitative extraction of non-rubber substances from latex is difficult, because almost any extraction treatment causes coagulation with the resultant formation of an impermeable mass of rubber. Previous workers have not usually been concerned with obtaining quantitative recovery of latex lipid, but Altman (1947) attempted this by coagulating the latex in alcohol (the term 'alcohol' is used to indicate redistilled industrial methylated spirit throughout this paper), making a sheet of the coagulum and re-extracting this with alcohol in a continuous extractor. This method was rejected in the present work, since experiments showed that only a small further amount of phosphatide was obtained by the re-extraction, and it was clear that quantitative recovery was not obtained. A method was therefore developed for obtaining a representative sample of the total phosphatide present in latex without attempting a complete recovery. The method was used, with suitable adjustment of conditions, for (i) the determination of total phosphatide phosphorus (phosphatide P) in latex; (ii) the preparation of representative samples of latex phosphatide for subsequent analysis; and (iii) the examination of changes in the properties of the phosphatide after alcohol coagulation. The technique also permitted for the determination of free choline present, either in the latex or liberated as a result of alcohol coagulation.

About 40 ml. latex were poured from the well-mixed bulk into a measuring cylinder. This was coagulated by dropwise addition to 3 vol. of alcohol with vigorous mechanical stirring in a Hamilton Beach fourfold aerator with a simple disk stirrer. The latex added was weighed. The use of larger quantities of latex resulted in low values being obtained in subsequent determinations of phosphatide P, presumably owing to trapping within the bulky coagulum (see Table 1): 40 ml. of latex was chosen as the minimum amount giving a reasonably large quantity of phosphatide for subsequent examination. Efficient stirring during the latex addition gave a more spongy coagulum, which also reduced trapping within the coagulum, thus resulting in higher determined values of phosphatide phosphorus (see Table 1). The addition of latex was made either into boiling alcohol, or into alcohol at a lower temperature, in which case the mixture was subsequently boiled for 15 min. When it was only required to determine phosphatide P, the former technique was preferred. The serum was filtered off while still hot, the coagulum being squeezed by hand. The volume of serum recovered was measured, and since the theoretical total volume of serum present could be calculated (with correction for losses due to evaporation), the percentage recovery of serum could also be calculated. The serum was evaporated in N_2 under reduced pressure, to leave an aqueous residue of about 15 ml., which was transferred to a stoppered centrifuge tube. The residue was extracted with four portions of 20 ml. of light petroleum (b.p. 56–70°). After each extraction, the layers had to be separated by centrifuging, since very stable emulsions were formed. The light petroleum extracts were siphoned off. The aqueous residues were evaporated to dryness in N_2 under reduced pressure, and twice extracted with 20 ml. quantities of boiling light petroleum. This further treatment was necessary for complete extraction (see Table 1). These extracts were clarified by centrifuging and added to the main light petroleum extracts. The phosphorus contents of samples of this solution were estimated and the phosphatide P in a given quantity of latex could therefore be calculated.

Table 1. *Effect of variations in the procedure used for the determination of phosphatide phosphorus in latex*

(The top line gives the procedure adopted for routine determinations. Each phosphatide P value given is a mean of duplicate determinations with an estimated standard error of the mean of ± 0.044 mg./10 g. latex. The minimum 5% significant difference between two such means is 0.14 mg./10 g. latex.)

Initial extraction from latex				Dried aqueous residue	
Latex used (ml.)	Solvent	Stirred	Boiled 15 min.	re-extracted with boiling light petroleum	Calculated phosphatide P (mg./10 g. latex)
40	Alcohol	+	+	+	1.84
40	Alcohol:ether (50:50, v/v)	+	+	+	1.90
40	Alcohol	+	+	-	1.76*
40	Alcohol	+	-	+	1.52
40	Alcohol	-	+	+	1.47
125	Alcohol	+	+	+	1.34

* Although the variation, due to the re-extraction with light petroleum, is small, it is a real effect, since the amount of phosphorus obtained by the second extraction was determined separately.

When required, the residue left after extraction with light petroleum was extracted with water, and the extract filtered and used for the determination of free choline.

There was no significant difference between results obtained after coagulation in boiling alcohol and after coagulation at 22–30° followed by 15 min. boiling of the mixture. Thus, using the former technique, six determinations on one latex gave a mean value for phosphatide P of 1.82 mg./10 g. latex with a range of 1.77–1.87 (S.E.M. ± 0.04). Using the latter technique with the same latex, thirty determinations gave a mean value of 1.85 mg./10 g. latex with a range of 1.62–2.03 (S.E.M. ± 0.02). The standard error of a single determination was estimated to be ± 0.10 mg./10 g. latex. In view of the variability inherent in this method of determination, all measurements of latex phosphatide P, given in this paper, will be expressed as mean values of replicate determinations with their standard errors.

