Biochem. J. (1971) **124**, 407–417
Printed in Great Britain

The Enzymes Forming Isopentenyl Pyrophosphate from 5-Phosphomevalonate (Mevalonate 5-Phosphate) in the Latex of *Hevea brasiliensis*

By D. N. SKILLETER and R. G. O. KEKWICK
Department of Biochemistry, University of Birmingham, Birmingham B15 2TT, U.K.

(*Received* 22 *March* 1971)

1. Phosphomevalonate kinase and 5-pyrophosphomevalonate decarboxylase have been purified from the freeze-dried latex serum of the commercial rubber tree *Hevea brasiliensis*. 2. The phosphomevalonate kinase was acid- and heat-labile and required the presence of a thiol to maintain activity. 3. The 5-pyrophosphomevalonate decarboxylase was relatively acid-stable and more heat-stable than the phosphokinase. 4. Maximum activity of the phosphokinase was achieved at pH 7.2 with 0.2mM-5-phosphomevalonate (K_m 0.042mM), 2.0mM-ATP (K_m 0.19mM) and 8mM-Mg^{2+} at 40°C. The apparent activation energy was 14.8kcal/mol. 5. Maximum activity of 5-pyrophosphomevalonate decarboxylase was achieved at pH5.5–6.5 with 0.1mM-5-pyrophosphomevalonate (K_m 0.004mM), 1.5mM-ATP (K_m 0.12mM) and 2mM-Mg^{2+}. The apparent activation energy was 13.7kcal/mol. The enzyme was somewhat sensitive to inhibition by its products, isopentenyl pyrophosphate and ADP.

Although the mechanism of isoprenoid biosynthesis in higher plants is fairly well understood, it being similar to that in animals, information about the detailed enzymology of the process is scant. There are three enzymes involved in the conversion of the first precursor unique to isoprenoid formation, mevalonate, into the isoprenoid monomer, isopentenyl pyrophosphate. Only preparations of the first enzyme in the sequence, mevalonate kinase, have been described from higher plants. The properties of preparations of this enzyme from the cotyledons of the pumpkin, *Cucurbita pepo* (Loomis & Battaile, 1963), and from the latex of the commercial rubber tree, *Hevea brasiliensis* (Williamson & Kekwick, 1965), have been reported.

Because the kinetic parameters of these enzymes may be relevant to the control of rubber biosynthesis in *H. brasiliensis* latex it was decided to extend the work of Williamson & Kekwick (1965) to a study of the properties of the phosphomevalonate kinase (EC 2.7.4.2) and of the 5-pyrophosphomevalonate decarboxylase (EC 4.1.1.33) of *H. brasiliensis* latex. A study of the latter enzyme was also of particular interest in view of the paucity of information about the properties of this enzyme from other tissues.

Preliminary accounts of this work have appeared (Skilleter, Williamson & Kekwick, 1965; Skilleter & Kekwick, 1968).

MATERIALS AND METHODS

Special chemicals and substrates. The source and details of these materials have been given by Williamson & Kekwick (1965). The radioactive substrates 5-phosphomevalonate (mevalonate 5-phosphate) and 5-pyrophosphomevalonate (mevalonate 5-pyrophosphate) were prepared from [^{14}C]mevalonate by the procedures described by Skilleter & Kekwick (1967).

Measurement of radioactivity. The radioactivity of enzyme products and substrates was measured in a Nuclear–Chicago model 720 β-scintillation spectrometer. Pieces of paper chromatograms were placed in vials containing 5ml of phosphor [0.3% 2,5-diphenyloxazole and 0.03% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene] and 5ml of light petroleum (b.p. 40–60°C). The efficiency was calculated by the channels-ratio procedure. Aqueous samples (0.1ml) were assayed in a dioxan-based phosphor (10ml) (Bray, 1960).

Determination of protein. The protein content of latex serum and enzyme preparations was determined by precipitating the protein with an equal volume of 40% (w/v) trichloroacetic acid at 4°C, washing the precipitate twice with cold 20% (w/v) trichloroacetic acid, dissolving it in 2M-NaOH and assaying the nitrogen by the micro-Kjeldahl procedure (Chibnall, Rees & Williams, 1943). The protein concentration of column eluates was determined by assuming that a protein solution containing 0.1mg of latex protein N/ml had an E_{280} of 0.6 (Williamson & Kekwick, 1965).

