Biochem. J. (1965) **96**, 862

The Formation of 5-Phosphomevalonate by Mevalonate Kinase in *Hevea brasiliensis* Latex

By I. P. WILLIAMSON and R. G. O. KEKWICK
Department of Biochemistry, University of Birmingham

(*Received* 23 *March* 1965)

1. Evidence has been produced for the formation of 5-phosphomevalonate from potassium DL-mevalonate by the latex of *Hevea brasiliensis* and by reconstituted freeze-dried serum obtained from this latex. 2. The enzyme, mevalonate kinase, catalysing the formation of 5-phosphomevalonate from potassium DL-mevalonate and ATP has been partially purified. 3. 5-Phosphomevalonate formed by the purified mevalonate kinase from potassium [2-^{14}C]mevalonate has been shown to be incorporated by latex into rubber to about 2·4 times the extent of DL-mevalonate. 4. The enzyme can utilize inosine triphosphate as effectively as adenosine triphosphate as a phosphate donor and is also slightly active with uridine triphosphate. 5. The enzyme was fairly stable to a range of pH values and temperatures, the activity being optimum at pH 7·5 and 60–70°. The energy of activation was 10·7 kcal./mole. The K_m values were 0·13 mM for potassium DL-mevalonate and 2·0 mM for ATP at 30°. 6. The enzyme required the presence of Mn^{2+} (1 mM) for maximum activity; this could be replaced by Mg^{2+} (4 mM), which was less effective, and by Ca^{2+}, which was far less effective. 6. Although the enzyme did not require cysteine or reduced glutathione for activation in aerobic conditions, it was inhibited by reagents known to react with thiol groups.

Mevalonic acid, originally shown to be precursor of the isoprene unit from which sterols are formed (Tavormina, Gibbs & Huff, 1956), has also been shown to be incorporated into rubber by the latex of *Hevea brasiliensis* (Park & Bonner, 1958; Kekwick *et al.* 1959). It is now well established that the 'active isoprene unit' which is the biological precursor of isoprenoid molecules is isopentenyl pyrophosphate (3-methylbut-3-enyl pyrophosphate) (Chaykin, Law, Phillips, Tchen & Bloch, 1958; Lynen, Eggerer, Henning & Kessel, 1958). Latex has been found to convert this substance into rubber with considerable efficiency (Henning, Möslein, Arreguin & Lynen, 1961; Archer, Audley, Cockbain & McSweeney, 1963).

The first stage in the conversion of mevalonate into isopentyl pyrophosphate is the formation of 5-phosphomevalonate catalysed by mevalonate kinase (ATP–mevalonate 5-phosphotransferase, EC 2.7.1.36). This enzyme has been purified to some extent from yeast (Tchen, 1958), pig liver (Levy & Popják, 1960), rabbit liver (Markley & Smallman, 1961) and *Cucurbita pepo* seedlings (Loomis & Battaille, 1963). As none of the enzymes concerned in the biosynthesis of rubber had been purified or characterized, the formation of 5-phosphomevalonate in *Hevea brasilensis* latex was investigated. An attempt was made to purify mevalonate kinase, and to compare its properties with similar enzymes reported from those other tissues, in which it is probably primarily concerned in the formation of sterols.

MATERIALS AND METHODS

Latex. Fresh latex was obtained by tapping 10-year-old *Hevea brasiliensis* trees grown in a greenhouse in the University of Birmingham Botanical Garden. Samples of freeze-dried latex serum, prepared by the procedure of Archer & Sekhar (1955), were obtained from the Rubber Research Institute of Malaya.

Special chemicals. DL-Mevalonic acid lactone was obtained from British Drug Houses Ltd., Poole, Dorset. DL-[2-^{14}C]Mevalonic acid lactone was obtained from The Radiochemical Centre, Amersham, Bucks. Both the labelled and unlabelled forms were converted into potassium DL-mevalonate before use by incubation with slightly more than 1 equiv. of KOH at 30° for 30 min. The specific activity of [2-^{14}C]mevalonate was adjusted to 0·2 μc/μmole by the addition of unlabelled mevalonate.

ATP (disodium salt) was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. It was dissolved in water and adjusted to pH 7·5 with 0·05 N-KOH before use. ATP labelled in the terminal phosphate group with ^{32}P was synthesized by the method of Glynn & Chappell (1964).

Chromatography. The paper-chromatographic separation of the metabolites of mevalonic acid was carried out as specified on either Whatman 3MM or no. 1 paper, with the following solvent systems: solvent 1, 2-methylbutan-2-ol-

acetic acid–water (4:1:2, by vol.) (ascending) (Henning, Möslein & Lynen, 1959); solvent 2, 2-methylpropan-2-ol–formic acid–water (20:5:8, by vol.) (descending) (Bloch, Chaykin, Phillips & de Waard, 1959); solvent 3, butan-1-ol–formic acid–water (73:13:10, by vol.) (ascending) (Tchen, 1958); solvent 4, isobutyric acid–aq. NH_3 (sp.gr.0·88)–water (66:13:30, by vol.) (descending) (de Waard & Popják, 1959); solvent 5, ethanol–aq. NH_3 (sp.gr.0·88)–water (8:1:1, by vol.) (descending) (Henning *et al.* 1959); solvent 6, methanol–aq. NH_3 (sp.gr.0·88)–water (6:1:3, by vol.) (Tchen, 1958). Radioautograms were prepared with Kodirex Brand Kodak X-ray film with an exposure time of 21 days.

The chromatographic separation of proteins was carried out on columns of Sephadex and DEAE-Sephadex obtained from Pharmacia (G.B.) Ltd., London, W. 13, prepared according to the manufacturer's instructions.

Purification of rubber. The rubber obtained from incubation of ^{14}C-labelled substrates with fresh latex was purified by the method of Kekwick *et al.* (1959).

Assay of radioactivity. The ^{14}C content of purified rubber was assayed by counting an infinitely thick layer beneath an end-window Geiger–Müller counter. An infinitely thick layer of poly[^{14}C]methyl methacrylate reference source of specific activity 1 μc/g. gave about 640 counts/min./cm.^{2} in the apparatus used.

