PHOSPHATIDES OF HEVEA LATEX

By

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The phosphatides, together with other lipin substances, were extracted from fresh latex in the form believed to be that in which they naturally occur. Analysis, after removal of water soluble impurities, gave evidence that the total lipin contained approximately 51% lecithin 10.5% inositol phosphatide, 3% phosphatidyl ethanolamine, 20% triglycerides and 15.5% unsaponifiables. An enzyme was found to be present in latex, capable of liberating choline from the lecithin component after coagulation by adding to alcohol below 40°C, by adding to formic acid or by freezing, but inactive in untreated latex. The enzyme was destroyed slowly on storing fresh latex and rapidly on adding ammonia or formaldehyde. The implications of these findings on the properties of commercially prepared rubber are discussed. Destruction of the phosphatide during storage of some untreated latices was observed but the effect was insufficiently consistent to account for the universal property of spontaneous coagulation.

The phosphatides in hevea latex, which were first definitely demonstrated by RHODES and BISHOP[1] in 1930, possess two quite dissimilar properties which render them of considerable practical interest. Firstly, they are highly surface active and consequently might be concerned in maintaining the colloidal stability of fresh latex. Secondly, they contain choline which has been shown by ALTMAN[2] to be a vulcanisation accelerator for rubber. The present investigation, which was devoted to obtaining fundamental knowledge about the composition and variations in composition of the phosphatides, has thrown some light on the more practical aspects.

EXTRACTION AND PURIFICATION

The method used by most previous workers for the preparation of latex lipin [1, 3, 4, 5] has consisted in coagulating fresh latex in alcohol at room temperature, removing and evaporating the serum and extracting the aqueous residue with a suitable lipin solvent. Phosphatides were isolated from the total lipin by acetone precipitation. This technique has been found to suffer from the following disadvantages.

Coagulation in alcohol at a temperature below about 40°C leads to a rapid enzymatic loss of choline from the lecithin forming the major constituent of the phosphatides (see later). The product obtained by coagulation at room temperature is therefore modified during the extraction procedure.

The degradation products formed in this way, although they can still be classified as phosphatides, are appreciably soluble in acetone. With such a mixture therefore, acetone precipitation is not a suitable method of separating phosphatides from other lipins.

Latex phosphatides, in common with most other phosphatides, possess the property of rendering water soluble substances soluble in lipin solvents. Only TRISTRAM[3], among previous workers with latex phosphatide, recognised this fact and took special steps to remove these contaminating substances.

In order to prepare latex lipin in the form in which it occurs in nature we coagulated fresh latex* in boiling alcohol, a procedure which destroys the choline liberating enzyme and which also results in more complete extraction of the phosphatides sparingly soluble in cold alcohol. The separated serum was evaporated to leave an aqueous residue and this was extracted with petroleum ether. Water soluble impurities were removed from the extracted lipin by dissolving it in the water poor phase from a mixture of chloroform, ethanol and water (80:20:2.5 parts by volume) and passing this solution through a column of cellulose powder slurried with the same solvent. This technique was first described by BEVAN, GREGORY, MALKIN and POOLE[6] and was believed by them to provide a method of separating phosphatide mixtures into choline containing phosphatides (passing through the column) and ethanolamine and serine containing phosphatides (retained on the column). Our experiments indicated that this separation did not occur, water soluble impurities, not phosphatides, were retained on the column and they were efficiently removed from the phosphatides which passed through. These impurities could be subsequently recovered from the column by washing with alcohol and water. Analysis of the lipin by chemical means and by paper chromatography, before and after passing through the column, and similar analysis of the water soluble substances recovered from the column, showed the impurities to include (*i*) substances containing nitrogen, including the amino acids glycine, α-alanine, valine, leucine (or isoleucine) and serine; (*ii*) substances containing phosphorus; (*iii*) substances containing metals (not soaps); (*iv*) glucose; (*v*) fructose; (*vi*) meso-inositol; (*vii*) quebrachitol; (*viii*) l-inositol.

COMPOSITION OF LATEX LIPIN

Simple fractionation of the purified lipin according to acetone and alcohol solubility did not result in clear cut separation of the various lipin components, but these were concentrated to different degrees in the different fractions.

* Seedling latex from the Experiment Station of the Rubber Research Institute of Malaya was used throughout the work.

TABLE 1 shows analytical results obtained on the total lipin and on the acetone/alcohol fractions obtained therefrom. Paper chromatographic analysis of hydrolysates of the lipin (*cf* RESING[5]) showed that (*i*) the non-choline nitrogen present consisted at least partially of ethanolamine but no serine was present except as water soluble impurity; (*ii*) the reducing sugar consisted of galactose, glucose and an unknown ketose. Partial hydrolysates contained substances in which glycerol was combined with one or more of these sugars or inositol.

