The Biosynthesis of Rubber from β -Hydroxy- β -methylglutaryl-Coenzyme A in *Hevea brasiliensis* Latex

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(Received 20 February 1969)

1. β -Hydroxy- β -methyl[3- 14 C]glutaryl-CoA is efficiently incorporated into rubber on incubation with *Hevea brasiliensis* latex, and the incorporation is diminished in the presence of unlabelled mevalonate. β -Hydroxy- β -methylglutaric acid is not utilized for rubber synthesis, but inhibits the formation of rubber from β -hydroxy- β -methylglutaryl-CoA. 2. The incorporation of β -hydroxy- β -methylglutaryl-CoA into rubber is stimulated equally by NADP⁺ and NADPH and less so by NAD⁺ and NADH. ATP is slightly stimulatory and CoA is inhibitory. 3. β -Hydroxy- β -methylglutaryl-CoA reductase is concentrated in the sediment (bottom fraction) formed by centrifuging latex at low speed and the enzyme is unstable in the absence of cysteine or GSH. The formation of NADPH takes place in the latex serum. 4. There is a marked seasonal variation in the extent of β -hydroxy- β -methylglutaryl-CoA incorporation into rubber in latex, but mevalonate incorporation is relatively constant. This observation, together with the finding that β -hydroxy- β -methylglutaryl-CoA reduction is the rate-limiting step in the formation of rubber from β -hydroxy- β -methylglutaryl-CoA, suggests that the conversion of β -hydroxy- β -methylglutaryl-CoA into mevalonate is of importance in the regulation of rubber synthesis. 5. Evidence suggesting that β -hydroxy- β -methylglutaryl-CoA lyase is present in *H. brasiliensis* latex has been obtained.

Hevea brasiliensis latex is a cytoplasmic system (Dickenson, 1964) that contains, besides rubber and the usual cell organelles, two characteristic bodies, the luteoid particle (Homans & van Gils, 1948) and the Frey-Wyssling complex (Dickenson, 1964). Centrifugation at low speed produces a sediment (bottom fraction), containing the luteoid particles and some Frey-Wyssling particles, and a supernatant (white fraction), containing most of the rubber. The role of these fractions in the biosynthesis of rubber from mevalonate is now reasonably well understood (Archer & Audley, 1967), but their effect on the formation of mevalonate itself has not been investigated. In animals and yeast, it is well established that mevalonate is formed by the reduction of HMG-CoA[†] (Knappe, Ringelmann & Lynen, 1959; Durr & Rudney, 1960; Linn, 1967a) and that this reaction is important in the regulation of sterol synthesis (Bucher, Overath & Lynen, 1960; Siperstein & Fagan, 1964; Linn, 1967b). There is, however, no direct experimental evidence that HMG-CoA is a precursor of rubber or any other isoprenoid synthesized by higher plants. The

purpose of this paper is to present results on the effect of white fraction and bottom fraction on the formation of rubber from HMG-CoA, and to consider the possible significance of reduction of HMG-CoA in the control of rubber biosynthesis.

MATERIALS AND METHODS

β -Hydroxy- β -methyl[3- 14 C]glutaric acid. This was obtained from the New England Nuclear Corp., Boston, Mass., U.S.A., and diluted with unlabelled β -hydroxy- β -methylglutaric acid before conversion into the anhydride.

[3- 14 C]HMG-CoA. This was synthesized by the method of Hilz, Knappe, Ringelmann & Lynen (1958) by using twice-crystallized β -hydroxy- β -methyl[3- 14 C]glutaric anhydride with a specific radioactivity of 0.347 μ C/ μ mole. The HMG-CoA was purified by ascending chromatography on Whatman no. 1 paper with ethanol-acetic acid (1:1, v/v), pH 4. In this solvent, HMG-CoA was separated from CoA disulphide and β -hydroxy- β -methylglutaric acid, but not from CoA. The HMG-CoA, which usually contained a small amount of CoA, was eluted with water, freeze-dried and stored in a desiccator at -20°. Solutions of HMG-CoA were adjusted to pH 5.5 with HCl and stored frozen at -20°. Under these conditions, Hilz *et al.* (1958) found that HMG-CoA decomposed to an extent of only 5% in 3 months. In the present work, labelled HMG-CoA was freshly prepared every 2 months. The average yield of HMG-CoA (based on CoA) was 57%.

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† Abbreviation: HMG-CoA, β -hydroxy- β -methylglutaryl-CoA.