To measure the $^{14}C/^{32}P$ atomic ratio in 5-phosphomevalonate, the total count of an infinitely thin sample was measured in the end-window counter in the presence and absence of a shield of aluminium foil sufficient to absorb all the emission from ^{14}C. The correction for the absorption of emission from ^{32}P by the shield was obtained by applying the same procedure to a standard $Na_2H^{32}PO_4$ specimen. The proportion of the total counts of the 5-phosphomevalonate due to ^{14}C and ^{32}P was then calculated and the atomic ratio obtained by reference to standards.

Protein estimation. The protein concentration of column eluates was determined by measuring the extinction at 280 mμ in a 1 cm. cell. The total protein contained in an enzyme preparation was determined by measuring the nitrogen content of the precipitate formed by adding 20% (w/v) trichloroacetic acid. An equal volume of ice-cold 20% trichloroacetic acid was added to the enzyme preparation, and the precipitate was centrifuged and washed with more ice-cold 20% trichloroacetic acid. The precipitate was then dissolved in N-NaOH and the total nitrogen estimated by the micro-Kjeldahl procedure. A solution containing 1 mg. of protein nitrogen/ml. was found to have an extinction value of 0·6–0·65 at 280 mμ in a 1 cm. cell.

Concentration of protein solutions. It was necessary to concentrate the large volumes of dilute protein solution obtained in column eluates before passing to the next stage of purification. Freeze-drying proved to be impracticable as, although solutions of mevalonate kinase retained their activity after freeze-drying, they no longer gave reproducible elutions from DEAE-Sephadex. Solutions were therefore concentrated by pressure dialysis through a cellophan membrane at 4°.

Assay of mevalonate-kinase activity. The enzyme was assayed either by a radiochemical procedure or by a spectrophotometric method. The radiochemical method was used only for measurements on crude enzyme preparations having enzyme contaminants likely to vitiate results obtained by the spectrophotometric procedure, where the phosphate donor was not ATP, or where the effect of compounds that would interfere with the additional enzymes used in the spectrophotometric procedure was being studied. A standard incubation mixture was used for this radiochemical assay containing: potassium DL-[2-^{14}C]mevalonate (1·0 μmole), ATP (3·0 μmoles), $MgCl_2$ (1·5 μmoles), 0·1M-tris–HCl buffer, pH 7·5 (0·23 ml.), and enzyme solution (0·1 ml.), in a total volume of 0·5 ml. The reaction was allowed to proceed for 1 hr., after which it was stopped by the addition of ethanol (1 ml.), any precipitated protein being removed by centrifugation. The mixture was freeze-dried and redissolved in water (35 μl.). Portions (10 μl.) were applied to Whatman 3MM filter paper and subjected to chromatography in solvent 2. The labelled compounds were detected by radioautography. The material having R_F 0·55, corresponding to that reported for 5-phosphomevalonic acid (Chaykin *et al.* 1958; Witting & Porter, 1959), was eluted, and its radioactivity assayed by counting as an infinitely thin sample under an end-window Geiger–Müller counter.

The spectrophotometric assay was essentially that originally proposed in 1958 by Lynen (1959) and subsequently used by Tchen (1958) and by Levy & Popják (1960). The ADP produced in the enzyme reaction was assayed by using it as a phosphate acceptor in the conversion of phosphoenolpyruvate into pyruvate catalysed by pyruvate kinase, and the pyruvate produced was converted into lactate by lactate dehydrogenase and NADH; the NADH oxidized, which was measured spectrophotometrically, was equivalent to the original ADP produced. The assay enzymes and substrates were obtained as test kit TC–K assembled for the assay of ADP in blood from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. The standard incubation mixture for the enzyme assay contained: ATP (10 μmoles), $MgCl_2$ (5·0 μmoles), potassium DL-mevalonate (3 μmoles), 0·1M-tris–HCl buffer, pH 7·5 (1·55 ml.), and enzyme (0·1 ml.) in a total volume of 2 ml. The reaction was stopped after 60 min. at 30° by the addition of ice-cold 3% (v/v) $HClO_4$ (2 ml.). The precipitated protein was centrifuged and a 3 ml. sample of the supernatant taken. To remove perchlorate, 1·0M-triethanolamine buffer, pH 9·6 (0·75 ml.), was added and the solution cooled to −10°; on thawing slowly the perchlorate precipitated. A 2 ml. sample of the perchlorate-free supernatant was taken for the ADP assay, and to this was added 0·15 ml. of phosphoenolpyruvate (10 mM), 0·1 ml. of NADH (5 mM) and 0·02 ml. of lactate dehydrogenase (1 mg. of enzyme protein/ml.). The initial concentration of NADH was was obtained by measuring the extinction at 366 mμ, where the extinction is approximately half that at the more frequently used wavelength, 340 mμ; this facilitated the measurement of large amounts of ADP produced by high concentrations of the enzyme. Pyruvate kinase solution (0·02 ml.), containing 1 mg. of enzyme protein/ml., was added, mixed well, and the extinction at 366 mμ measured until the reaction had ceased.

The assay differs from the continuous assay procedure used by other workers, the ADP being measured after the original enzyme solution had been removed: this necessitated the measurement of the ADP concentration after several different periods of enzyme action to ensure that ADP production was linear. It was found that the enzyme continued to act at the initial rate after incubation for 1 hr.

By assaying the ADP present in a protein-free superna-

tant contamination of the assay enzymes by those present in the original ADP-producing system was avoided. This ensures that all the NADH oxidation observed arises from the action of the lactate dehydrogenase added for the assay. The NADH-oxidase activity present in the latex serum therefore has no effect on the assay. The ADP in the protein-free supernatant may, however, arise from sources other than mevalonate kinase, and it was always necessary to assay a control incubation containing no mevalonate. When the results of the two assay procedures were compared it was found possible to obtain agreement to within about 5% with both reconstituted latex and the purified enzyme. The utilization of ATP for the metabolism of 5-phosphomevalonate by reconstituted freeze-dried latex appeared to be negligible compared with that used for its formation, in the radiochemical assay conditions.