The phosphatides likely to be present in the mixture can be put into one of two classes irrespective of the main structure of the molecule, according to the substituent groups combined with the phosphate radical. These are (*i*) nitrogenous phosphatides giving no ash but possessing a N:P atomic ratio of 1:1, and (*ii*) metal phosphatidates containing no nitrogen but giving an ash containing all the phosphorus present (*i.e.* possessing an ash P: total P ratio of 1:1). Thus in a phosphatide mixture containing substances falling into both of these classes and free of nitrogenous or metallic impurities, the N:P atomic ratio and the ash P: total P ratio would give the proportions of phosphatide phosphorus present as nitrogenous phosphatide and metallic phosphatidate respectively. The sum of these two ratios would be unity. The experimentally determined figures for this sum (after correction for a small amount of acetone soluble non-phosphatide nitrogen) for the total purified lipin and fractions obtained therefrom are given in TABLE 1. All are close to unity implying that the lipin is substantially free of nitrogenous or metallic impurities.

It appears probable from the manner in which the various phosphatide constituents distributed themselves in the acetone/alcohol fraction, that most of the phosphatide in the lipin consisted of (*i*) an essentially alcohol soluble lecithin combined with reducing sugar and (*ii*) an essentially alcohol insoluble metal phosphatidate containing inositol and also combined with reducing sugar. In addition the chromatographic detection of ethanolamine implied the presence of phosphatidyl ethanolamine. The non-phosphatide constituents consisted of unsaponifiables and triglycerides (no free fatty acid was present). On the basis of these results and making a number of assumptions, it is possible to calculate the following approximate composition of the total lipin:

Lecithin containing combined reducing sugar 51%,

Metal phosphatidate containing combined inositol and reducing sugar 10.5%,

Phosphatidyl ethanolamine 3%,

Triglycerides 20%,

Unsaponifiables 15.5%.

TABLE I: PROPERTIES OF PURIFIED TOTAL LATEX LIPIN AND OF FRACTIONS OBTAINED THEREFROM ACCORDING TO ACETONE AND ALCOHOL SOLUBILITY

Lipin/Fractions	*Percentage of total lipin*	*Total P %*	*Total N %*	*Ash P %*	*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 %*
Total lipin (total ash 1.71 %)	—	1.82	0.70*	0.33	0.59	0.85	0.72	0.18	1.03	1.30	8.6	55	14
Acetone soluble	25	0.40	0.18*	0.03	0.17	1.02	0.92	0.07	1.09	0.07	4.4	34	51
Acetone insoluble· Alcohol soluble	62	2.20	0.91	0.15	0.88	0.92	0.89	0.07	0.99	0.54	12.2	61	1
Alcohol insoluble	13	2.48	0.43	1.62	0.22	0.38	0.20	0.65	1.03	8.17	11.2	56	10

* These values and the corresponding derived ratios have been corrected for a small amount of non-phosphatide acetone soluble nitrogen found to be present.

CHOLINE LIBERATING ENZYME IN LATEX

When fresh latex was coagulated in alcohol (3 vol) at a temperature higher than about 40°C, it was found that virtually all the choline present in the latex was recovered in a combined form in the phosphatide. At a lower alcohol temperature coagulation was immediately followed by a more or less rapid liberation of choline into the free form.