Enzyme assays. The enzymes were assayed either by a spectrophotometric procedure or by a radiochemical

procedure. The spectrophotometric assay of phosphomevalonate kinase was carried out on the ADP produced in a standard incubation mixture consisting of 5-phosphomevalonate (0.2mM), ATP (2.5mM), $MgCl_2$ (8mM) and 0.3ml of 0.5M-potassium phosphate buffer, pH7.2, in a total volume of 1.0ml. The corresponding standard incubation mixture for measuring the ADP produced by 5-pyrophosphomevalonate decarboxylase contained 5-pyrophosphomevalonate (0.25mM), ATP (2.5mM), $MgCl_2$ (5mM) and either 0.3ml of the 0.5M-phosphate buffer, pH7.2, or 0.3ml of 0.1M-tris–maleate buffer, pH7.2, in a total volume of 1.0ml. Incubations were normally carried out at 30°C. The total ADP produced by either enzyme was assayed at the end of the reaction by the method of Lynen (1959), details of which were given by Williamson & Kekwick (1965). For the determination of K_m values, however, this procedure was modified and the ADP produced during the course of the reaction was measured. To the standard incubation mixture was added KCl (40μmol), phosphoenolpyruvate (0.4μmol), NADH (0.3μmol), lactate dehydrogenase (50μg of protein, 18 units of enzyme activity) and 0.3ml of 0.05M-potassium phosphate buffer, pH7.2. The mixture was incubated in a cuvette at 30°C and the E_{340} measured. After 3min the extinction was checked, and provided it had remained constant, pyruvate kinase (20μg of protein, 25 units of enzyme activity) was added, the E_{340} was again measured, checked, and shown to be constant, the enzyme to be assayed was then added to give a final volume of 2ml and the decrease in E_{340} was measured over a 10min period. A control incubation mixture containing no 5-phosphomevalonate or 5-pyrophosphomevalonate was used to assay adenosine triphosphatase activity; with purified preparations this activity was negligible. The continuous assay and the total ADP assay agreed to within 2%.

The nucleotide and metal ion specificity of phosphomevalonate kinase was investigated by a radiochemical procedure. The contents of the incubation mixtures are described in the Results section. The incubations at 30°C were terminated, and the protein precipitated, by the addition of an equal volume of ethanol, which was then evaporated by boiling. The precipitate was removed by centrifugation, washed twice and the washings were combined with the original supernatant. The combined supernatants were freeze-dried and the resulting material was dissolved in ethanol–NH_3 (sp.gr. 0.88)–water (7:2:1, by vol.) and separated by paper chromatography. After development the regions containing 5-phosphomevalonate and 5-pyrophosphomevalonate were identified by radioautography; the radioactive areas were cut out and assayed in the liquid-scintillation counter. The proportion of the original substrate converted into 5-pyrophosphomevalonate was calculated from the ratio of the radioactivity (c.p.m.) from 5-pyrophosphomevalonate to the total radioactivity on the paper. The results were reproducible but a 2–4% breakdown of 5-pyrophosphomevalonate to 5-phosphomevalonate was observed during the chromatography; nevertheless the assay agreed with the total ADP assay to within 2%.

The activity of 5-pyrophosphomevalonate decarboxylase was assayed radiochemically by measuring the $^{14}CO_2$ produced from 5-pyrophospho[1-^{14}C]mevalonate. The incubations were carried out in Warburg flasks, the $^{14}CO_2$ produced being trapped and assayed by the procedure of Snyder & Godfrey (1961). 5-Pyrophospho-[1-^{14}C]mevalonate (0.3ml, 0.25mM, 0.1μCi/μmol) was placed in one side arm of the Warburg flask, 0.5ml of 3% $HClO_4$ was placed in the other and Hyamine hydroxide (0.2ml) in the centre well. To start the incubation at 30°C the mevalonate 5-pyrophosphate was tipped in, and it was stopped by tipping the $HClO_4$. At the conclusion the flask was kept closed for 2h; the Hyamine was then removed quantitatively and assayed for radioactivity in the liquid-scintillation counter. The assay was more sensitive than the spectrophotometric ADP assay, with which it agreed to within 2%.

Enzyme preparations. The enzymes were prepared from freeze-dried latex serum prepared by the method of Archer & Sekhar (1955) in the Rubber Research Institute of Malaya, Kuala Lumpur, Malaya. All protein preparations were carried out at 4°C unless otherwise stated. Ion-exchange chromatography was carried out with an exponential gradient of increasing KCl concentration at a given pH, by the procedure of Cherkin, Martinez & Dunn (1953). After application to the column the protein was washed in with 30–40ml of buffer, followed by a gradient elution (120ml in each vessel).