Enzyme units. The unit of enzyme activity was defined as that which catalysed the production of 1 μmole of 5-phosphomevalonate/min. at 30° measured by either assay procedure.

RESULTS

Formation of 5-phosphomevalonic acid

The metabolites produced from mevalonate by fresh latex were investigated by incubating 1 μM-mevalonate with 0·2 ml. of fresh latex at 30° for 2 hr. Rubber was removed by coagulation with 2 vol. of boiling ethanol, and the soluble material was subjected to ascending chromatography on Whatman no. 1 paper in solvent 1. Three principal zones of radioactivity were obtained (R_F values 0·58, 0·29 and 0·14) in addition to those due to mevalonic acid and its lactone. As it seemed probable, by comparison with the results of Henning *et al.* (1959), that the spot at R_F 0·29 contained 5-phosphomevalonic acid, material from this spot was eluted and subjected to chromatography in those other solvents in which the mobility of 5-phosphomevalonic acid had previously been reported, and the chromatographic characteristics of this substance in these solvents agreed well with those reported for 5-phosphomevalonic acid (Table 1). Similar experiments in which [1-^{14}C]mevalonate was the substrate also produced components having R_F values 0·29 and 0·14 in solvent 1.

This substance having the same chromatographic characteristics as 5-phosphomevalonate was also produced when freeze-dried latex serum, reconstituted to its original strength with water, was incubated with [2-^{14}C]mevalonate. When this metabolite was separated chromatographically and was mixed with synthetic 5-phosphomevalonate synthesized by the method of Robinson & Wittreich (1961), the two materials behaved as a single substance in solvents 2, 3, 4 and 5.

This material having similar chromatographic characteristics to 5-phosphomevalonate also had similar properties when subjected to conditions that might cause hydrolysis. When the substance was heated in N-hydrochloric acid for 7 min. or for 30 min. there was no detectable change of the R_F of the material after chromatography in solvent 1, although after this acid treatment the spot was somewhat elongated. This effect was also observed by Tchen (1958). Incubation of the material with a preparation of calf-intestinal alkaline phosphatase for 4 hr. at pH 7·8 and at 37° converted the unknown entirely into material having the chromatographic behaviour of mevalonate.

The possible phosphorus content of the substance was investigated by incubating fresh latex (0·2 ml.) with $Na_2H^{32}PO_4$ (1 μmole) and [2-^{14}C]mevalonate (0·3 μmole) at 30° for times from 18 min. to 2 hr. Fresh latex has been found to contain mitochondria, and although the concentration is low compared with that in the latex vessel itself (P. B. Dickenson, personal communication) some incorporation of $Na_2H^{32}PO_4$ into ATP might be expected, giving rise to [^{32}P]ATP, which could be used to phosphorylate mevalonate. To separate the metabolites derived from mevalonate, rubber was removed as described above and the material soluble in 60% (v/v) ethanol was subjected to two-dimensional chromatography with first solvent 1 and then solvent 6. The material containing ^{14}C and ^{32}P, having R_F 0·1–0·3 in solvent 1 and 0·7–0·9 in solvent 6, was eluted and rechromatographed in solvent 6 to remove any inorganic phosphate. The material having R_F about 0·8 in this solvent was again eluted and chromatographed once more in solvent 2: ^{14}C and ^{32}P activity was found in material having R_F 0·55, the R_F reported by Chaykin *et al.* (1958) for 5-phosphomevalonate in this solvent. When this material was eluted and the ^{32}P and ^{14}C content measured the proportion of ^{32}P was found to increase with the time of incubation, becoming constant after 2 hr. It was, however, not possible from these experiments to obtain an

Table 1. *R_F values in different solvent systems of the metabolite of mevalonic acid having R_F 0·29 in solvent 1*

Experimental details and the compositions of solvents 1–5 are given in the text.

	R_F		
Solvent system	Observed	Reported for 5-phosphomevalonic acid	Reference
Solvent 2	0·55	0·53–0·61	Bloch *et al.* (1959)
Solvent 3	0·15	0·15	Tchen (1958)
Solvent 4	0·41	0·37	de Waard & Popják (1959)
Solvent 5	3 cm. in 24 hr.	4 cm. in 24 hr.	Henning *et al.* (1959)

analysis of the phosphorus content of the unknown in atoms of P/mevalonate molecule as the concentration of inorganic phosphate and ATP in the original latex was not known.

The phosphorus content of the suspected 5-phosphomevalonate was measured finally by using a partially purified preparation of the synthesizing enzyme. The enzyme obtained by the first (stepwise) elution from DEAE-Sephadex (see below), having a specific activity of 0·32 unit/mg. of protein, was used. This mevalonate-kinase preparation (1 mg. of protein) was incubated with [2-^{14}C]mevalonate (4 μmoles), [^{32}P]ATP labelled in the terminal phosphate group (10 μmoles), magnesium chloride (8 μmoles) and 0·1 M-tris–hydrochloric acid buffer, pH 7·5 (0·7 ml.), in a total volume of 2·0 ml. for 1 hr. at 30°. The incubation was terminated by the addition of 5 ml. of ethanol, 2 ml. of water was added and the volume of the incubation mixture was reduced to about 0·5 ml. under reduced pressure. The concentrated solution was diluted to 10 ml. with water, filtered through a Whatman no. 1 filter paper and applied to a Dowex 1 (formate form) column (0·8 cm. × 12 cm.). The material was eluted with a gradient of formic acid and ammonium formate by the procedure of Hurlbert, Schmitz, Brumm & Potter (1954), as used by Bloch *et al.* (1959) for the isolation of the phosphorylated derivatives of mevalonic acid. The fraction having radioactivity that was eluted in the conditions corresponding to those found by Bloch *et al.* (1959) to elute inorganic phosphate, ADP and 5-phosphomevalonic acid was freeze-dried and extracted with 90% (v/v) ethanol to remove ADP. The ethanolic solution was then applied to Whatman 3MM filter paper and subjected to chromatography in solvent 6, as used by Tchen (1958) to separate inorganic phosphate, which has R_F 0·1 in this solvent. The material having R_F 0·8 was eluted and the ^{14}C/^{32}P atomic ratio was found to be 1:1·18.