The value of the method in giving a true absolute determination of phosphatide P and a representative sample of total phosphatide depends upon the assumptions that (i) all the phosphatides are extracted by alcohol and (ii) there is a uniform distribution of phosphatide between the free solvent and the solvent associated with the coagulum. Regarding the first assumption, the use of alcohol as a solvent requires some justification, since certain phosphatides are not readily soluble in this solvent. They are, however, fairly soluble in hot alcohol, particularly when present in a mixture with alcohol-soluble phosphatides, and latex lipid extracted according to the above procedure contains a considerable amount (about 15% of the total) of alcohol-insoluble P. Furthermore, extraction of phosphatides by coagulating latex in a mixture of equal volumes of alcohol and ether gives little if any improvement in the yield of phosphatide P over extraction with alcohol alone, provided that the latter mixture is boiled immediately before the filtration of the serum (Table 1). In view of its greater convenience, alcohol alone was adopted for the extraction. The second assumption may not be strictly correct, but since the present work required the determination of only relative values, it was considered that the above technique was more satisfactory than any attempt to achieve a complete recovery. There is some evidence that the figures obtained by this method are correct, or nearly so. Thus, the calculation of phosphatide choline in a given amount of latex rests on the same assumptions as does the calculation of phosphatide P. Now figures for total choline,

obtained from the sum of free choline and this calculated phosphatide choline, are virtually constant for one sample of latex, whether the phosphatide is undegraded or whether the majority of the choline has been liberated in a free form (see, for example, Fig. 1). This would not be so if the calculated phosphatide choline figures were not nearly correct. By analogy, it appears that the phosphatide P figures must also be nearly correct. Further evidence is provided by the fact that phosphatide added to the coagulation mixture is recovered in approximately theoretical amounts (see Table 3).

Preparation of latex serum. It was desired to obtain a rubber- and phosphatide-free fraction from latex in which the factor responsible for choline liberation could be studied in greater detail. Two methods were examined. (1) Latex was collected at ambient temperature, rapidly frozen in shallow pans and kept at -20° for 2 weeks. On thawing, a clear rubber-free serum was separated from the coagulum. This serum contains about 50% of the protein present in the latex. (2) Latex, which had been collected into vessels surrounded by freezing mixture, was thawed below 10° and centrifuged at 21 000 rev./min. in the Spinco refrigerated ultracentrifuge. By this means, latex was separated into three main fractions: rubber, clear serum and a white precipitate at the bottom of the tube (Cook & Sekhar, 1953). For these experiments, a mixture of clear serum and bottom white precipitate was separated from the rubber and is subsequently referred to as total centrifuged serum. This total serum is almost free of rubber and contains very little phosphatide.

Examination of choline liberation from phosphatides. When the liberation of choline from latex phosphatide on storage of the latex was examined the phosphatide was extracted as described previously, but since it was necessary to avoid any secondary changes after extraction, the coagulation was carried out in boiling alcohol to destroy any choline-liberating factor present. This method was satisfactory, and with fresh latex the virtual absence of any free or water-soluble combined choline from the alcohol serum indicated that there was no liberation of choline from the phosphatide during the extraction procedure. When it was required to examine choline liberation after coagulation of latex (fresh or stored) in alcohol, the same procedure was used, but the alcohol was at a lower temperature, and the liberation reaction was stopped after suitable periods by boiling the mixture for 15 min. and rapidly filtering it. Boiling, not

followed by filtration, greatly slowed the reaction but did not stop it, apparently because the rubber mass, being a poor conductor of heat, protected any of the choline-liberating factor associated with it. This difficulty was not encountered on coagulating in boiling alcohol, since the latex was added dropwise, and each drop was efficiently heated before a massive coagulum was formed.

