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Rubber Coagulation by Enzymes of Hevea brasiliensis Latex

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It has been shown that latex contains an enzyme system which can coagulate rubber dispersions under certain conditions in vitro. *The enzymic activity is found in the liquid phase of the latex (C-serum). It is distinct from phospolipase-D which is also located in C-serum and is not essential for rubber coagulation. These findings are discussed in relationship with the hypotheses that spontaneous and acid coagulation of latex are partly mediated by endogenous enzymes and that the same enzymic activity may contribute to the formation of plugs of coagulum in latex vessels* in vivo.

Hevea latex as obtained by tapping is generally accepted as dilute cytoplasm from the latex vessels (SOUTHORN, 1961). In keeping with this view, latex contains in addition to sub-cellular particulate structures, a variety of enzymes. A few examples are the much-studied enzymes of rubber biosynthesis (LYNEN, 1969), the enzymes of glycolysis (BEALING, 1969; D'AUZAC AND JACOB, 1969; TUPY AND RESING, 1969; MOIR, 1969) and the 'coagulase' which is the topic of interest in the present paper.

Coagulation of rubber particles in suspension is a matter in which the physico-chemical forces between the particles and the stabilising properties of the envelope coating the rubber particles are both of importance. All workers are agreed on the physico-chemical reduction in stability brought about by acidification or the addition of cations. There are still questions about the relative importance of bacteria and native enzymes in bringing about spontaneous coagulation and about the possible role of enzymes in acid coagulation.

DE VRIES (1920) in his book quoted the results of classical experiments which showed that a drop of fresh latex has coagulating effects on a heated stable latex. He discussed the possibility that an enzyme might be important in the commercial acid coagulation of latex and the name 'coagulase' was introduced for this enzyme. MCMULLEN (1951) was interested in spontaneous coagulation and his investigations suggested that enzymes found in latex were the coagulating agents after they had been activated by calcium ions or a calcium-protein complex released from the lutoids. SMITH (1953) reported the finding of a choline-releasing enzyme (phospholipase-D) in latex, but concluded that 'it does not (seem to) play any part in the processes leading to the spontaneous coagulation of latex.' A later report (RUBBER RESEARCH INSTITUTE OF MALAYA, 1966) implicated phospholipase-D in coagulation, but detailed evidence was not presented. PUJARNISCLE (1968) studied the hydrolases of the lutoids of latex, but phospholipase-D was not included in his study. SOUTHORN AND EDWIN (1968) have shown that there is a rubber-flocculating system involving the B-serum proteins, but this appears to work electrostatically rather than enzymically. Moreover, the flocculation studied by SOUTHORN (1961) does not amount to mass coagulation, which probably requires extensive rubber-to-rubber contact between particles.

The present work deals with the enzymic coagulation of rubber at pH 4.5 and the evidence for the hypothetical 'coagulase.' The possible role of 'coagulase' *in vivo*

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and in spontaneous and acid coagulation is discussed. Phospholipase-D was found to be located in C-serum, and not in B-serum as earlier suggested (RUBBER RESEARCH INSTITUTE OF MALAYA, 1966); it was also found to be distinct from 'coagulase' and inessential for coagulation.

MATERIALS AND METHODS

Latex

Latex obtained by tapping was collected in flasks chilled in melting ice. The tapping cut was rinsed with a 5% alcohol solution and the first 10 ml of latex was discarded. Latex was collected for the next half an hour. Large volumes of latex were obtained by pooling the collections from several trees of the same clone. Most of the latex used was taken from clone RRIM 600 growing in the R.R.I.M. Experiment Station, Sungei Buloh, although latex of other clones was occasionally examined.

Latex Serum

Fresh latex samples were centrifuged in a refrigerated centrifuge (Measuring and Scientific Equipment Ltd, England) for 30 min at 2500 rev [1400 *g* (max)] and 5°C to give a two-layer separation. The lighter 'white' fraction was used for preparing rubber dispersions (see below). A three-layer separation discovered by COOK AND SEKHAR (1953) and improved by MOIR (1959) was obtained by centrifuging fresh latex samples in rotor 21 of a Spinco Model L centrifuge, for 60 min at 20 000 rev [53 620 *g* (max)] and 0 – 5°C. The liquid middle layer is known as 'C-serum' (centrifuge serum). The 'bottom fraction,' consisting mainly of lutoids, was treated by repeated freezing and thawing followed by high speed centrifugation to give a second serum, known as 'B-serum' [(*i.e.* 'bottom fraction serum') (HSIA, 1958)].