Units of enzyme activity. These are expressed as μmol of product formed/min at 30°C, unless otherwise stated. The product assayed varied with the assay procedure but, as is shown, the stoicheiometry was established for each enzyme. Specific activities are given in units/mg of protein.

RESULTS

Purification of the enzymes

Phosphomevalonate kinase. Preliminary experiments showed that a starting material with an enhanced specific activity could be obtained by dialysing reconstituted freeze-dried latex serum (Archer & Sekhar, 1955) against 0.05M-potassium phosphate buffer, pH7.0, containing 4mM-*N*-acetylcysteine and 5mM-magnesium chloride. To obtain effective chromatography of this material on Sephadex ion-exchange resins, prior fractionation on a column of Sephadex G-200 was necessary. Reconstituted freeze-dried latex serum (6ml, 96mg of protein), previously dialysed against the potassium phosphate buffer described above, was applied to a Sephadex G-200 column (2cm × 15cm), at 4°C, and inactive protein was eluted by the same buffer in the void volume. The fractions eluted immediately after this excluded material contained the phosphomevalonate kinase activity (Fig. 1*a*). Combination of the active fractions resulted in a solution containing 54% of the protein and 90% of the activity applied to the column, giving a 1.5-fold increase in specific activity. The preparation was then concentrated by pressure dialysis and applied to a DEAE-Sephadex column (1.3cm × 12cm), previously equilibrated with the potassium phosphate buffer, pH7.0, used in the previous stage. Active protein was eluted (Fig. 1*b*) by adding potassium chloride to the eluting buffer in

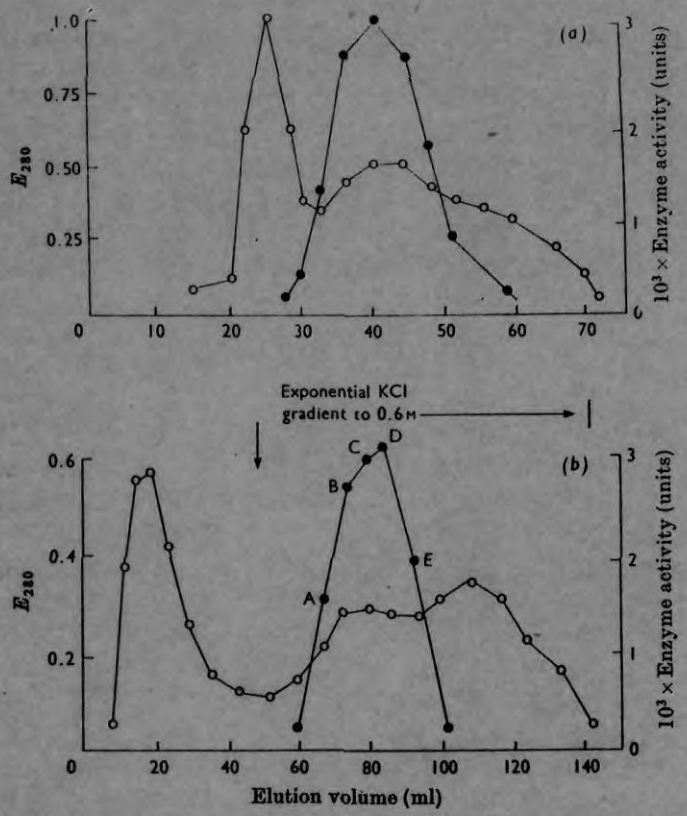

Fig. 1. Purification of phosphomevalonate kinase: (*a*) by gel filtration on Sephadex G-200; (*b*) chromatography of active fractions from (*a*) on DEAE-Sephadex. Experimental details are given in the text. ○, Protein (E_{280}); ●, enzyme activity.

Table 1. *Products formed from 5-phospho*[2-^{14}C]*mevalonate by active fractions from DEAE-Sephadex*

The products were identified by their elution characteristics from Dowex 1 (formate form) (see Bloch *et al.* 1959). Each fraction was incubated for 1 h at 30°C with 2 mM-5-phospho[2-^{14}C]mevalonate (specific radioactivity 0.086 μCi/mol), ATP (4 mM), $MgCl_2$ (4 mM), potassium phosphate buffer (pH 7.0, 0.17 M) and active fraction (0.3 ml) in a total volume of 1.0 ml.