The choline-liberating activity of latex serum (frozen and centrifuged) was examined by adding samples of latex phosphatide or egg lecithin, either in alcoholic solution or in aqueous suspension. The egg lecithin used was a commercial preparation (British Drug Houses Ltd.) which contained no free choline, but possessed a choline N/P ratio of only 0.75. Although this was clearly not very pure, it was for the present experiments, considered suitable without special purification. The reaction in the alcoholic medium was stopped when required by boiling for 10 min., the mixture was filtered and the filtrate worked up, in the same way as the alcoholic serum samples from latex, to give values for phosphatide P, phosphatide choline and free choline. The reaction in the aqueous medium was stopped by immersing in a boiling-water bath for 10 min. or by adding trichloroacetic acid to a final concentration of 4% (w/v), the product was filtered and free choline was determined in the filtrate.

RESULTS

Variations in the phosphatide on latex storage

A variety of latex samples from different sources was examined after different storage periods. In no case was there any significant decrease in phosphatide P on storage up to 4 hr. Of seven different samples of latex studied, however, three showed a decrease on from 4 to 8 hr. storage, while the remainder showed no such decrease. A maximum period of storage of 8 hr. was examined since the latices usually coagulated shortly after this time. The results given in Table 2 are representative of values obtained in these experiments. Latex sample 1 in this table showed the biggest decrease in phosphatide P of any of the samples examined. Latex sample 2 showed no phosphatide decrease, even when collected into an open tapping cup in the commercial manner, a procedure which resulted in gross bacterial contamination and spontaneous coagulation of the latex 7.5 hr. after collection.

Determinations of lipid choline N/P ratio and free choline N in the above latices after different periods of storage, of which the results given in Table 2 are representative examples, showed that there was little or no free choline present in fresh latex, and that there was no liberation of choline from the phosphatide on storage except as a result of general breakdown of the phosphatide (e.g. latex sample 1). The choline N/P ratio of the lipid varied from 0.68 to 0.77 for the different latices examined, and under no circumstances did it fall appreciably on storage. There was, in fact, sometimes a small rise, the reason for which is not known.

Table 2. *Variations in certain properties of latex on storage at 30°*

(Each phosphatide P value given is a mean of four determinations with an estimated standard error of the mean of ± 0.03 mg./10 g. latex. The minimum 5% significant difference between two such means is 0.08 mg./10 g. latex.)

Storage time at 30° (hr.)	Latex sample 1				Latex sample 2			
	Collected in bottles surrounded by freezing mixture		Collected in bottles surrounded by freezing mixture		Collected in bottles surrounded by freezing mixture		Collected in open tapping cups	
	Phosphatide P (mg./10 g. latex)	Choline N/P atomic ratio of lipid	Free choline N (mg./10 g. latex)	Phosphatide P (mg./10 g. latex)	Choline N/P atomic ratio of lipid	Free choline N (mg./10 g. latex)	Phosphatide P (mg./10 g. latex)	Free choline N (mg./10 g. latex)
0	1.98	0.75	0.00	1.56	0.72	0.01	1.60	0.02
3	•	•	•	•	•	•	1.55	0.03
4	1.93	0.72	0.00	1.49	0.70	0.03	•	•
6	•	•	•	•	•	•	1.53	0.02
8	1.56	0.77	0.05	1.52	0.73	0.02	•	•
							Coagulated	

Liberation of choline from latex phosphatide after coagulation of the latex in alcohol

The liberation of free choline from the phosphatide, illustrated in Fig. 1, is not accompanied by a significant decrease in total phosphatide P. In this experiment, the phosphatide P values (means of duplicate determinations) obtained were 2.12, 2.02, 2.10 and 2.16 mg./10 g. latex for 0, 3, 12 and 24 hr. storage, respectively. The estimated standard error of each of these means was ± 0.044 mg./10 g. latex, and the minimum 5% significant difference between two such means was 0.17 mg./10 g. latex.

Fig. 1. Variations in the distribution of choline after coagulation of fresh latex in 3 vol. of alcohol at a temperature of 22° and subsequent storage at this temperature. A, total choline N; B, free choline N; C, lipid-bound choline N.

Fig. 2. Effect of temperature on the percentage liberation of choline from latex phosphatide after coagulation of the latex in 3 vol. of alcohol. A, after 3 hr. storage; B, after 24 hr. storage.

Fig. 2 shows the effect of temperature on this choline-liberation reaction. There is a fairly wide temperature range over which the reaction rate is a maximum or nearly so, but above 40° there is little or no reaction.

The effects of adding alcohol-soluble latex lipid (prepared by coagulation of latex in boiling alcohol, and therefore possessing a high choline N/P ratio), or of egg lecithin (British Drug Houses Ltd.) to the alcohol coagulation mixture are shown in Table 3. These lipids were dissolved in the alcohol in which the latex was to be coagulated; otherwise the procedure was as before. The results indicate that choline is liberated from both latex lipid and egg lecithin without any accompanying decrease in phosphatide P when these substances are respectively added to the alcohol/latex coagulation mixture at 22°.