'Coagulase' Assay

A rubber dispersion of 9% d.r.c. was prepared by modifying the method of SPENCE (1938). The white layer obtained by centrifuging latex at 1400 *g* max was diluted with water and heated at 70°C for 15 minutes. It was then filtered through muslin to remove flocs, if any. The heated rubber dispersion was adjusted to pH 4.5. $CaCl_2$ (0.25 ml of 0.1 M) was added to 4 ml portions of the dispersion to give a final amount of 25 μ moles of calcium ions per tube. This mixture was stable for at least one week at room temperature (ca 24°C), since — as will appear — the enzyme protein had been destroyed by the heat treatment.

Assays were started with the addition of latex sera to the basic mixture. The tubes were examined visually for signs of coagulation (*i.e.* thickening). Usually a rubber coagulum could be seen to form immediately following thickening. The time for the coagulum formation was recorded and this was the basis of the test. In some of the experiments an alternative check on the assay was used. This was accomplished by weighing the wet coagulum at a predetermined time after the start of the incubation and calculating this weight as a percentage of the total weight of coagulable rubber in the original mixture. Reproducibility of both methods of assay was good if they were done on the same preparation of rubber and on the same day.

Phospholipase-D Assay

The assay was based on a well-known procedure (DAVIDSON AND LONG, 1958) with the difference that all measurements were made with an ethanol solution of ovolecithin (BDH Chemicals Ltd, England). Cleavage of the terminal phosphate ester bond of the phosphoglyceride with the formation of phosphatidic acid was started by the addition of 1 ml of the latex serum under study to

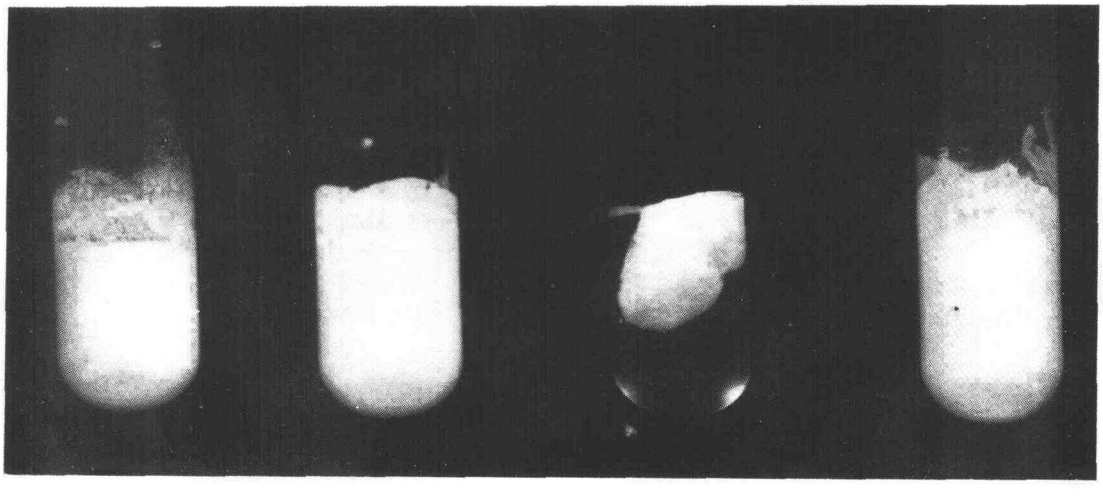
1 ml of solution of ovolecithin containing 20 μ moles of choline. This mixture was supplemented with 0.25 ml of a 1 M $CaCl_2$ solution. After incubating the mixture at 30°C for 20 min, 1 ml of a 30% solution of trichloroacetic acid was added. The precipitated protein was filtered off and the supernatant was titrated with dilute alkali to a phenolphthalein end-point (pH 8.3 – 10).