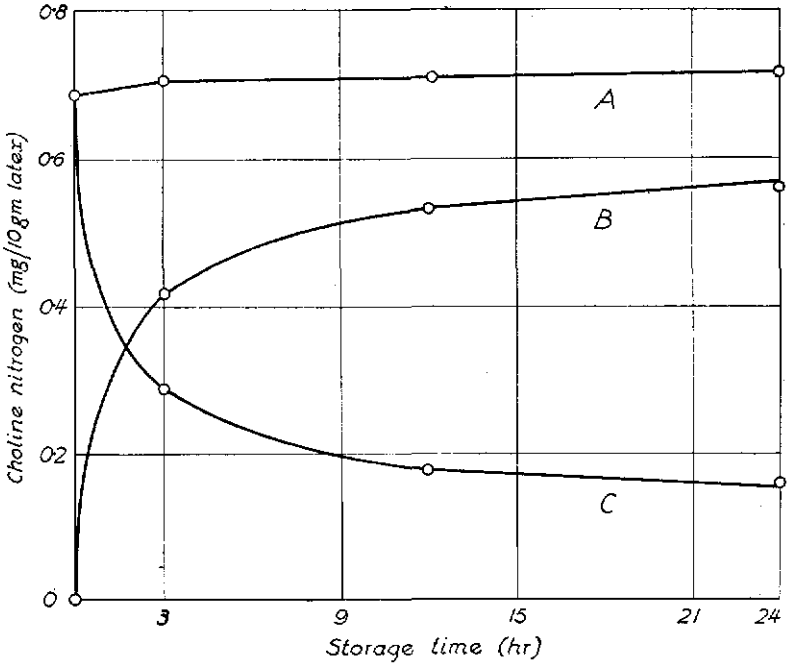

Figure 1. Variations in the distribution of choline after coagulation of fresh latex in 3 vol alcohol at a temperature of 22°C and subsequent storage at this temperature. A — total choline nitrogen. B — free choline nitrogen. C — lipin bound choline nitrogen.

At temperatures between about 5°C and 22°C the liberation rate was optimum or nearly so. *Figure 1* illustrates the change in the distribution of choline on storage of a coagulation mixture prepared by adding fresh latex (tapped directly into a bottle surrounded by freezing mixture, in order to minimise any changes which might occur during the time of tapping, and thawed only just before beginning the experiment) to 3 vol alcohol at 22°C. This breakdown was shown to be due to the action of an enzyme, inactive in untreated latex but activated on alcohol coagulation. The splitting off of choline from the lecithin molecule appeared to be the only change catalysed by the enzyme. Part of the free phosphatidic acid formed in this way combined with metal ions present to form salts. The enzyme was destroyed

in the alcoholic medium above 40°C but was exceptional in being active up to this temperature—most enzymes are rapidly denatured by alcohol at 20°C or lower. The enzyme was also activated by coagulating latex with formic acid at a pH of about 4 or by freeze coagulating. The former treatment led to the liberation of the majority of the choline into a free form within 10 minutes of adding the acid.

The action of the enzyme was studied in more detail by centrifuging latex at a high speed (21,000 r.p.m.). In this way latex was separated into rubber, clear serum and a white precipitate at the bottom of the tube (COOK and SEKHAR[7]). A mixture of the latter two fractions was almost free of rubber and contained little latex phosphatide but it was highly active in liberating choline from added egg lecithin both in solution in 75% alcohol and in aqueous suspension. The reaction in aqueous medium, unlike that in alcohol, was fairly resistant to temperature, some breakdown still occurring at 60°C although the optimum temperature was about 30°C. The optimum pH was about 6.0. Now fresh latex has a pH of about 6.5. (*i.e.* nearly optimum) which probably means that one of three alternatives is operative since the enzyme is inactive in 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 alternative would fit in with the COCKBAIN[8] theory of latex stabilisation in which the rubber particles are considered to be covered by an inner layer of lipin material and an outer layer of protein. The fact, that the destruction of latex colloidal stability by the addition of alcohol, formic acid or by freezing is accompanied by the creation of conditions suitable for the choline liberation reaction to occur, provides some evidence that this view is correct.

The findings described above indicate that when recently tapped fresh latex is coagulated with formic acid in the normal commercial manner (*i.e.* at a pH of about 4.5) conditions favourable to the choline liberating enzyme are created. This was confirmed experimentally and it was found that about 95% of the phosphatide choline was liberated into a free and water soluble form in less than 2 hours after coagulation in this way. It has been found however that the enzyme is progressively destroyed as fresh latex is stored after tapping. When latex kept for 8 hours is coagulated in alcohol there is often a maximum liberation of only about 15% of the choline into a free form (compared with about 75% for recently tapped latex), although the actual amount of enzyme destruction seems to depend upon the degree of bacterial contamination and consequently varies from sample to sample. In an aqueous medium the enzyme is more active than in alcohol and even when it has been destroyed to a marked extent the residual

activity is sufficient to cause considerable choline liberation. *Figure 2* shows the percentage of choline liberated from the phosphatide during a period of 2 hours subsequent to the formic acid coagulation of latex stored for different periods after tapping. If, as in commercial practice, the coagulation mixture was kept for at least 4 hours before the rubber was sheeted, then almost complete liberation of choline was found to occur whatever the prior latex storage period. Thus, although on theoretical grounds it might be expected that increased latex storage would result in less of the phosphatide being degraded after coagulation, in practice the effect is too small to be appreciable. In general it appears that rubber prepared from fresh latex (whatever the time of prior storage of the latex) must contain little choline, since most is removed with the serum in the free and water soluble form.