The results given in Fig. 3 show how the factor responsible for the liberation of choline from the phosphatide is progressively inactivated on latex storage. A similar decrease in activity was observed with eight out of nine latex samples examined. The atypical sample, which showed no such decrease, was relatively free from bacteria, and when a further sample of the same latex was collected into an open tapping cup (with consequent gross bacterial contamination) it then conformed with the majority. It appears probable, therefore, that this inactivation is due to bacterial action. It is responsible for the apparent paradox that, if the lipid is to be prepared by coagulation of latex in alcohol at room temperature, a product closer to the unchanged phosphatide is obtained if the latex is stored for some hours before coagulation.

Fig. 3. The effect of prior latex storage on variations in the distribution of choline after coagulation of the latex in 3 vol. of alcohol at a temperature of 30°, and subsequent storage at this temperature. Continuous lines: total choline N (free + lipid-combined choline). Broken lines: free choline N. O, fresh latex; Δ, latex stored for 4 hr. at 30°; □, latex stored for 8 hr. at 30°.

Table 3. *Effect of added phosphatide on the liberation of choline by latex*

(Each phosphatide phosphorus value given is a mean of duplicate determinations with an estimated standard error of the mean of ± 0.07 mg./10 g. latex. Since there is no significant difference between these values obtained at 0 and 24 hr. storage the percentage recovery of added lipid phosphorus is derived from the mean.)

Additions to the alcohol used for coagulation	Time of storage of coagulation mixture at 22° (hr.)	Determined total phosphatide P (mg./10 g. latex)	Recovery of added lipid P (%)	Lipid choline N (mg./10 g. latex)	Free choline N (mg./10 g. latex)	Choline N liberated during 24 hr. storage (mg./10 g. latex)	
						Measured as increase of free choline N	Measured as decrease of lipid choline N
No addition	0	1.98	.	0.66	0.01	0.55	0.51
	24	2.08	.	0.15	0.56		
Egg lecithin containing P and choline N equivalent to 0.71 and 0.24 mg./10 g. latex respectively	0	2.86	111 (s.e. ± 10)	0.89	0.02	0.65	0.62
	24	2.77		0.27	0.67		
Latex lipid containing P and choline N equivalent to 1.06 and 0.45 mg./10 g. latex respectively	0	3.14	100 (s.e. ± 9)	1.05	0.04	0.74	0.75
	24	3.04		0.30	0.78		

Liberation of choline in formic acid coagulated latex

When 50 ml. fresh latex were added to 10 ml. 1 % (w/v) formic acid giving a pH of about 4 (a procedure resulting in immediate thickening and slow coagulation of the latex), it was found that within 2 hr., approximately 95 % of the choline, originally present in the latex in combination with the phosphatide, was liberated in a free form. Attempts to stop the reaction by adding 3 ml. 80 % (w/v) trichloroacetic acid were not very successful, owing to the difficulty of obtaining adequate dispersal in the rubbery mixture which completely coagulated on addition of trichloroacetic acid. The best mixing was obtained by adding the trichloroacetic acid to the formic acid before addition of fresh latex. Even so, 25 % choline liberation occurred before the reaction was completely stopped by filtration of the serum (taking about 15 min.). Addition of trichloroacetic acid immediately after coagulation of the latex in formic acid, followed by filtration, resulted in the liberation of 75 % of the choline. The initial rate of phosphatide degradation must therefore be high. When mixtures of formic acid and fresh latex were stored at 30° for 0.5, 1, 2 and 6 hr. before addition of trichloroacetic acid and filtration, the percentages of choline liberated were found to be 90, 94, 96 and 96 respectively. When latex which had been stored at 30° for 6 hr. was coagulated in formic acid, trichloroacetic acid added immediately, and the mixture filtered, only 9 % liberation occurred, a result which confirmed that the choline-liberating factor is inactivated when latex is stored. This effect is further illustrated by the results given in Fig. 4, where it is seen that, as the latex is stored, there is a progressive decrease between the second and the eighth hour in the percentage of choline liberated in a period of 2 hr. after the addition of formic acid.

Neither formic acid nor trichloroacetic acid in the concentrations used caused any liberation of free choline from an aqueous suspension of latex lipid.