Mevalonic acid. [2-¹⁴C]Mevalonolactone (specific radioactivity 3.6 $\mu\text{C}/\mu\text{mole}$) was obtained from The Radiochemical Centre, Amersham, Bucks. It was diluted with unlabelled lactone [Sigma (London) Chemical Co. Ltd., London S.W.6] to a specific radioactivity of 0.347 $\mu\text{C}/\mu\text{mole}$ and converted into sodium mevalonate by heating at 70° for 30 min. at pH 9. The *NN'*-dibenzylethylenediammonium salt of mevalonic acid was purchased from Anderman and Co. Ltd., London S.E.1.

Potassium [3-¹⁴C]acetoacetate. This was prepared by the method of Davies (1943) from labelled ethyl acetoacetate (The Radiochemical Centre) and had a specific radioactivity of 5.8 $\mu\text{C}/\mu\text{mole}$.

Other chemicals. ATP, NADP⁺, NADPH, NAD⁺, NADH and β -hydroxy- β -methylglutaric acid were obtained from the Sigma (London) Chemical Co. Ltd., CoA was from the Boehringer Corp. (London) Ltd., London W.5, and cysteine and GSH were from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Silica gel G was purchased from Anderman and Co. Ltd., and Hyflo Supercel from Johns-Manville Ltd., London S.E.1.

Fractionation of latex. Fresh *Hevea brasiliensis* latex was obtained by pricking the trunks of 7–8-year-old seedlings growing in the tropical greenhouse attached to these laboratories. Usually 2 vol. of latex was collected into 1 vol. of ice-cold 0.11 M-tris-HCl buffer, pH 7.2, containing 0.56 M-mannitol (tris-mannitol buffer), but in some experiments 0.05 M-sodium phosphate buffer, pH 7.0, containing 0.47 M-mannitol (phosphate-mannitol buffer) was used. The latex was separated into white fraction and bottom fraction by centrifuging at 600g for 3 min. at 4° and a small amount of residual bottom fraction removed from the white fraction by recentrifuging at 1750g for 5 min.

Preparation of lysed bottom fraction and washed bottom fraction. Bottom-fraction lysate was prepared by adjusting the volume of the unwashed bottom fraction to the original volume of the undiluted latex with either ice-cold 0.11 M-tris-HCl buffer, pH 7.2 (tris-HCl buffer), or 0.05 M-sodium phosphate buffer, pH 7.0, and then freezing and thawing once. 'Double-strength' lysate was prepared by adjusting the final volume of the suspension to half the volume of the undiluted latex.

Washed bottom fraction was prepared by stirring bottom fraction with an equal volume of ice-cold phosphate-mannitol buffer, and centrifuging at 600g for 3 min. at 4°.

Preparation of serum and bottom-fraction extract. These were prepared by centrifuging white fraction and bottom-fraction lysate, respectively, at 175000g_{max} for 2 hr. at 0° in a Spinco model L ultracentrifuge.

Incubations. Duplicate incubations were performed at 30 ± 1°. The incorporations reported are the mean values for each pair of incubations. Individual results usually did not differ from the mean by more than 10%. Incorporations were calculated on the assumption that the amount of endogenous substrate was negligible.

Radioactivity assay. Radioactivity was measured by standard procedures in a Tracerlab Corumatic scintillation counter with an external standard for determination of counter efficiency. The phosphor used was 5-(4-biphenyl)-2-(4-*tert*-butylphenyl)-1-oxa-3,4-diazole (Ciba Ltd., Duxford, Cambs.) in toluene at a final concentration of 0.6% (w/v). Radioactivity is usually expressed as d.p.s. (disintegrations/sec.).

Purification of radioactive rubber. Labelled rubber was

isolated and purified by the method of Archer, Audley, Cockbain & McSweeney (1963). The resulting coagulum was dissolved in 5 ml. of 0.4% (w/v) trichloroacetic acid in toluene by stirring at 65° and the solution quantitatively transferred to a scintillation vial for counting.