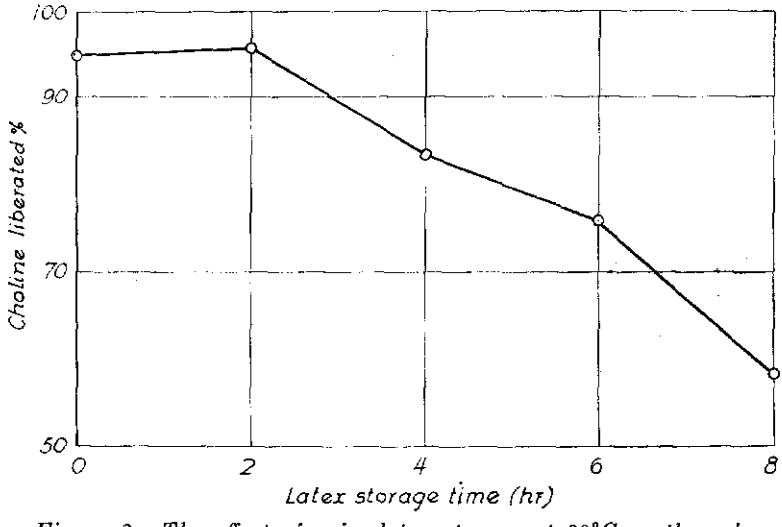

Figure 2. The effect of prior latex storage at 30°C on the subsequent percent liberation of choline from the phosphatide when the latex (5 vol) is coagulated with 1 % (w/v) formic acid (1 vol) and stored for 2 hours at 30°C.

Further factors must be taken into account when preserved latex is considered. When 0.7% ammonia or 0.6% formaldehyde were added to fresh latex, and coagulation with formic acid was carried out immediately in the normal commercial manner, little or no choline was found in a free form in the serum. Similar coagulation of latex ammoniated to 0.2% (a concentration frequently used for temporary preservation) still resulted in considerable choline liberation—in one experiment 84% compared with 95% for fresh latex. When, however, the latex was kept for one day before coagulation, no choline liberation occurred. It appears that the enzyme is destroyed rapidly by 0.7% ammonia and 0.6% formaldehyde and more slowly by 0.2% ammonia.

These results mean that rubber prepared from latex temporarily preserved with these substances is likely to contain the phosphatide more or less undegraded by the choline liberating enzyme and consequently probably contains choline (up to about 0.15% on the rubber) in a combined form. In latex ammoniated to 0.7% and stored for a prolonged period, the phosphatide was slowly destroyed and in two weeks only about 1.5% remained. This destruction, which is quite different from the degradation produced by the choline liberating enzyme, is only one of a variety of reactions occurring in stored ammoniated latex. It indicates that rubber prepared from ammoniated latex which has been stored for about two weeks or longer, must contain little or no phosphatide (or presumably choline).

It is impossible to say at present whether variations in the amount of phosphatide, or in the choline content of the phosphatide, in rubber prepared under different conditions affect the properties of the rubber. It has been shown in this laboratory that choline chloride exerts a marked activating influence on the rate of cure when added to a standard M.B.T. mix in an amount equivalent to about 0.15% choline on the rubber. It is not known, however, whether choline combined in the phosphatide would have a similar effect. So many other factors are likely to be involved that any effect due to choline differences may well be completely masked.

PHOSPHATIDE CHANGES ON STORING FRESH LATEX

Latex in the tree is a complex colloidal system in which the rubber particles are considered by some authorities (Cockbain[8]) to be stabilised by an interfacial coating comprising an inner layer of lipin material and an outer layer of protein. Anyway, 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. It has been previously mentioned that the choline liberating enzyme is inactive in untreated latex so that this cannot be responsible for changes leading to spontaneous coagulation. The possibility of phosphatide destruction from other causes was examined using a number of latex samples from different sources. The total phosphatide phosphorus in these latices was determined immediately after tapping (the latex having been collected into a vessel surrounded by freezing

mixture) and after various periods of storage at room temperature. The former determinations gave values of about 0.015 to 0.022% for different latices (corresponding to about 0.5 to 0.8% phosphatide). In some latices examined this value fell after 8 hours storage by as much as 20%. 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[9]. However, in other latices examined no such breakdown was observed on storage. In general, all latex samples collected under non-sterile conditions undergo spontaneous coagulation and this was so in our experiments, 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, although it may contribute towards it in special cases.

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The majority of the work outlined in this paper has been described in greater detail in a series of three papers submitted to the *Biochemical Journal*, published by the Cambridge University Press. At the time of writing, the first of these papers is in press.