Fig. 4. The effect of prior latex storage at 30° on the subsequent percentage liberation of choline from the phosphatide when the latex (5 vol.) is coagulated with 1 % (w/v) formic acid (1 vol.) and stored for 2 hr. at 30°.

Liberation of choline from phosphatide by latex serum

No capacity for liberating choline from either latex lipid or egg lecithin could be detected in the serum which separated after latex had been maintained in a frozen condition for 2 weeks. However, the latex from which this serum was prepared contained 0.67 mg. lipid choline N/10 g. latex, but no free choline, while the frozen serum contained free choline N equivalent to 0.65 mg./10 g. of the latex. It is evident, therefore, that virtually complete choline liberation occurred during the period in which the latex was kept frozen.

The choline-liberating activity of total centrifuged serum was tested on egg lecithin, both in alcoholic solution and as an aqueous suspension. Fig. 5 shows the activity in 75% (v/v) alcohol. The centrifuged serum sample examined in this experiment contained 0.04 mg. phosphatide P/ml., which was equivalent to about 7% of the total present in the latex. It appeared, from the presence of some free choline and from the fact that the extracted phosphatide, when no lecithin was added, possessed a choline N/P ratio of only 0.33, that some liberation of choline had already occurred in the period—which had been made as short as possible—between centrifuging and the start of the experiment. In the course of the experiment this liberation continued to completion. The addition of egg lecithin to the serum was followed by a further large liberation of choline. The change, in both the presence and absence of egg lecithin, was not accompanied by any decrease of phosphatide P; the values for phosphatide P after adding egg lecithin containing 2.50 mg. phosphorus to 10 ml. serum were 2.90,

Fig. 5. Variations in the distribution of choline after the addition of 10 ml. total centrifuged latex serum to 3 vol. of alcohol (with and without the addition of egg lecithin containing 0.85 mg. choline N) at 22° followed by storage at this temperature. A, no additions, free choline N; B, no additions, lipid-combined choline N; C, egg lecithin added, free choline N; D, egg lecithin added, lipid-combined choline N.

2.78, 2.82 and 2.86 mg. for 0, 0.5, 2 and 24 hr., respectively, and for 10 ml. serum in the absence of lecithin, 0.42, 0.39 and 0.41 mg. for 0, 2 and 24 hr., respectively. Thus, the only degradation suffered by the lecithin appears to have been a loss of choline.

Fig. 6 shows the rate of liberation of choline by centrifuged serum, with and without the addition of an aqueous suspension of egg lecithin. There was some free choline formed in the serum alone, presumably from residual latex phosphatide, but the amount was much increased by the addition of egg lecithin. The serum must therefore be responsible for the rapid liberation of choline from the latter.

Fig. 7 illustrates the effect of temperature on this liberation. The optimum temperature is in the region of 30° (cf. liberation in an alcoholic medium, Fig. 3). The values plotted in Fig. 7 have been calculated, taking into account the choline formed from the residual latex phosphatide present in the serum. Thus, for example, after 2 hr. storage at 30°, the 10 ml. sample of serum with no added lecithin contained 0.103 mg. free choline N, while the sample with egg lecithin contained 0.440 mg. free choline N. The difference between these figures was taken as the choline N liberated from the egg lecithin.

The same type of correction was applied in calculating the values given in Fig. 8, in which the effect of pH on the reaction is illustrated. The pH of the mixture was adjusted by the addition of formic acid or sodium hydroxide. Buffers were not considered necessary as the serum itself possessed buffering properties. Trichloroacetic acid, which was used to arrest the liberation at a suitable time

Fig. 6. Liberation of free choline at 30° in 10 ml. centrifuged total latex serum adjusted to pH 4.2 with formic acid. A, 10 ml. water added; B, with addition of 10 ml. aqueous egg lecithin suspension containing 0.22 mg. combined choline N.

interval, was itself shown to cause no liberation of choline from egg lecithin. The pH of optimum reaction at 30° is in the region of 6.0.

Changes in latex lipid properties associated with the change in choline content

Appearance. Light petroleum extracts of latex lipid, prepared as described previously, were evaporated under N₂ and the residues were com-

pletely dried under vacuum. Lipid with a high choline N/P ratio was a waxy solid: lipid in which this ratio was low was a viscous, oily liquid.