Free choline was then estimated in the supernatant by the Glick method (GLICK, 1944). The choline was specifically precipitated by ammonium reineckate in ethanol. The choline reineckate complex was allowed to crystallise in the cold overnight. The crystals were washed with n-propanol and finally dissolved in acetone. Optical absorption at 526 nm was measured with a Bausch and Lomb Spectronic 20. Results were calculated by comparison with standard solutions containing known concentrations of choline chloride.

RESULTS

Rubber Coagulation by Latex C-serum

The role of an enzyme system in rubber coagulation was demonstrated in experiments like that illustrated in *Figure 1.* The four tubes shown in the figure were photographed 18 h after the addition of the substances indicated under each tube. In the tube (third from the left) which contained fresh C-serum and calcium ions, the rubber coagulum was visible within 10 minutes. No rubber coagulum was observed in the other three tubes after as long as 18 hours. These three tubes were treated as indicated in *Figure 1;* the control (first tube on the left) contained, in addition to rubber, 1 ml of water and 0.25 ml of a 0.1 M $CaCl_2$. The second tube from the left contained C-serum but without calcium ions and the fourth tube



Control without C-serum | C-serum without Ca^{++} | C-serum + Ca^{++} | Boiled C-serum + Ca^{++}

Figure 1. Coagulation of heated rubber dispersion by C-serum in the presence of calcium. Each tube contained 4 ml of heated rubber dispersion (9% d.r.c; pH 4.5) with the additions indicated. Tubes were photographed 18 h after mixing.

contained boiled C-serum and calcium ions (concentration as above).

It can be concluded that C-serum contains a component which, in the presence of calcium ions, brings about rapid coagulation of heated rubber particles at pH 4.5, and that this component is rendered inactive if the C-serum is boiled, or if calcium ions are not added to the mixture. This is considered to confirm the presence of an enzyme system corresponding to the 'coagulase' discussed by DE VRIES (1920). The above experiment shows for the first time that 'coagulase' is located in C-serum and requires the presence of calcium ions for it to be active.

Supporting evidence for the role of 'coagulase' in rubber coagulation is found in the results of *Table 1*. C-serum prepared in the usual way was subjected to a second centrifugation at 40 000 rev [144 000 *g* (max)] for 120 min at 0 – 5°C in rotor 40 of the Spinco Model L ultracentrifuge, and this preparation was used as the source of 'coagulase.' The test consisted of adding two levels of C-serum (twice centrifuged), namely 1 ml and 0.1 ml, to each of ten tubes of rubber dispersion. The time taken for the rubber to coagulate was noted. All twenty tubes showed coagulation, and the agreement with respect to time of coagulation within each of the two groups was reasonably good. However, the time of coagulation appeared to show an inverse relationship to the amount of C-serum. This observation cannot be explained at present: one possibility is that 'coagulase' is accompanied in C-serum by inhibitors.

TABLE 1. VALIDITY OF THE COAGULASE EFFECT

Samples replicated[a]	Coagulation time (min)	
	1 ml twice centrifuged C-serum	0.1 ml twice centrifuged C-serum
1	27	20
2	30	23
3	32	27
4	34	29
5	27	21
6	32	25
7	33	28
8	32	27
9	29	21
10	34	29

[a] Each sample contained 4 ml of heated rubber dispersion (9% d.r.c; pH 4.5), 0.25 ml of 0.1 M $CaCl_2$ and 0.1 or 1 ml of serum.

Location of 'Coagulase' Among Latex Phases

All the 'coagulase' assays just described were conducted with C-serum and it therefore seemed that C-serum was the site of the enzyme activity. It was of interest, however, to relate this to the findings of others (SOUTHORN AND EDWIN, 1968) that B-serum contains a destabilising system which is released into the aqueous medium of latex when lutoids containing the B-serum disrupt. SOUTHORN AND YIP (1968) considered that this destabilisation was electrostatic rather than enzymic. The results in *Table 2* show again that an enzymic destabilising system, 'coagulase,' exists in C-serum. Practically all the rubber in the test system had coagulated within 20 min in the presence of dialysed C-serum at pH 4.5, while, under similar conditions, less than 2% of the rubber had coagulated in the presence of dialysed B-serum. The latter value was in agreement with that of the control test which contained water instead of either of the two types of sera. It follows that B-serum is essentially devoid of 'coagulase,' as defined in this paper, and hence that the activity in C-serum originates in C-serum and