To investigate the incorporation of the 5-phospho-[2-^{14}C]mevalonate into rubber a preparation of this substance was made by using the same purified sample of mevalonate kinase as was used for the synthesis of the substance labelled with both ^{14}C and ^{32}P. The enzymic synthesis and subsequent purification were carried out under the same conditions and procedure as were used to produce the compound labelled with ^{32}P and ^{14}C except that the ATP used was not labelled with ^{32}P. A comparison was then made of the incorporation of this purified material into rubber with that of mevalonate by the samples of fresh latex from the same tapping. Each incubation was carried out with 0·1 ml. of fresh latex, incubated with solid substrate in the absence of added cofactors for 3 hr. at 30°. When the incorporation of 250 mμmoles of 5-phospho-[2-^{14}C]mevalonate was compared with that of an equal amount of [2-^{14}C]mevalonate, 29 mμmoles of 5-phospho[2-^{14}C]mevalonate were incorporated into rubber compared with 12 mμmoles of [2-^{14}C]mevalonate. The addition of 500 mμmoles of ATP as the solid to the incubation mixture resulted in the incorporation of 54 mμmoles of 5-phospho[2-^{14}C]-mevalonate and 40 mμmoles of [2-^{14}C]mevalonate. Thus in the absence of added ATP 2·4 times as much 5-phospho[2-^{14}C]mevalonate was incorporated as [2-^{14}C]mevalonate, whereas in the presence of 5 mM-ATP the incorporation of 5-phospho-[2-^{14}C]-mevalonate was only 1·4 times that of [2-^{14}C]-mevalonate. This last result is lower than would be expected when allowance is made for the fact that the 5-phosphomevalonate must have been composed of the natural isomer whereas the mevalonate was a racemic mixture.

Purification of mevalonate kinase

A search for a suitable initial step giving a high yield and a reasonable increase in the specific activity of the enzyme from reconstituted freeze-dried latex serum showed that neither ammonium sulphate fractionation at neutral pH and at room temperature nor organic solvent fractionation with ethanol or acetone over the pH range 5·5–7·0 at 0° gave a clear fractionation, both types of system giving many fractions having specific activities not much greater than that of the starting material. As Archer & Cockbain (1955) had observed that a large proportion of the protein of latex serum was precipitated at acid pH values, the enzyme activity of the supernatants remaining after adjusting the serum to pH values from 6·0 to 4·0 with 2 N-acetic acid was measured. The supernatant obtained at any pH value in the range had a higher specific activity than that of the original serum: the maximum specific activity was obtained by precipitating 80% of the serum protein at pH 4·5, leaving 45% of the original enzyme in a solution having about twice the specific activity of the serum. Although the recovery obtained by this procedure was low it was adopted as the initial step because it produced the highest increase in specific activity of any of the many precipitation procedures tried. The yield of enzyme was independent of the temperature of the precipitation between 0° and 18°.

Purification of the supernatant from the pH 4·5 *precipitation.* Preliminary experiments in which the supernatant from the pH 4·5 precipitation, dialysed against 0·02 M-phosphate buffer, pH 6·0, was applied to a DEAE-cellulose column equilibrated in this buffer showed that no enzyme, and very little protein (about 8% of that applied), could be eluted by applying a solution of potassium chloride, the concentration of which increased in a gradient to 1·0 M, to the column. Similar results