DEAE-Sephadex fraction (see Fig. 1*b*)	Radioactivity in metabolite (c.p.m.)		
	5-Phosphomevalonate	5-Pyrophosphomevalonate	Isopentenyl pyrophosphate
A	36700	0	9580
B	17050	0	26420
C	24810	18780	3840
D	23520	24110	0
E	34280	11350	

concentrations increasing in a concave exponential gradient to 0.6 M. When fractions judged to be active from the spectrophotometric assay procedure were assayed for their products with 5-phospho[2-^{14}C]mevalonate as substrate it was found that the fractions eluted at low salt concentrations formed both 5-pyrophosphomevalonate and isopentenyl pyrophosphate (Table 1), but those eluted by higher salt concentrations formed only 5-pyrophosphomevalonate. Some degree of resolution of phosphomevalonate kinase and 5-pyrophosphomevalonate decarboxylase activity was thus obtained. In practice it was found that the material eluted in the fractions after those having a maximum activity

combined (phosphomevalonate kinase and 5-pyrophosphomevalonate decarboxylase), assayed by the spectrophotometric procedure, contained only the phosphomevalonate kinase activity. A preparation of the enzyme was obtained by combining those fractions that had a specific activity of about eight times that of the starting material and about 27 times that of whole serum. This material catalysed the formation of 0.13μmol of 5-pyrophosphomevalonate/min per mg of protein at

Table 2. *Purification of phosphomevalonate kinase from* Hevea *latex serum*

Enzyme activity was measured by the spectrophotometric assay of ADP; hence the values quoted for steps 1–3 include 5-pyrophosphomevalonate decarboxylase activity.

Purification stage	Total activity (units)	Total protein N (mg)	$10^3 \times$ Specific activity (units/mg of protein)
1. Reconstituted latex serum, pH 7.5	1.11	19.2	9.2
2. Dialysed serum containing *N*-acetylcysteine	3.34	19.2	33
3. Elution from Sephadex G-200	3.01	9.96	48
4. Elution from DEAE-Sephadex	6.20	0.785	126
5. Most active fractions eluted from DEAE-Sephadex	2.60	0.262	159

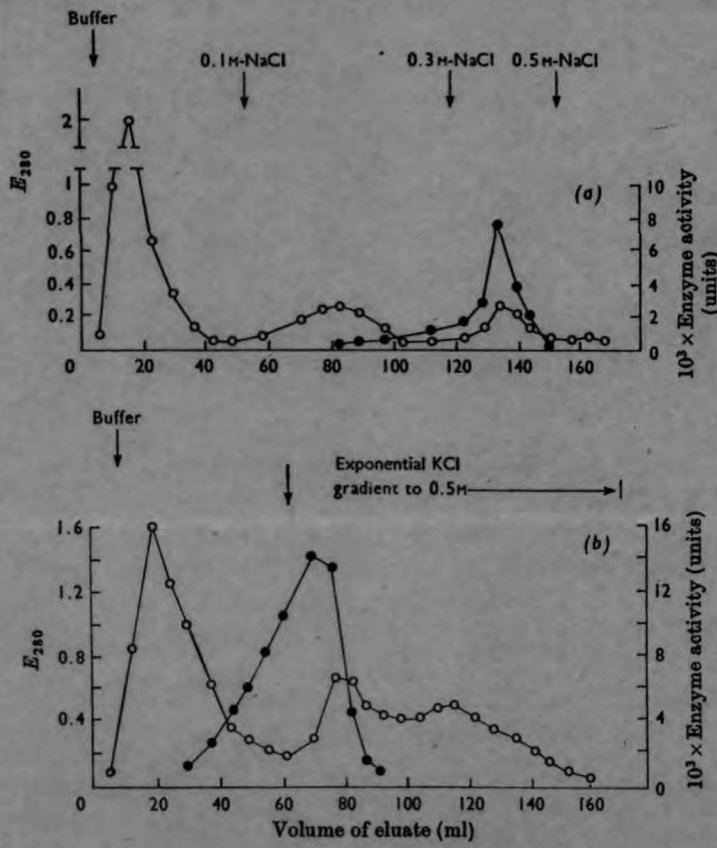

Fig. 2. Purification of 5-pyrophosphomevalonate decarboxylase: (*a*) chromatography of pH 5.5 supernatant on CM-Sephadex; (*b*) chromatography of active fractions from (*a*) on DEAE-Sephadex. Experimental details are given in the text. ○, Protein (E_{280}); ●, enzyme activity.