Ozonolysis of labelled rubber. Samples of purified rubber (total weight approx. 200 mg.) were dried to constant weight *in vacuo* at 50° and their radioactivity was measured. Immediately after the radioactivity measurement, the rubber was precipitated from the pooled phosphor solutions by the slow addition of methanol to a final concentration of 60% (v/v) and the suspension chilled for 2–3 days at 4°. After being washed twice with methanol, the rubber was extracted with boiling ethanol overnight to remove any residual phosphor, dried to constant weight and its specific radioactivity was again determined. Ozonolysis was carried out by the method of McSweeney (1968), and the resulting laevulinic acid isolated as the crystalline 2,4-dinitrophenylhydrazone (yield, 80% of theoretical). This was subjected to combustion by the oxygen-flask method (Schöniger, 1955); the labelled CO₂ was absorbed in a solution of freshly distilled ethanolamine in 2-methoxyethanol (1:8, v/v) (Jeffay & Alvarez, 1961) and the radioactivities of samples of the solution were counted.

Determination of radioactive mevalonate. The isolation and purification of mevalonate was based on the method of Linn (1967a). After incubation, 3 mg. of carrier sodium mevalonate was added to the latex followed immediately by 4 vol. of ethanol. The mixture was evaporated to dryness and the residue extracted twice with 0.5 ml. of 0.1 M-KOH and twice with 0.25 ml. of hot water. The extracts were pooled, acidified with conc. HCl, saturated with NaCl and extracted with 4 vol. of ether eight times. The ether was removed and the residue chromatographed on a 600 μm . layer of Hyflo Supercel-CaSO₄ mixture (3:1, w/w) with benzene as developing solvent. The mevalonolactone, which ran at the solvent front, was eluted with acetone and chromatographed in ethanol on a 250 μm . layer of silica gel G. The position of the mevalonolactone was located by radioautography and the area containing the radioactive spot scraped into a scintillation vial containing 3 ml. of ethanol. After 30 min., 12 ml. of phosphor solution was added, the suspension allowed to clear and the sample assayed. The overall recovery of mevalonate was between 55 and 65%.

Detection of HMG-CoA lyase in latex fractions. [3-¹⁴C]-HMG-CoA and latex serum or bottom-fraction lysate were placed in separate areas of the outer well of a Conway unit. The inner well contained two 13 mm.-diam. Whatman GF/A glass paper discs, each carrying 20 μl . of 0.93 M-semicarbazide hydrochloride in 0.9 M-sodium acetate. The lid was sealed on with silicone grease and the contents of the outer well were well mixed by gentle tipping. After incubation, for 3 hr. at 30°, 60 μl . of 0.35 M-H₂SO₄ and 0.5 ml. of 3.2 M-aniline hydrochloride in 0.2 M-sodium acetate buffer, pH 5.0, were rapidly added to the outer well to convert any acetoacetate formed into acetone (Barkulis & Lehninger, 1951). The lid was replaced and the unit left at room temperature overnight. The glass-paper discs were then dried at 65° for 15 min. and each pair of discs was placed in a vial with 15 ml. of phosphor solution and their radioactivity was counted (Pinter, Hamilton & Miller, 1963). Alongside each set of incubations, standards were run with [3-¹⁴C]acetoacetate to determine the overall

recovery of acetone, which was between 35 and 45%. This method gives an indication of the maximum activity of HMG-CoA lyase since any radioactive volatile carbonyl material formed from HMG-CoA would be included in the assay.

Spectrophotometry. Extinction values were measured with a Hitachi-Perkin-Elmer 139 spectrophotometer.

RESULTS

Incorporation of HMG-CoA into rubber. Fig. 1 shows that labelled rubber is formed on incubating [$3\text{-}^{14}\text{C}$]HMG-CoA with latex and cofactors and that incorporation is maximal at 2 hr. In another experiment the optimum concentration for the incorporation of *S*-HMG-CoA was found to be about 0.12 mM.

Purity of labelled rubber. Samples of rubber isolated from incubations of latex with [$3\text{-}^{14}\text{C}$]-

HMG-CoA and cofactors were precipitated, to remove any radioactive low-molecular-weight material that might have been present, and then degraded to laevulinic acid. The results of these experiments established that the rubber was radiochemically pure (Table 1).

Effect of pH. To study the effect of pH on the formation of rubber from HMG-CoA, latex was collected undiluted and separated into white fraction and bottom fraction as described above. The white fraction was then diluted with half its own volume of water and double-strength bottom-fraction lysate was prepared by adding water to the bottom fraction, followed by freezing and thawing. 0.3 M-sodium phosphate and 0.3 M-tris-hydrochloric

Fig. 1. Incorporation of HMG-CoA into rubber. Each incubation mixture contained 0.15 ml. of white fraction, 0.1 ml. of bottom-fraction lysate, 0.1 μ mole of MgSO_4 , 3 μ moles of ATP, 0.2 μ mole of NADP^+ and 77 nmoles of (*RS*)-[$3\text{-}^{14}\text{C}$]HMG-CoA (995 d.p.s.); total volume 0.30 ml., pH 7.2. Incubation was at 30°.