Behaviour on precipitation by acetone. Lipid samples with a high choline N/P ratio gave a flocculent white precipitate when 6 vol. of acetone were added to 1 vol. of a solution in light petroleum. Samples in which this ratio was low formed a turbid suspension settling slowly as an oily liquid. The change from a high to a low choline N/P ratio was accompanied by an increase in the acetone solubility. The relationship is shown in Fig. 9. Hanahan & Chaikoff (1947*b*) found similar variations with soya-bean lecithin degraded to a greater or lesser extent by the choline-liberating enzyme which they extracted from carrots.

Ash phosphorus. Variations in the ash P/total P ratio with variations in choline N/P ratio are also shown in Fig. 9. The values obtained for the former ratio do not give a true measure of the metal phosphatides, owing to the presence of water-soluble metallic impurities, which are taken into the light petroleum solution by the phosphatides. That this is so is indicated by the fact that the sum of the ash P/total P ratio and the choline N/P ratio is always greater than unity for unpurified lipid extracts. Furthermore, water washing of the solutions of lipid in light petroleum results in a decrease of the ash P/total P ratios. For example, an unpurified lipid with a choline N/P ratio of 0.17 possessed an ash P/total P ratio of 0.93. After water washing, the latter ratio was reduced to 0.74.

Fig. 7. Variations with temperature in the percentage choline liberated from 10 ml. of an aqueous suspension of egg lecithin (containing 0.49 mg. choline N) added to 10 ml. total centrifuged serum, and adjusted to pH 4.2 with formic acid. A, after 5 min.; B, after 2 hr.

Fig. 8. Variation with pH in the percentage choline liberated from 10 ml. of an aqueous suspension of egg lecithin (containing 0.36 mg. choline N) added to 10 ml. total centrifuged serum at 30° and incubated for 1 hr.

Fig. 9. Relationship between choline N/P ratio of unpurified latex lipid and: A, ash P/total P ratio; B, percentage acetone-solubility of the phosphatide (measured under standardized conditions, see p. 241). The plotted values were derived from a number of experiments on different latices.

DISCUSSION

The results show that virtually all the choline present in fresh latex is in a combined form with the phosphatide, and that if the choline-containing phosphatide which is present is of a normal lecithin type structure, approximately 68–77% of the phosphatide phosphorus is in this form. No evidence has been obtained in the present work concerning the nature of the remaining 23–32%, primarily owing to the difficulty of removing water-soluble impurities from the light petroleum extracts of lipid. Thus, in addition to the metallic impurities which give rise to falsely high figures for ash P, the light petroleum extracts prepared as described earlier, contain other water-soluble impurities, in particular nitrogenous ones, not normally soluble in light petroleum but rendered soluble by the presence of the phosphatides. This is a well-known property of phosphatides (see, for example, McKibbin & Taylor, 1949). However, it has been shown that there is no water-soluble choline present in the unpurified extracts, while the amount of water-soluble phosphorus impurity is very low (less than 5% of the total light petroleum soluble phosphorus), so that results for lipid choline and phosphorus are valid even for unpurified extracts. In view of the large number of small samples which had to be examined, and the fact that the principal object of the present work was served by the measurement of these two properties, the difficult removal of water-soluble impurities was not attempted as a routine measure. Since total nitrogen determinations were found to be quite valueless without rigorous purification, no information on the composition of the phosphatides could be derived by the determination of this property on the unpurified extracts. The problem of the purification of latex lipid will be dealt with fully in a later paper.

The liberation of choline which occurred after coagulation of latex in alcohol below 40° was not accompanied by a decrease in phosphatide phosphorus. This implies that, if the choline-containing phosphatide in latex is of a normal lecithin type, the degradation which it suffers consists in a simple loss of choline without the rest of the molecule being affected. Such a degradation might be expected to give rise to free phosphatidic acids, which would then form salts with any metallic ions present. This was indicated by an observed increase in ash phosphorus/total phosphorus ratio as the choline nitrogen/phosphorus ratio decreased (Fig. 9), although, since metallic impurities were present, deductions from these determinations are not entirely reliable.

It follows from these observations that the lipid extracts obtained by previous workers (Rhodes &

Bishop, 1930; Tristram, 1942; Altman, 1946; Resing, 1951) by coagulation of latex in alcohol at room temperature, have, in fact, been artifacts in which the choline content of the phosphatide was reduced to a greater or lesser extent during the extraction procedure. This is implied in the results obtained by Tristram (1942) in that his product contained only about 35% of crude lecithin. These workers all used acetone precipitation to separate the phosphatides from the other lipids. It has been shown in the present work, however, that the latex phosphatide is appreciably soluble in this solvent, particularly when the choline nitrogen/phosphorus ratio is low. Consequently some loss and probable fractionation of the phosphatide mixture may be expected to occur on acetone precipitation. For this reason we have made no attempt to isolate the phosphatides, and have made all analyses on the total lipid. Since the substances determined are characteristic of phosphatides and do not occur in other lipids, this procedure was considered to be justified.