TABLE 2. EFFECT OF DIALYSED C- AND B-SERA ON HEATED RUBBER DISPERSIONS [a]

Incubation time (min)	Coagulation (%)		
	Control	C-serum	B-serum
10	1.2	55.4	1.5
20	1.0	94.1	1.4
30	1.8	95.2	5.5

[a] Assay conditions as given in *Table 1*. 1 ml of C- or B-serum was used in each tube. Control tube contained 1 ml of H_2O.

cannot be an artefact of leakage of enzyme from bottom fraction.

Additional evidence that the enzyme activity of C-serum is not an artefact of leakage from bottom fraction is found in the results of *Table 3*. Latex was collected at 5-min intervals after tapping. C- and B-sera were then prepared from each flow fraction numbered 1 to 6. The 'coagulase' activities of the two sera from each fraction were compared. If the 'coagulase' originated in bottom fraction, its activity could be expected to be greatest during initial flow and least towards the end of flow. This follows from the 'dilution reaction' (FREY-WYSSLING, 1952) and its effect on lutoids (PAKIANATHAN *et al.*, 1966) which results in lutoid damage being greatest in the initial latex flow. As anticipated, the 'coagulase' activity index corresponding to six flow fractions *(Table 3)* failed to show any marked differences; furthermore, B-serum fractions were virtually devoid of 'coagulase' activity. These results were interpreted as additional proof that 'coagulase' was non-particulate in origin.

Effect of Calcium Ions

The fact that calcium ions were required for the C-serum to act is shown by the data of *Figure 1* and is also clear in *Table 4*. The tubes which contained the full admixture including C-serum and calcium ions gave 100% coagulation within 30 minutes. Without the addition of C-serum, calcium ions alone were incapable of causing the rubber to coagulate. The requirement for calcium ions for activation of the 'coagulase' was a further observation in support of the

TABLE 3. EFFECT OF C- AND B-SERA FROM SUCCESSIVE FLOW FRACTIONS ON RUBBER COAGULATION [a]

Latex flow fraction	Coagulation time (min)	
	C-serum	B-serum
1	9	–
2	15	94
3	14	92
4	8	97
5	8	96
6	13	87

[a] Assay conditions as given in *Table 1*. 1 ml of C- or B-serum was used in each tube.

TABLE 4. EFFECT OF CALCIUM CHLORIDE ON THE COAGULATION OF HEATED RUBBER DISPERSIONS BY C-SERUM [a]

moles of $CaCl_2$ per incubation	Coagulation at 30 min (%)	
	C-serum	Control
0	0	0
20	5.1	1.7
40	38.6	1.2
80	97.6	1.8
160	100.2	2.3
240	102.8	3.5

[a] Assay conditions as given in *Table 1* except for the amounts of $CaCl_2$.

conclusion that 'coagulase' has the characteristics of an enzyme.

The Nature of 'Coagulase'

There have been suggestions (RUBBER RESEARCH INSTITUTE OF MALAYA, 1966) that a choline-releasing enzyme may play a role in rubber coagulation. This has been generally supposed to be present in the readily sedimentable fraction of latex, possibly in B-serum. The results described above show that 'coagulase' activity does not reside in B-serum. However, it was also found that the major choline-releasing activity occurs in C-serum rather than B-serum *(Figure 2)* so that the possibility was now considered that 'coagulase' activity might be accounted for by a choline-releasing enzyme.