Fig. 2. Effect of pH on the incorporation of HMG-CoA into rubber. Each incubation mixture contained 0.1 ml. of white fraction, 0.05 ml. of double-strength bottom-fraction lysate, 0.05 ml. of 0.3 M buffer, 0.1 μ mole of MgSO_4 , 3 μ moles of ATP, 0.2 μ mole of NADP^+ and 81 nmoles of (*RS*)-[$3\text{-}^{14}\text{C}$]HMG-CoA (1040 d.p.s.); total volume 0.30 ml. Incubation was at 30° for 2 hr. ●, Sodium phosphate buffer; ○, tris-HCl buffer.

Table 1. *Ozonolysis of labelled rubber*

LADNP, laevulinic acid 2,4-dinitrophenylhydrazone. N.D., not determined.

Expt. no.	Sp. radioactivity of rubber (d.p.s./mg.)		Wt. of LADNP burned (mg.)	Total radioactivity of burned LADNP (d.p.s.)		Calc. sp. radioactivity of rubber (d.p.s./mg.)
	Before precipitation	After precipitation		First crystallization	Second crystallization	
1	4.4	4.7	98	115	N.D.	5.2
			96	85	N.D.	3.9
2	N.D.	12.0	72.4	219	N.D.	13.3
			70.2	N.D.	219	13.7

acid buffers were used to adjust the pH of the incubation mixtures. Fig 2 shows that the optimum pH for the incorporation of HMG-CoA into rubber is about 7.

Effect of cofactors. The effect of ATP, NADP⁺, NADPH, NAD⁺ and NADH on the incorporation of HMG-CoA into rubber is shown in Table 2. In these experiments the relatively high concentration of ATP was used because bottom-fraction lysate has a high phosphatase activity (Archer *et al.* 1963).

Effect of mevalonate. If mevalonate is an intermediate in the formation of rubber from HMG-CoA, then the incorporation of HMG-CoA should be diminished in the presence of unlabelled mevalonate. Table 3 shows that this is so, but that a relatively high mevalonate/HMG-CoA ratio is required. However, if all the *R*-mevalonate present in the incubation mixture were converted into rubber, no dilution of label would be observed. This appeared to have occurred in the above experiment when 0.5 μ mole (or less) of *R*-mevalonate was added to the latex, since this amount of *R*-[2-¹⁴C]mevalonate was completely converted into rubber when incubated under the conditions described in Table 3.

Non-incorporation of β -hydroxy- β -methyl[3-¹⁴C]-glutaric acid into rubber. It has been shown that β -hydroxy- β -methylglutaric acid can be converted into HMG-CoA in rat kidney mitochondria and that the reaction is stimulated by NAD⁺ and α -oxoglutarate (Burch, Rudney & Irias, 1964). It was therefore decided to test whether β -hydroxy- β -methylglutaric acid could be utilized by latex for rubber synthesis. No labelled rubber was formed

when latex was incubated with β -hydroxy- β -methyl[3-¹⁴C]glutaric acid in the presence of α -oxoglutarate, NAD⁺, NADP⁺, ATP and Mg²⁺. However, unlabelled β -hydroxy- β -methylglutaric acid significantly decreased the incorporation of [3-¹⁴C]HMG-CoA into rubber.

Effect of fractionation of latex on the conversion of HMG-CoA into rubber. Removal of the bottom fraction from the latex considerably decreased the amount of HMG-CoA converted into rubber. This effect was observed in both tris-mannitol and phosphate-mannitol buffers. Moreover, increasing the proportion of bottom fraction above that normally found in the latex greatly stimulated the formation of rubber from HMG-CoA (Fig. 3). Bottom fraction washed in phosphate-mannitol buffer had little stimulatory effect, even when the washing buffer contained 10mM-GSH (see below). Heating the bottom-fraction lysate for 1 min. at 100° completely abolished its activity, indicating that the stimulatory material was enzymic.

Effect of latex fractions on NADPH formation. To ascertain if the stimulatory effect of bottom fraction was due to its containing enzymes or substrates necessary for NADPH formation, the effect of bottom-fraction extract and latex serum on NADP⁺ reduction was examined. The results of this experiment established that the formation of NADPH takes place in the serum and that bottom fraction has no effect on this process (Fig. 4).