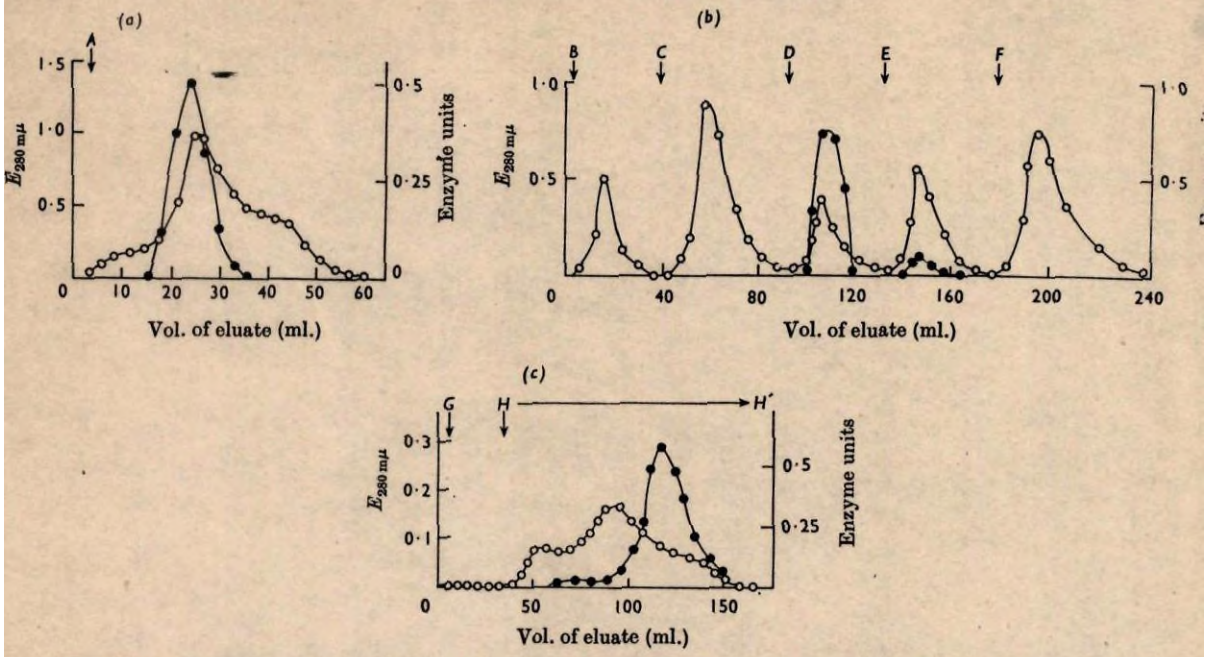

Fig. 1. Chromatographic purification of mevalonate kinase from *Hevea* latex serum. (*a*) Fractionation of the supernatant from the pH 4·5 precipitation of serum by elution from Sephadex G-200: *A*, 0·05 M-phosphate buffer, pH 7·5. (*b*) Fractionation of the active peak from (*a*) by elution from DEAE-Sephadex A-50 by steps of increasing KCl concentration: *B*, 0·05 M-phosphate buffer, pH 6·0, followed by the same buffer containing KCl at 0·10 M (*C*), 0·20 M (*D*), 0·30 M (*E*) and 0·50 M (*F*). (*c*) Fractionation of the active peak from (*b*) by elution from DEAE-Sephadex A-50 by a gradient of increasing KCl concentration: *G*, 0·02 M-phosphate buffer, pH 6·0, followed by the same buffer containing a continuous KCl gradient up to 0·3 M ($H \rightarrow H'$). ○, Protein concentration measured by $E^{1\,cm.}_{280\,m\mu}$; ●, units of enzyme activity measured spectrophotometrically.

were obtained when elution of serum proteins from a similar DEAE-cellulose column at pH 7·5 was attempted with a similar potassium chloride gradient solution. Although adsorption on calcium phosphate gel has been successfully used as a purification technique for both yeast (Tchen, 1958) and pig-liver (Levy & Popják, 1960) mevalonate kinase, the enzyme present in the pH 4·5 supernatant could not be successfully eluted from hydroxyapatite columns. Good recoveries of enzyme activity were, however, obtained when the supernatant from the pH 4·5 precipitation was applied to a Sephadex G-200 column (1·5 cm. × 20 cm.) in 0·05 M-phosphate buffer, pH 7·5. Elution with this buffer produced the result shown in Fig. 1(*a*): 91% of the enzyme activity applied to the column was eluted in an active peak that had a threefold increase in specific activity over the applied solution and a sevenfold increase in specific activity over the original serum.

Although it had been found impossible to recover mevalonate-kinase activity after serum had been applied to DEAE-cellulose columns, similar experiments with DEAE-Sephadex showed that about 95% of the enzyme activity could be eluted by potassium chloride solution when serum, dialysed against 0·02 M-phosphate buffer, pH 6·0, was applied to a column equilibrated with this buffer.

The active fraction from a Sephadex G-200 column was therefore concentrated to 5 ml. by ultrafiltration, and was applied to a DEAE-Sephadex A-50 column (1·5 cm. × 15 cm.) in 0·02 M-phosphate buffer, pH 6·0. Stepwise elution with the phosphate buffer containing increasing concentrations of potassium chloride gave the elution pattern shown in Fig. 1(*b*), in which most of the enzyme was present in the fraction eluted at 0·2 M-potassium chloride. This procedure produces a further fourfold increase in specific activity, and Table 2 shows that 91% of the enzyme activity was eluted from the column, the total protein recovery being 93%.

To obtain a further enrichment of the enzyme, the fraction eluted from DEAE-Sephadex at 0·2 M-potassium chloride was concentrated by ultrafiltration, dialysed against 0·02 M-phosphate buffer,

Table 2. *Purification of* Hevea *mevalonate kinase from reconstituted freeze-dried latex serum*

The enzyme activity was assayed spectrophotometrically at 30°. Experimental details are given in the text.

Purification step	Total enzyme activity obtained (units)	Total protein N (mg.)	Specific activity of enzyme (unit/mg. of protein)
Reconstituted serum pH 7·5	5·65	82	0·011
Precipitation of inactive protein at pH 4·5	2·55	16·5	0·024
Filtration on Sephadex G-200	2·32	5·0	0·075
Elution from DEAE-Sephadex A-50 with 0·2 M-KCl	2·06	1·0	0·33
Gradient elution from DEAE-Sephadex A-50	1·94	0·2	1·55

pH 6·0, and applied to a second DEAE-Sephadex (A-50) column (1 cm. × 10 cm.), at pH 6·0. The protein was eluted by applying a potassium chloride gradient to 0·3 M, giving the elution pattern shown in Fig. 1(*c*). The peak containing the enzyme activity had an enrichment of about 4·7 times that of the applied material, corresponding to an increase in specific activity of about 140 times over the original reconstituted serum (the most active fraction had a specific activity 160 times that of the starting material). The enzyme recovery at this stage was 92% (Table 2), which was accompanied by a total protein recovery of 91% for this last stage.

Purity of the enzyme preparation

The enzyme preparation obtained from the first DEAE-Sephadex column, having a 30-fold enrichment, was incubated with mevalonate and ATP under standard conditions. Chromatographic examination of the reaction products revealed only the presence of 5-phosphomevalonic acid in addition to the starting material. This suggests that there is no phosphomevalonate-kinase activity in the final product. The spectrophotometric assay carried out on an incubation mixture containing the purified enzyme and all the standard components showed that the adenosine-triphosphatase activity present in the starting material had been removed. Studies of nucleotide specificity showed that the preparation of mevalonate kinase from the first DEAE-Sephadex column could not utilize ADP instead of ATP and therefore contained no myokinase. Further, nucleotides that were not substrates (CTP and GTP) did not give any activity in the presence of ADP, nor was the enzyme activity against the substrate UTP enhanced in the presence of ADP, thus showing that there was no nucleotide diphosphate-kinase activity present in the preparation.

As the most highly purified enzyme preparations were only available in very small amounts it was difficult to assess the molecular homogeneity of these preparations. To obtain an indication of the purity of the enzyme preparation from the first DEAE-Sephadex column having a 30-fold enrichment, immunoelectrophoresis of a 0·1 ml. sample of this material containing 1 mg. of protein was carried out in 1% agar made up in 0·05 M-glycine buffer, pH 9·2, for 6 hr. at 1 v/cm. A rabbit antiserum raised against the original reconstituted latex serum from which the enzyme was prepared was then allowed to diffuse against the antigens separated by electrophoresis: four precipitin lines developed, all fairly near the starting point of the run, showing that the preparation contained at least four protein components of somewhat similar charge.