The problem was resolved by fractionation of the enzyme activity in C-serum using ethanol. Two volumes of ethanol were added to one volume of C-serum resulting in a precipitate. The precipitate was extracted repeatedly with water to give three aqueous fractions. Each fraction was assayed for

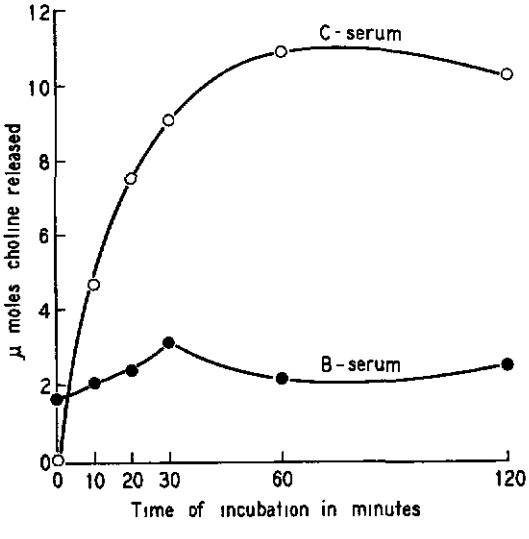


Figure 2. Choline-releasing activity of C- and B-sera with ovolecithin as substrate (20 μ moles). Assays were done as described in the text with 1 ml of serum.

TABLE 5. ALCOHOL-PRECIPITATED FRACTION OF C-SERUM SHOWING 'COAGULASE' WITHOUT CHOLINE-RELEASING ACTIVITY

Successive aqueous extracts of alcohol precipitate	Choline release [a]	Coagulation time (min)[b]	
		Not heated	Heated
1	0	31	NC
2	0	11	NC
3	0	15	NC

[a]Measured with evolecithin (20 μ moles) as substrate, as described in the text. 1 ml of aqueous extract was used in the assay.

[b]Aqueous extract was tested before and after heating. Assay conditions as given in *Table 1*. 1 ml of extract was used in the assay.

NC: Not coagulated at 100 minutes.

coagulase and choline-releasing activity. The results in *Table 5* show conclusively that rubber was coagulated enzymically in the absence of choline-releasing activity. The possibility of destabilising effects of a choline-releasing enzyme is not excluded by this experiment, but it does show that other destabilising enzymes ('coagulase') are present which are not choline-releasing. That the coagulation was enzymic was again confirmed by the results appearing in the last column *(Table 5)* which showed that heat destroyed the 'coagulase.'

'Coagulase' Assay with Commercial Enzymes

In order to get an idea of the chemical mechanism of 'coagulase' activity, a number of likely commercial enzymes were tested to see whether they would reproduce the effects of 'coagulase.' Results on the activity of the seven selected enzymes are given in *Table 6*. Each test contained about 5 mg of enzyme preparation. The assay was conducted as described above. The optimum pH for the action of these enzymes may not be that which was employed, but for the purpose of this experiment they were assayed at the same pH as that in the assay for

TABLE 6. 'COAGULASE' ACTIVITY OF SOME COMMERCIAL ENZYMES [a]

Enzyme	Source	Coagulation time (min)
Lipase	Wheat germ; Worthington Biochemical Corporation	75
Lysozyme	Nutritional Biochemicals Corporation	NC
Papain	B.D.H. Chemicals Ltd.	55
Pectinase	Nutritional Biochemicals Corporation	240
Proteinase (Subtilisin)	*Bacillus subtilis;* Koch-Light Laboratories Ltd.	15
Trypsin	Worthington Biochemical Corporation	25
Chymotrypsin	Sigma Chemical Company	85

[a]Assay conditions as given in *Table 1* at an enzyme concentration of 5 mg per millilitre.
NC: Not coagulated.

latex 'coagulase,' namely pH 4.5. *Table 4* shows that the proteinase, subtilisin, from *Bacillus subtilis* is relatively the most active in rubber coagulation and its reactivity is comparable to that of C-serum 'coagulase.' Trypsin is next in order of activity followed by one other proteolytic enzyme, papain; chymotrypsin is unexpectedly less active. Lipase shows a little activity, but this may be due to proteinase impurities. Pectinase is relatively unreactive and lysozyme is completely inactive. These results suggest that C-serum 'coagulase' activity is probably proteolytic in nature.