Effect of CoA on the incorporation of HMG-CoA and mevalonate into rubber. Bottom fraction has little effect on the incorporation of mevalonate into rubber provided ATP is present (Archer *et al.* 1963),

Table 2. *Effect of cofactors on the incorporation of HMG-CoA into rubber*

Each incubation mixture contained 0.15ml. of white fraction, 0.1ml. of bottom-fraction lysate, 0.1 μ mole of MgSO₄, 81 nmoles of (*RS*)-[3-¹⁴C]HMG-CoA (1040 d.p.s.) and cofactors as shown; total volume 0.31 ml., pH 7.2. Incubation was at 30° for 2 hr.

Expt. no.	Cofactors added (μ moles)	Incorporation of <i>S</i> -HMG-CoA into rubber (nmoles)
1	—	0.3
	ATP (3.0)	2.3
	ATP (3.0), NADP ⁺ (0.2)	16.8
2	ATP (3.0)	6.8
	ATP (3.0), NADP ⁺ (0.2)	35.1
	ATP (3.0), NAD ⁺ (0.2)	8.5
	ATP (3.0), NADH (0.2)	7.5
3	ATP (3.0)	0.2
	ATP (3.0), NADP ⁺ (0.2)	7.5
	ATP (3.0), NADPH (0.2)	6.6

Table 3. *Effect of unlabelled mevalonate on the incorporation of [3-¹⁴C]HMG-CoA into rubber*

Each incubation mixture contained 0.15ml. of white fraction, 0.1 ml. of double-strength bottom-fraction lysate, 0.1 μ mole of MgSO₄, 3 μ moles of ATP, 0.2 μ mole of NADP⁺, 80 nmoles of (*RS*)-[3-¹⁴C]HMG-CoA (1030 d.p.s.) and (*RS*)-mevalonate as indicated; total volume 0.32 ml., pH 7.2. Incubation was for 2 hr. at 30°. Mevalonate was added as the sodium salt except in Expt. 2, when the *NN'*-dibenzylethylenediammonium salt was used.

Expt. no	Unlabelled mevalonate added (μ moles)	Incorporation of <i>S</i> -HMG-CoA into rubber (nmoles)
1	0	6.6
	0.8	7.2
2	0	21.2
	1.0	18.3
	3.0	2.9
3	0	16.7
	4.0	4.9

Fig. 3. Effect of bottom fraction on the incorporation of HMG-CoA into rubber. Each incubation mixture contained 0.15 ml. of white fraction, 0.1 μ mole of MgSO_4 , 3 μ moles of ATP, 0.2 μ mole of NADP^+ and 74 nmoles of (*RS*)-[3- ^{14}C]-HMG-CoA (950 d.p.s.). Double-strength bottom-fraction lysate was added as shown; total volume 0.53 ml., pH 7.2. Incubation was at 30° for 2 hr.

Fig. 4. Reduction of NADP^+ by latex fractions. The cuvettes contained amounts of latex serum and bottom-fraction extract equivalent to 1 ml. of undiluted latex and the reaction was started by the addition of 1 μ mole of NADP^+ ; total volume 1.7 ml., pH 7.2, temp. 20°. ○, Serum; ●, serum + bottom-fraction extract; ▲, bottom-fraction extract.

(Ferguson, Durr & Rudney, 1959; Lynen, Knappe, Eggerer, Henning & Agranoff, 1959), is concentrated in this fraction. However, the results obtained with HMG-CoA and mevalonate were not strictly comparable, since the incubation mixtures used in the HMG-CoA experiments contained relatively large amounts of CoA. The effect of CoA on the incorporation of these precursors into rubber was therefore examined. It was found in these experiments that, although CoA inhibited the incorporation of both precursors to a small extent, the effect of bottom fraction on the incorporation of HMG-CoA and mevalonate into rubber was unaltered by the presence of CoA.