The liberation of choline which occurred when fresh latex was coagulated in cold alcohol appeared to be due to the presence of an enzyme in the latex, despite the fact that the liquid phase of the coagulation mixture contained about 80% alcohol. The liberation was also found to occur in latex coagulated with formic acid at pH 4, and in latex coagulated by freezing. Evidence for the enzymic nature of the reaction is as follows. (a) Latex serum, obtained largely free from phosphatide by high speed centrifuging, was active in liberating choline from added egg lecithin, both in alcohol solution and in aqueous suspension. The activity of this serum was destroyed by immersion in boiling water, or by the addition of trichloroacetic acid. (b) The effect of temperature on the rate of liberation of choline in latex coagulated by alcohol (Fig. 2) was typical of an enzyme reaction except that the optimum temperature (about 10–15°) was lower than is usual for enzyme reactions in aqueous media, and in that complete inactivation occurred above about 40°. This was presumably due to the denaturing effect of the alcohol; the optimum reaction temperature for latex serum on egg lecithin in an aqueous medium was found to be about 30° (Fig. 7). (c) The degradation of egg lecithin by latex serum in an aqueous medium was shown to depend on pH in a manner typical of an enzyme reaction (Fig. 8).

Although this enzyme manifested itself in the above ways, it was not active in liberating choline from the latex phosphatide in untreated latex kept at 30°, during the period between tapping and spontaneous coagulation. Since fresh latex has a pH of about 6.5 (i.e. the pH was nearly optimum), this probably means that one of three alternatives is operative in the untreated latex. (a) The enzyme is

present as an inactive precursor. (b) An inhibitor is present. (c) The phosphatide is protected from contact with the enzyme. The last possibility would agree with the Cockbain (1948) theory of latex stabilization, in which the rubber particles are considered to be covered by an inner layer of lipid material and an outer layer of protein. The fact that the destruction of the colloidal stability of latex by the addition of alcohol, formic acid, or by freezing is accompanied by the creation of conditions suitable for the choline-liberating reaction to occur, provides some evidence that this view is correct. Whatever the reason for the lack of activity in untreated latex, it seems that the choline-liberating enzyme does not play any part in the processes leading to the spontaneous coagulation of latex.

The possibility of phosphatide degradation, other than that involving the choline-liberating enzyme, but rather leading to a decrease of phosphatide phosphorus, was also investigated. We made no attempt to collect the latex under sterile conditions. The bacteria in latex almost certainly play an important part in its spontaneous coagulation, and when latex is collected under sterile conditions it remains largely uncoagulated for an indefinite time (McMullen, 1949, 1951). Thus, a study of changes likely to lead to spontaneous coagulation must take bacterial contamination into account. It would clearly be desirable to identify the bacteria normally present in latex, and to examine the effects of pure cultures on sterile latex, but this was outside the scope of the present work and would involve a lengthy and independent investigation. It was found that, in some latices examined, there was a decrease in phosphatide phosphorus in the period between tapping and spontaneous coagulation. Such a decrease must be the result of a breakdown of the phosphatide, and consequently might be responsible for a change in latex stability, either directly, because it would involve the destruction of a component of the protective layer around the rubber particles, or indirectly, as a result of the formation of free fatty acids which could lead to destabilization, according to the theory of van Gils (1947). However, in other latices examined, no such breakdown was observed during storage. In general, all latex samples collected under non-sterile conditions undergo spontaneous coagulation; this was so in this investigation, even in samples showing no signs of phosphatide breakdown. It seems improbable, therefore, that phosphatide breakdown is a factor which generally plays a part in the process of spontaneous coagulation. This does not support the theory of van Gils (1947), although it is possible that the fatty acids required by this theory are provided by the non-phosphatide glycerides in latex, a possibility which we did not examine. Altman (1947) has reported a decrease in

total lipid when latex is stored. In only one experiment, however, could the observed decrease be dissociated from experimental errors due to premature flocculation of the latex, and in this case a maximum decrease of only 6% was observed. The method of determination involved alcohol extraction from a rubber coagulum, a procedure well known to give variable results. Since no indication of the reproducibility of the determinations was given, these observations are regarded as being of doubtful value.