Properties of Hevea *mevalonate kinase*

The characteristics of *Hevea* mevalonate kinase were investigated with a preparation obtained from the first (stepwise) elution from the DEAE-Sephadex column at 0·2 M-potassium chloride. The preparation had a 30-fold enrichment over the original serum and catalysed the phosphorylation of 0·32 μmole of potassium DL-mevalonate/min./mg. of protein under the standard conditions of the spectrophotometric assay.

Effect of substrate concentration. When the rate of phosphorylation of potassium DL-mevalonate by the purified enzyme (0·13 mg. of protein) was studied at mevalonate concentrations varying from 0·1 to 5 mM under conditions that were standard, except for the ATP concentration (6 mM) and the magnesium chloride concentration (4 mM), by the spectrophotometric procedure, the rate of reaction was maximal at 2 mM-potassium DL-mevalonate. Similar experiments carried out under standard conditions in which the ATP concentration was varied showed that the enzyme was saturated with ATP at 6 mM concentration. The K_m values for the action of the enzyme on the two substrates were obtained from reciprocal plots (Lineweaver & Burk, 1934) as exemplified by Figs. 2(*a*) and 2(*b*) for a number of temperatures between 3° and 40°. The results shown in Table 3 indicate that the enzyme has about ten times the affinity for mevalonate that it has for ATP; also, although the K_m values for both substrates are almost constant over the range of temperatures studied, that for mevalonate decreases very slightly with increase in temperature whereas that for ATP increases.

The reciprocal plot of $1/v$ against $1/s$ was always linear, indicating the presence of only one enzyme

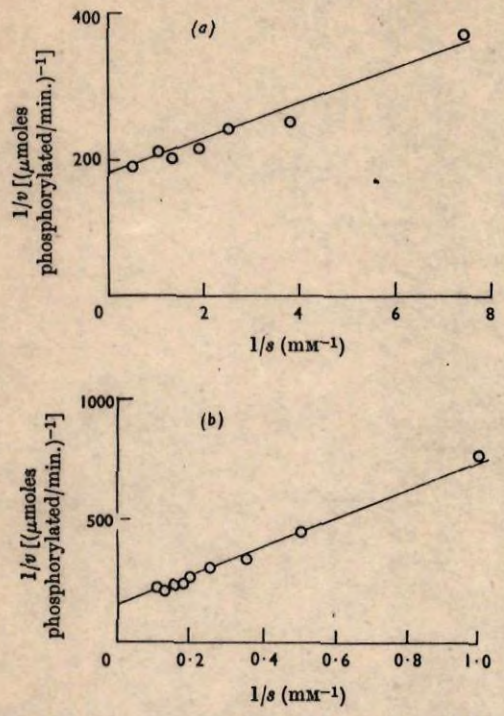

Fig. 2. Relation between $1/v$ and $1/s$ for the phosphorylation of mevalonate by *Hevea* mevalonate kinase assayed spectrophotometrically: (*a*) at constant ATP concentration (6mM) and various mevalonate concentrations; (*b*) at constant mevalonate concentration (1·5mM) and various ATP concentrations.

phosphorylating mevalonate. When the enzyme reaction was allowed to go to completion in conditions where the amount of product formed was limited only by the amount of mevalonate present the reaction ceased when only 50% of the substrate had been phosphorylated; this suggested that, like the preparation of mevalonate kinase from other sources, the *Hevea* enzyme is specific for only one optical enantiomorph of mevalonate.

Nucleotide specificity. The efficiency of ITP, CTP, GTP and UTP as phosphate donors at three different concentrations was compared with that of ATP, at optimum concentration, in the radiochemical assay. The results given in Table 4 show that ITP was as effective as ATP at similar concentrations and that the only other nucleotide that was acted on by the enzyme was UTP. ADP could not replace ATP as a phosphate donor and the preparation therefore contained no myokinase; the nucleotides GTP and CTP, which were inactive alone, were also inactive in the presence of ADP, showing that the preparation was also free of nucleoside diphosphate kinase. The addition of

Table 3. *K_m values of* Hevea *mevalonate kinase acting on mevalonate and ATP at various temperatures*

Experimental details are given in the text.

Substrate	K_m (mM)				
Temp.	3°	15°	25°	30°	40°
DL-Mevalonate	0·17	0·14	0·13	0·13	0·13
ATP	1·1	—	—	2·0	2·2

Table 4. *Nucleotide specificity of* Hevea *mevalonate kinase*

The enzyme activity was assayed by the radiochemical procedure. Experimental details are given in the text.

Nucleotide	Concn. (mM)	5-Phosphomevalonate formed (mμmoles)
ATP	6·0	115
ITP	2·5	71
	5·0	106
	10·0	120
CTP	2·5	0
	10·0	0
GTP	2·5	0
	10·0	0
UTP	2·5	4·5
	5·0	12·0
	10·0	16·5
ADP	5·0	0
+CTP	5·0	0
+GTP	5·0	0
+ITP	5·0	104
+UTP	5·0	18·5

ADP did not increase the small amount of activity obtained with UTP.

Metal ion requirement. Initial experiments with reconstituted freeze-dried latex serum showed that the production of 5-phosphomevalonate was stimulated by the addition of Mg^{2+}. Because of this observation and those of other workers on the metal ion activation of mevalonate kinase from different sources, the metal ion activation of the partially purified enzyme preparation was investigated. Activation by Mn^{2+} and Mg^{2+} was measured by the spectrophotometric assay procedure, whereas that produced by Ba^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+} and Ca^{2+}, all at 1mM and 5mM concentration, was measured by the radiochemical procedure.