Formation of mevalonate from HMG-CoA in bottom-fraction lysates. Direct evidence that bottom fraction contains HMG-CoA reductase was obtained by demonstrating mevalonate formation on incubation of the fraction with HMG-CoA and NADPH in the presence of EDTA to inhibit the metabolism of mevalonate. A 0.2 ml. sample of bottom-fraction lysate was prepared in 0.05 M-sodium phosphate buffer, pH 7.0, containing 12 mM-GSH, and incubated for 2 hr. with 77 nmoles of (*RS*)-[3- ^{14}C]HMG-CoA (995 d.p.s.), 200 nmoles of NADPH and 3.5 μ moles of EDTA (total volume, 0.47 ml.). After incubation, carrier mevalonate was added and the lactone extracted and chromatographed as described in the Materials and Methods section. Radioautography of the chromatogram on silica gel G showed a radioactive spot with the same R_F and shape as the carrier mevalonolactone, which was detected by spraying the plate with hydroxylamine and ferric chloride (Abdel-Akher & Smith, 1951). Further evidence that the radioactive material was mevalonolactone was obtained by treating a sample of the purified compound with alkali and incubating the neutralized product with white fraction and ATP: 62% of the material was converted into rubber. A quantitative experiment showed that bottom fraction was more effective than white fraction in converting HMG-CoA into mevalonate. Further, the incorporation of HMG-CoA into rubber in whole latex (white fraction plus bottom fraction) in the absence of EDTA was approximately equivalent to the amount of mevalonate formed in the bottom fraction, implying that the reduction of HMG-CoA to mevalonate is the rate-limiting step in the formation of rubber from HMG-CoA (Table 4).

Effect of cysteine and GSH on bottom-fraction lysates. Bottom-fraction lysates lost all their stimulatory activity on standing for 1 hr. at 0° or at -10°, but this was prevented by 10 mM-GSH or -cysteine. Lysate stored at -70° in 10 mM-GSH lost 25% of its activity in 24 hr. These observations indicate that the HMG-CoA reductase of latex, like those of yeast and liver (Kirtley & Rudney, 1967;

and consequently the above findings suggested that HMG-CoA reductase (EC 1.1.1.34), which catalyses the formation of mevalonate from HMG-CoA

Table 4. *Formation of mevalonic acid and rubber from HMG-CoA in latex fractions*

Incubation mixtures contained latex fractions, EDTA, ATP and MgSO_4 as shown, $2\mu\text{moles}$ of GSH, $0.4\mu\text{mole}$ of NADPH and 165nmoles of $(RS)\text{-}[3\text{-}^{14}\text{C}]\text{HMG-CoA}$ (2120 d.p.s.); total volume 0.67ml. , pH 7.0, temp. 30° , incubation time, 2 hr. In this experiment the latex was collected in phosphate-mannitol buffer and the bottom fraction lysed in 12mM -GSH in 0.05M -sodium phosphate buffer. Results of individual incubations are given.

Latex fraction (ml.)		EDTA (μmoles)	ATP (μmoles)	Mg^{2+} (μmole)	Total radioactivity (d.p.s.)	
White fraction	Bottom-fraction lysate				In mevalonate*	In rubber
0.3	—	5	—	—	11	—
0.3	—	5	—	—	20	—
0.3	—	—	6	0.2	—	17
0.3	—	—	6	0.2	—	14
—	0.2	5	—	—	163	—
—	0.2	5	—	—	236	—
0.3	0.2	—	6	0.2	—	191
0.3	0.2	—	6	0.2	—	229

* Corrected for 60% recovery.

Linn, 1967a), is labile in the absence of low-molecular-weight thiols.

Seasonal variation in incorporation of HMG-CoA into rubber. During the 3-year period when the work was carried out, it was observed that the extent of HMG-CoA incorporation into rubber depended strongly on the time of year when the latex was tapped. Each year, from June to October, incorporations were almost always very low whereas between January and March high incorporations were readily obtained. In contrast, when mevalonate was incubated with latex under virtually identical conditions, its incorporation was between 90 and 100% of the maximum value throughout the year, suggesting that changes in the activity of HMG-CoA reductase were responsible for the variation in HMG-CoA incorporation. This explanation was supported by the observation that an increase in the bottom-fraction content of an incubation mixture during the period of low incorporation stimulated the synthesis of rubber from HMG-CoA (Table 5). It is unlikely that the low HMG-CoA reductase activity was due to its stability *in vitro* being decreased because of a decrease in the concentrations of endogenous GSH or cysteine, since HMG-CoA incorporation was still very low in the June–October period, even when bottom fraction lysed in buffer containing 10mM -GSH was used in the incubations.