Inactivation of the choline-liberating enzyme took place on latex storage in almost all the samples examined. This may have been due to bacterial action, but whatever the cause, the change might be responsible for variations in the choline content of commercially prepared rubber, with variations in the time that the latex is kept before acid coagulation. Thus, the older the latex, the less phosphatide breakdown after acid coagulation, and consequently the less loss of choline into the serum in a water-soluble form. Choline is an accelerator of rubber vulcanization (Altman, 1948), and its concentration might therefore be expected to affect the properties of the final rubber product.

SUMMARY

1. A method is described for the determination of phosphatide phosphorus in the latex of *Hevea brasiliensis*. Different latices gave values, immediately after tapping, varying from about 0.015 to 0.022%. In some cases this fell, after 8 hr. storage, by as much as 20% but in other cases there was no decrease.

2. The phosphatide contains virtually all the choline present in fresh latex and possesses a choline nitrogen/phosphorus ratio of about 0.68–0.77. The presence has, however, been demonstrated of an enzyme which is capable of causing the liberation of choline from latex phosphatide without any other apparent degradation. The liberation has been shown to occur after coagulation of the latex by the addition of alcohol below 40°, by the addition of formic acid, or by freezing; it does not occur in untreated latex.

3. The total serum obtained by high speed (21 000 rev./min.) centrifuging of latex contains the choline-liberating enzyme free from most of the latex phosphatide. This serum is highly active in liberating choline from egg lecithin in aqueous suspension or in alcoholic solution. In the former case an optimum temperature of about 30° and an optimum pH of about 6.0 were observed.

4. The degradation of latex phosphatide by this enzyme is accompanied by (i) the total lipid becoming more liquid; (ii) an increase in the solubility of the phosphatide in acetone; and (iii) the formation

of metal phosphatidates, as evidenced by an increase in the ash phosphorus/total phosphorus ratio of the lipid.

5. The enzyme is progressively inactivated in the period between tapping and spontaneous coagulation of the latex, probably owing to bacterial action.

This work forms part of a programme of research on natural rubber undertaken by the Rubber Research Institute of Malaya. The author wishes to thank Mr Yee Shin Meng for able practical assistance and Mr D. R. Westgarth for the statistical analysis.

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The Turnover Rates of the Phosphate Groups of Flavin-Adenine Dinucleotide and Adenosine Triphosphate during Oxidative Phosphorylation

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(Received 16 July 1953)

The nature of the reactions by which inorganic phosphate is converted into pyrophosphate groups of adenosine triphosphate (ATP) when reduced diphospho- or triphospho-pyridine nucleotides are oxidized is still unknown. An approach to the problem is a search, with the help of radioactive phosphate, for the formation of any phosphorylated compound in an enzyme system which synthesizes ATP from adenosine diphosphate (ADP) and phosphate. If radioactive phosphate is added to a phosphorylating system any intermediate on the way from phosphate to ATP must become radioactive, its specific activity being at least as high as that of ATP.

Hummel & Lindberg (1949), working with rabbit liver, reported that flavin-adenine dinucleotide (FAD) becomes radioactive about as rapidly as ATP, and they concluded that FAD is closely associated with oxidative phosphorylation. The present paper is a re-investigation of the problem with improved methods of separation of the phosphate fractions. Whilst the experimental observations of Hummel & Lindberg have been repeated

with similar results, evidence is presented which suggests that their technique fails to separate FAD quantitatively from phosphate, and that FAD does not become radioactive under conditions where ATP shows a high turnover rate.

Materials

A commercial preparation of the sodium salt of ATP (Schwarz Laboratories Inc., New York) was used in most experiments. The distribution of total phosphorus was: ATP 68.4, ADP 20.4, AMP 1.44, inorganic pyrophosphate 4.18, and inorganic orthophosphate 5.58% (methods of Eggleston & Hems (1952) and Krebs & Hems (1953)). [³²P] ATP was prepared by R. Hems (Hems & Bartley, 1953); after dilution with carrier, its activity was 1.7×10^6 counts/min./ml. 0.02M ATP solution. Flavin mononucleotide (FMN), of about 95% purity, was supplied by the Sigma Chemical Co. Flavin-adenine dinucleotide was prepared from baker's yeast or brewer's yeast according to Warburg & Christian (1938) up to the stage of the decomposition of the silver salt with H₂S. After aeration to remove H₂S, the solution containing the mixture of nucleotides (about 100 ml.) was neutralized, transferred to phenol, brought back to water (30 ml.), and run on to a charcoal