The results of the spectrophotometric assay of the activation produced by Mn^{2+} and Mg^{2+} on the enzyme reaction at optimum ATP concentration

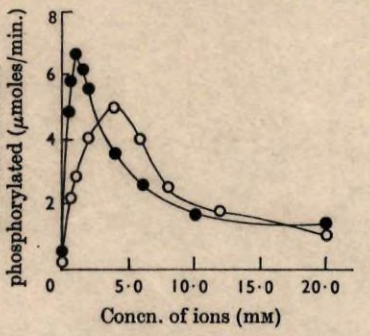

Fig. 3. Activity of *Hevea* mevalonate kinase measured spectrophotometrically in the presence of various concentration of Mg^{2+} (○) and Mn^{2+} (●).

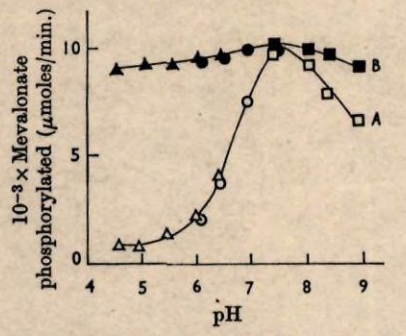

Fig. 4. Curve *A* shows the effect of pH on the activity of *Hevea* mevalonate kinase. Curve *B* shows the effect of subjecting the enzyme to various pH values at 30° for 1 hr. on the activity subsequently measured at pH 7·5 and 30°. All activities were measured by the spectrophotometric method. The buffers used were at 0·05 M concentration: △ and ▲, acetate–phosphate; ○ and ●, phosphate; □ and ■, tris–HCl.

Table 5. *Activation of* Hevea *mevalonate kinase by metal ions*

The enzyme activity was assayed by the radiochemical procedure. Experimental details are given in the text.

Salt	Concn. (mM)	5-Phosphomevalonate formed (mμmoles)
$MgCl_2$	2·5	120
	4·0	153
$CaCl_2$	1·0	8
	5·0	46
	10·0	34
$Co(NO_3)_2$	1·0	1·5
	5·0	10
$FeSO_4$	1·0	3·0
	5·0	6·0
$NiSO_4$	1·0	0
	5·0	0
$BaCl_2$	1·0	4·6
	10·0	7·5

(6 mM) and potassium DL-mevalonate (2·5 mM) are shown in Fig. 3. Maximum activation was given by Mn^{2+} at 1 mM concentration; Mg^{2+} gave a lower stimulation: the maximum at 4 mM was 77% of that given by Mn^{2+}. A higher concentration of either ion was inhibitory, the decrease in activity at higher concentrations being most pronounced with Mn^{2+}. Incubations containing suboptimum amounts of ATP (4 mM) and various concentrations of Mn^{2+} or Mg^{2+} showed maximum activation at the same concentration of Mn^{2+} but at a slightly lower concentration of Mg^{2+} (3·5 mM); there was again marked inhibition with excess of either ion.

Of the other ions investigated (Table 5) only Ca^{2+} produced any marked stimulation, giving 30% of that given by Mg^{2+} at 4 mM; Co^{2+}, Fe^{2+} and Ba^{2+} all gave a small stimulation of activity.

As would be expected the enzyme reaction was inhibited by EDTA. Radiochemical assays carried out on incubation mixtures containing the standard components, but with 6 mM-ATP, 4 mM-Mg^{2+} and EDTA concentrations ranging from 0·01 to 10 mM, showed that the residual activity was very close to that which would be expected from the calculated free Mg^{2+}, assuming a dissociation constant 10^{-9} for Mg–EDTA.

pH-*dependence of enzyme activity*. The variation in the initial reaction rates catalysed by the partially purified enzyme was studied over the range pH 4·0–pH 9·0 by using the buffers shown in the legend to the resultant pH–activity curve (Fig. 4): there was a pronounced optimum at pH 7·5. The stability of the enzyme at various pH values at 30° was investigated; samples of purified enzyme were subjected to pH values from 4·5 to 9·0 for 1 hr. and were then brought to pH 7·5 with N-sodium hydroxide or N-hydrochloric acid, and the residual enzyme activity was then assayed by the standard spectrophotometric procedure. The results, shown in Fig. 4, show that the enzyme was stable over the pH range studied.

Temperature-dependence of enzyme activity. Initial velocities obtained from a series of progress curves were plotted for the enzyme reaction assayed by the spectrophotometric procedure in standard incubation mixtures held at temperatures between 28° and 80°. The concentrations of potassium DL-mevalonate and ATP were increased to 3 mM and 9 mM respectively to obviate any decrease in the observed rates from the maximum arising from a possible change in K_m. Such measured velocities

Table 6. *Inhibition of* Hevea *mevalonate kinase by thiol reagents*

The enzyme activity was assayed by the radiochemical procedure. Experimental details are given in the text.

Reagent	Concn. (mM)	Inhibition (%)
Iodoacetamide	0·1	0
	1·0	5
N-Ethylmaleimide	0·1	66
	1·0	94
p-Chloromercuribenzoate	0·1	95
	1·0	100
$HgCl_2$	0·1	90
	1·0	94
$ZnSO_4$	0·1	90
	1·0	94
$CuSO_4$	0·1	56
	1·0	77

could therefore be assumed to be directly proportional to the velocity constant for the breakdown of the enzyme–substrate complex and be substituted in the Arrhenius (1889) equation for the determination of activation energy. Thus when the log (initial velocity) was plotted against $1/T$ a straight line was obtained for measurements made below 50°; from the slope of this graph the critical increment (activation energy) was calculated to be 10·7 kcal./mole. The enzyme preparation showed maximum activity between 60° and 70°, and appeared to be fairly stable at high temperatures, since little inactivation was found to have occurred when the enzyme was assayed under standard conditions after it had been held for 1 hr. at 50° and after 1 hr. at 80° 40% of the original activity still remained.

Sensitivity to thiol reagents. Unlike the mevalonate-kinase preparations obtained from pig liver (Levy & Popják, 1960) and rabbit liver (Markley & Smallman, 1961), *Hevea* mevalonate kinase was not activated by cysteine or reduced glutathione. The enzyme was, however, found to be sensitive to thiol alkylating reagents (Table 6): the enzyme was markedly inhibited by *N*-ethylmaleimide, 100% inhibition being produced at 10 mM concentration, but iodoacetamide was much less effective and gave only a 15% inhibition at 10 mM. Mercaptide-forming reagents were inhibitory (Table 6), as were Cu^{2+} ions, which produced 96% inhibition at 10 mM.