Degradation of HMG-CoA in latex. Serum was prepared from undiluted white fraction and the small amount of sediment formed was redispersed in the serum (serum fraction). The bottom fraction was lysed in a volume of 0.05M -sodium phosphate buffer, pH 7.0, equivalent to one-third of the volume of latex collected. These fractions were then incubated with $[3\text{-}^{14}\text{C}]\text{HMG-CoA}$ and any acetoacetate formed was converted into acetone as

Table 5. *Effect of bottom fraction on the conversion of HMG-CoA into rubber during a period of low incorporation (August)*

Each incubation mixture contained 0.15ml. of white fraction, $0.1\mu\text{mole}$ of MgSO_4 , $3\mu\text{moles}$ of ATP, $0.2\mu\text{mole}$ of NADP^+ and 82nmoles of $(RS)\text{-}[3\text{-}^{14}\text{C}]\text{HMG-CoA}$ (1045 d.p.s.). Double-strength bottom-fraction lysate was added as shown; total volume 0.41ml. , pH 7.2. Incubation was for 2 hr. at 30° .

Vol. of lysate added (ml.)	Incorporation of <i>S</i> -HMG-CoA into rubber (nmol)
0	0.2
0.05	1.3
0.20	7.5

described above. Table 6 shows that the largest amount of radioactive volatile carbonyl material was produced in the incubation with the serum fraction. Boiled serum fraction and bottom-fraction lysate had much less activity. It is very likely that some, if not all, of this volatile material is acetone produced by the decarboxylation of acetoacetate, since the enzyme catalysing the formation of acetoacetate from HMG-CoA (HMG-CoA lyase, EC 4.1.3.4) is widespread in animal tissues (Coon, Kupiecki, Dekker, Schlesinger and del Campillo, 1959) and might be expected to occur in plants also.

DISCUSSION

Label from $[3\text{-}^{14}\text{C}]\text{HMG-CoA}$ is incorporated into rubber on incubation with *H. brasiliensis* latex and it has been proved by ozonolysis of the labelled rubber that the radioactive carbon is located in the

Table 6. *Degradation of HMG-CoA by latex fractions*

Each incubation mixture contained 65nmols of (*RS*)-[3-¹⁴C]HMG-CoA (835d.p.s.) and either 0.2ml. of serum fraction or 0.2ml. of lysed bottom fraction; total volume 0.26ml., pH 7.0. Incubation was in Conway units at 30° for 3hr. Any acetoacetate produced was converted into acetone which was trapped in semicarbazide and assayed as described in the Materials and Methods section. The figures in the right-hand column show the amount of acetoacetate formed, assuming that all the trapped radioactivity is due to acetone semicarbazone and that the recovery of acetone was 42%.

Expt. no.	System	Radioactivity	
		trapped (d.p.s.)	Acetoacetate formed (nmols)
1	Serum fraction	38	7.0
	Serum fraction boiled for 5 min.	7.5	1.4
	Lysed bottom fraction	0.7	0.13
2	Serum fraction	26.5	4.9

polyisoprene chains. The fact that high incorporations can be obtained, and that acetate, acetyl-CoA and acetoacetate are poor precursors of rubber *in vitro* (Teas & Bandurski, 1956; Kekwick, 1964; see Archer & Audley, 1967), shows that it is HMG-CoA that is utilized directly for rubber synthesis. Evidence has been obtained by Brodie, Wasson & Porter (1963, 1964) and Rudney, Stewart, Majerus & Vagelos (1966) that HMG-CoA (or β -hydroxy- β -methylglutaric acid) bound to a carrier protein may be the actual intermediate reduced to mevalonate in liver and yeast. If this is so, then in latex HMG-CoA itself must be readily converted into the bound form.

β -Hydroxy- β -methylglutaric acid is not utilized for rubber synthesis *in vitro*, presumably because the activating system for this acid found by Burch *et al.* (1964) in rat kidney mitochondria is not present in tapped latex. However, unlabelled β -hydroxy- β -methylglutaric acid diminishes the incorporation of labelled HMG-CoA into rubber, indicating that this acid inhibits an enzyme required for rubber synthesis from HMG-CoA. Isler, Rüegg, Würsch, Gey & Pletscher (1959) observed a similar effect of β -hydroxy- β -methylglutaric acid on the incorporation of [¹⁴C]acetate into cholesterol in rat liver homogenates.