DISCUSSION

The present study was centred on testing the theory that enzymes play an important role in latex coagulation. In order to study this, it was necessary to denature the enzymes in the latex and at the same time retain the dispersion of rubber particles. This was accomplished by first centrifuging off the easily sedimentable particles, diluting the white fraction and finally heating it. This procedure gave a dispersion of rubber particles, which would also contain the denatured enzymes and other proteins. The mixture when adjusted to pH 4.5 and supplemented with calcium ions remained stable. The zeta potential of this mixture was not measured, but judging from the results of SOUTHORN AND YIP (1968) it is reasonable to suppose that the zeta potential would be near zero and in spite of this the mixture had not coagulated. When as little as 0.1 ml or 1 ml of fresh C-serum was added to 4 ml of the mixture *(Table 1)*, it was rapidly converted into an irreversible coagulum. In the absence of added calcium ions or if boiled C-serum was added instead, the mixture remained stable and uncoagulated. Thus, what has been observed is an enzymic process of rubber coagulation *in vitro*.

A series of papers by Southorn and co-workers deals with the problems of latex flow and the mechanism of cessation. Earlier theories suggesting that cessation of latex flow is caused by the formation of plugs of bacteria, Frey-Wyssling particles or lutoids have been proved by these authors to be untenable. Evidence for the presence of multiple plugs of rubber coagulum at various sites in the latex vessels (SOUTHORN, 1968) raised the question of how these are formed. In this connection SOUTHORN AND EDWIN (1968) investigated the role of lutoids and

the release of their contents. They demonstrated an electrostatic interaction between B-serum and rubber particles which induced flocculation *in latex in vitro*. The flocs would be capable of blocking latex vessels. SOUTHORN AND YIP (1968) speculated on the possibility that this might be supplemented by other mechanisms then unknown in which enzymes might play a part. SOUTHORN AND EDWIN (1968) noted that C-serum, which promotes colloid stability at pH 6.7 and above, acquires destabilising activity when acidified to pH 5.5. SOUTHORN AND YIP (1968) remarked that electrostatic destabilisation brings about flocculation but not necessarily coagulation, so that the commercial method of coagulating latex by acidification may involve additional factors, such as the acid activation of lytic enzymes in latex. Present results support the enzymic theory, but the enzyme activity has been observed in C-serum instead of arising from lutoids. The idea that rubber coagulation as observed *in vitro* may also operate in (1) the latex vessels and (2) acid or spontaneous coagulation is discussed next.

For the purpose of discussing ways by which the observed rubber plugs are formed, it is worthwhile to relate C-serum, obtained by ultracentrifugation of latex, to latex vessel cytology. It is in fact the liquid phase of the latex vessels, and therefore 'coagulase' is a soluble non-particulate enzyme system freely in contact with the rubber particles. Tapping or wounding the tree sets in motion the several conditions favourable for coagulation. It is reasonable to suppose that a small proportion of the lutoids near the wound undergo shearing effects and osmotic shock (SOUTHORN AND EDWIN, 1968); YIP AND SOUTHORN, 1968). These lutoids either burst open or become leaky giving up their acidic contents containing a high concentration of calcium ions (RUBBER RESEARCH INSTITUTE OF MALAYA, 1966). Thus, it may be speculated that 'coagulase' activity observed on rubber particles under certain conditions *in vitro* may, also in the vicinity of the disrupted lutoids, be operative in the formation of rubber plugs. To test this further work would be necessary, since 'coagulase' as defined in this paper is active against heated rubber particles and its effects on native, unheated rubber particles are not known.

It may be further postulated that 'coagulase' is also operative in acid coagulation and in spontaneous coagulation of latex. These phenomena have been known for a long time, but the mechanism of coagulation is still in doubt. It is reasonable to expect that 'coagulase' might play a role in such coagulation. However, since the 'coagulase' effect was observed under different conditions, with heated rubber dispersions, further experimental work on this is necessary.

Finally it may be asked: what is the mechanism of 'coagulase' action? The most logical primary site for the 'coagulase' to react with would be the protein layer of the envelope surrounding the rubber particles. If hydrolysis took place rubber surfaces could be exposed, and contact between such surfaces would result in coagulation. The proteinase subtilisin, an endopeptidase, which according to HILL (1965) degrades protein more extensively than, say, trypsin or chymotrypsin, has been shown to be as effective as 'coagulase.' Trypsin and papain are also capable of coagulating rubber although less efficiently. Thus, it may be postulated that 'coagulase' operates as a proteolytic enzyme in the hydrolysis of the protein layer surrounding the rubber particles.

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