DISCUSSION

Perhaps the most striking feature of the enzyme described is the close similarity of its properties to those of the mevalonate kinase purified from pig liver by Levy & Popják (1960). The main point of difference between the enzymes in *Hevea* latex and pig liver is that the *Hevea* enzyme is insensitive to atmospheric oxygen, and does not require cysteine or reduced glutathione for activation; in this respect the enzyme resembles the mevalonate-kinase preparations from yeast (Tchen, 1958) and *Cucurbita pepo* (Loomis & Battaille, 1963). Nevertheless, *Hevea* mevalonate kinase, like all the mevalonate-kinase preparations so far reported, is highly sensitive to reagents known to react with thiol groups.

The kinetic characteristics of *Hevea* mevalonate kinase resemble those of the pig-liver enzyme more closely than any of the other preparations described. The pig-liver enzyme has K_m 0·05 mM for natural (+)-mevalonate, and *Hevea* mevalonate kinase has K_m 0·13 mM for potassium DL-mevalonate at 30°; the pH optima of the rabbit-liver (pH 7·5), pig-liver (pH 7·3) and *Hevea* (pH 7·5) enzymes are very similar, whereas the yeast and *Cucurbita* enzymes have optima at more acid pH values. The *Hevea* enzyme also resembles the pig-liver enzyme in its restricted nucleotide specificity; both can utilize ATP and ITP equally effectively at similar concentrations, but, whereas the pig-liver enzyme can utilize these two nucleotides only, the *Hevea* enzyme also shows a little activity with UTP, and in this respect is like the rabbit-liver enzyme. The metal ion activation of the *Hevea* enzyme differs slightly from the pig-liver enzyme; *Hevea* mevalonate kinase and all the other mevalonate-kinase preparations except the pig-liver enzyme are activated by Mn^{2+} to a greater extent than by Mg^{2+}.

A further difference between the properties of *Hevea* mevalonate kinase and those of the pig-liver enzyme is in their sensitivity to acid; the pig-liver enzyme is completely inactivated after a few minutes at pH 5·2 (Hellig & Popják, 1961), whereas little of the activity of the *Hevea* enzyme is lost after 1 hr. at this pH.

The specific activity of the reconstituted freeze-dried *Hevea* latex serum used as the source of the enzyme is of interest in relation to the activity of the sources of the other mevalonate-kinase preparations; the only source having a comparable specific activity is pig liver, reported by Levy & Popják (1960) to phosphorylate 8·8 mμmoles of mevalonate/min./mg. of protein at 37°, whereas the reconstituted *Hevea* serum phosphorylated 11 mμmoles of mevalonate/min./mg. of protein at 30°. The relatively high specific activity may reflect at high capacity of latex for rubber biosynthesis, but there do not appear to be any very reliable data on the quantitative capacity of latex or indeed of individual *Hevea* trees for rubber biosynthesis.

We thank the Natural Rubber Producers Research Association for a maintenance grant to I.P.W. We are indebted to Professor J. Heslop-Harrison for greenhouse

acilities and are very grateful to Mr W. G. Rutter for growing the rubber trees.

REFERENCES

Archer, B. L., Audley, B. G., Cockbain, E. G. & McSweeney, G. P. (1963). *Biochem. J.* **89**, 565.
Archer, B. L. & Cockbain, E. G. (1955). *Biochem. J.* **61**, 508.
Archer, B. L. & Sekhar, B. C. (1955). *Biochem. J.* **61**, 503.
Arrhenius, S. (1889). *Z. phys. Chem.* **4**, 226.
Bloch, K., Chaykin, S., Phillips, A. H. & de Waard, A. (1959). *J. biol. Chem.* **234**, 2595.
Chaykin, S., Law, J., Phillips, A. H., Tchen, T. T. & Bloch, K. (1958). *Proc. nat. Acad. Sci., Wash.*, **44**, 998.
de Waard, A. & Popják, G. (1959). *Biochem. J.* **73**, 410.
Glynn, I. M. & Chappell, J. B. (1964). *Biochem. J.* **90**, 147.
Hellig, H. & Popják, G. (1961). *J. Lipid Res.* **2**, 235.
Henning, U., Möslein, E. M., Arreguin, B. & Lynen, F. (1961). *Biochem. Z.* **333**, 534.
Henning, U., Möslein, E. M. & Lynen, F. (1959). *Arch. Biochem. Biophys.* **83**, 259.
Hurlbert, R. B., Schmitz, H., Brumm, A. F. & Potter, V. R. (1954). *J. biol. Chem.* **209**, 23.
Kekwick, R. G. O., Archer, B. L., Barnard, D., Higgins, G. M. C., McSweeney, G. P. & Moore, C. G. (1959). *Nature, Lond.*, **184**, 268.
Levy, H. R. & Popják, G. (1960). *Biochem. J.* **75**, 417.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Loomis, W. D. & Battaille, J. (1963). *Biochim. biophys. Acta*, **67**, 54.
Lynen, F. (1959). In *Ciba Found. Symp.: Biosynthesis of Terpenes and Sterols*, p. 95. Ed. by Wolstenholme, G. E. W. & O'Connor, M. London: J. and A. Churchill Ltd.
Lynen, F., Eggerer, H., Henning, U. & Kessel, I. (1958). *Angew. Chem.* **70**, 738.
Markley, K. & Smallman, E. (1961). *Biochim. biophys. Acta*, **47**, 327.
Park, R. B. & Bonner, J. (1958). *J. biol. Chem.* **233**, 340.
Robinson, F. M. & Wittreich, P. (1961). *U.S. Patent* 3014057.
Tavormina, P. A., Gibbs, M. H. & Huff, J. W. (1956). *J. Amer. chem. Soc.* **78**, 4498.
Tchen, T. T. (1958). *J. biol. Chem.* **233**, 1100.
Witting, L. A. & Porter, J. W. (1959). *J. biol. Chem.* **234**, 2841.