At the single concentration tested (Table 2) NADP⁺ stimulated the conversion of HMG-CoA into rubber much more than did NAD⁺, suggesting that the reduction of HMG-CoA in latex requires NADPH, as in yeast and liver (Knappe *et al.* 1959; Durr & Rudney, 1960; Linn, 1967a). A similar

experiment showed that NADP⁺ was as stimulatory as NADPH and it appears that the concentration of NADP⁺, rather than the availability of hydrogen for its reduction, is a limiting factor in the formation of rubber from HMG-CoA *in vitro*.

Unlabelled mevalonate decreases the incorporation of [3-¹⁴C]HMG-CoA into rubber, but only at relatively high concentrations and this is, to some extent, a consequence of the rapid rate of utilization of mevalonate in latex. The incomplete equilibration of added mevalonate with that formed from HMG-CoA may also be involved, for significant amounts of [3-¹⁴C]HMG-CoA are converted into rubber even when the pool of unlabelled mevalonate is large (Expts. 2 and 3, Table 3).

The HMG-CoA reductase of latex is concentrated in the bottom fraction and is thus particulate, whereas the enzymes converting mevalonate into isopentenyl pyrophosphate are in the soluble fraction of the latex (Archer *et al.* 1963; Chesterton & Kekwick, 1968). This distribution is similar to that found in animal cells (Popják, 1959). The predominant component of the bottom fraction is the lutoid particle, a vacuolar body containing hydrolytic enzymes characteristic of lysosomes (Wiersum, 1957; Pujarniscle, 1968). *A priori* it is unlikely that HMG-CoA reductase is located within such particles, and this view is supported by the observation that washing the bottom fraction in iso-osmotic buffer (containing GSH to prevent deactivation of the reductase) greatly diminishes its stimulatory action, but does not remove the hydrolases of the lutoid particle (Audley, 1964; Pujarniscle, 1968). The Frey-Wyssling complex is also an unlikely site for the reductase because a considerable proportion of these bodies remains in the white fraction after centrifugation. In animal cells the enzyme is found almost exclusively in the microsomes (Bucher *et al.* 1960; Siperstein & Fagan, 1966; Linn, 1967a) and it is reasonable to suppose that the HMG-CoA reductase activity of latex bottom fraction is due to the presence of such particles. If this is so, the microsomes (or endoplasmic reticulum from which they are derived) must be carried down with the lutoid particles on centrifugation, probably by entanglement with lutoid-particle aggregates, which are abundant in tapped latex, or by adsorption on their surface. However, it is also possible that microsomes and lutoid particles sediment together because there is an intimate association of endoplasmic reticulum and lutoid particles *in vivo*.

Lynen (1967) has assayed most of the enzymes catalysing the transformation of acetate into rubber in *H. brasiliensis* latex. The activity of HMG-CoA reductase was exceptionally low and Lynen (1967) therefore suggested that the reduction of HMG-CoA to mevalonate is the rate-limiting step in the formation of rubber from acetate. The latex used

in his experiments contained cysteine and had been frozen at -70° for at least 24 hr. before assay. The measurements were made on the supernatant produced by centrifuging the latex at 20 000g. Our results show that the HMG-CoA reductase of latex sediments at 600g and that the enzyme is partially inactivated after 24 hr. at -70° , even when GSH is present. Thus, even if freezing and thawing 'solubilized' all of the enzyme, Lynen's (1967) value for its activity is certainly too low. Nevertheless his proposal is probably valid, for we also find that the rate of formation of rubber from HMG-CoA is controlled by the rate of mevalonate synthesis. Our observation that the activity of HMG-CoA reductase varies throughout the year, whereas under the same conditions the activities of the enzymes converting mevalonate into rubber remain relatively constant, is consistent with this view, since this finding suggests that HMG-CoA reductase activity can vary in response to environmental changes.

Our limited measurements show that a significant quantity of HMG-CoA is degraded in latex, probably by the action of HMG-CoA lyase. In liver homogenates the activity of this enzyme is so much greater than that of HMG-CoA reductase (Bucher *et al.* 1960) that the conversion of HMG-CoA into squalene and cholesterol does not take place (Rudney, 1959; Popják & Cornforth, 1960; Siperstein & Fagan, 1966). It seems likely therefore that the high incorporations of HMG-CoA into rubber that we have observed may be due to the relatively low lyase activity of tapped latex.

This work forms part of the research programme undertaken by the Natural Rubber Producers' Research Association. The authors thank Dr G. P. McSweeney for carrying out the ozonolyses, Miss J. M. Cox and Mr P. J. Naylor for technical assistance, and Dr E. G. Cockbain for helpful discussion.

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