Table 3. *Purification of 5-pyrophosphomevalonate decarboxylase from* Hevea *latex serum*

Enzyme activity was measured by the spectrophotometric assay of ADP.

Purification stage	Activity (units)	Total protein N (mg)	$10^3 \times$ Specific activity (units/mg of protein)
1. Reconstituted latex serum, pH 7.5	26.0	96.0	43.2
2. Supernatant from pH 5.5 precipitation dialysed at pH 6.0	19.0	63.0	48.4
3. Elution from CM-Sephadex	13.6	13.5	16
4. Elution from DEAE-Sephadex	7.8	2.30	542
5. Most active fraction eluted	1.7	0.220	1240

pH 7.0 and 30°C in the presence of 2mM-5-phosphomevalonate and 2.5mM-ATP. The details of a typical purification of the enzyme are summarized in Table 2. The purified preparation lost considerable activity on storage at 4°C for 4–5 days but retained maximum activity when stored at −10°C for at least 1 month.

5-Pyrophosphomevalonate decarboxylase. To remove phosphomevalonate kinase activity and to produce a suitable preparation for ion-exchange chromatography, inactive protein was precipitated from reconstituted freeze-dried latex serum by adjusting it to pH 5.5 with 2.0M-acetic acid at room temperature. The precipitate was removed by centrifugation and the active supernatant dialysed against 0.01M-potassium phosphate buffer, pH 6.0.

A sample of this material (5ml, 63mg of protein) was then applied to a CM-Sephadex column (1.5cm × 12cm) previously equilibrated with the dialysis buffer at 4°C. About 80% of the protein was then eluted from the column by this buffer in a fraction containing mevalonate kinase but no 5-pyrophosphomevalonate decarboxylase. Addition of sodium chloride to the eluting buffer to a concentration of 0.3M resulted in the elution of 54% of the activity applied, the combined active fractions having a specific activity about four times that of the starting material (see Fig. 2*a* and Table 3).

The active fraction was concentrated by pressure dialysis, and then dialysed against 0.05M-potassium phosphate buffer, pH 7.2, before application to a DEAE-Sephadex column (1.5cm × 12cm) previously equilibrated with this buffer. Inactive protein was eluted from the column by the dialysis buffer, and the enzyme was eluted with a potassium chloride concentration gradient increasing exponentially to 0.5M. The active fractions were eluted by buffer containing 0.15–0.2M-potassium chloride (see Fig. 2*b*). About 70% of the 5-pyrophosphomevalonate decarboxylase activity was eluted in these fractions, which when combined had a specific activity about four times that of the material applied to the column. Such preparations, which contained neither mevalonate kinase nor phosphomevalonate kinase and were free of phosphatase action on ATP, ADP or isopentenyl pyrophosphate, had a specific activity about 12 times that of the reconstituted freeze-dried latex serum, and catalysed the formation of 0.7μmol of isopentyl pyrophosphate/min per mg of protein at 30°C and pH 7.2 in the presence of 0.25mM-5-pyrophosphomevalonate and 2.5mM-ATP. The details of a typical purification of this enzyme are summarized in Table 3. Enzyme preparations were concentrated by pressure dialysis and stored at −10°C. There was little loss of activity after 2–3 months although samples did lose activity after repeated thawing and freezing.

Identity of the products of enzyme action. The product of the action of phosphomevalonate kinase on 5-phosphomevalonate had the same characteristics as 5-pyrophosphomevalonate on paper chromatography in the four solvent systems previously used (Skilleter & Kekwick, 1967). The $^{32}P/^{14}C$ ratio of 5-pyrophosphomevalonate was measured with [^{32}P]ATP and 5-phospho[2-^{14}C]mevalonate as substrates. Phosphomevalonate kinase (60μg of protein) was incubated with 1mM-5-phospho[2-^{14}C]mevalonate (sp. radioactivity 0.086μCi/μmol), 2mM-[γ-^{32}P]ATP and 8mM-magnesium chloride in a solution containing 0.3ml of 0.05M-potassium phosphate buffer, pH 7.2, in a total volume of 1ml for 2h at 30°C. The incubation products were then separated by chromatography in ethanol–aq. NH_3 (sp.gr. 0.88)–water (7:2:1, by vol.) and the material in the position corresponding to 5-pyrophosphomevalonate was eluted from the paper in 20% (v/v) NH_3 solution. This material was further purified by chromatography on the Dowex 1 (formate form) column by the procedure of Bloch, Chaykin, Phillips & de Waard (1959) and NH_4^+ was removed by passage through Dowex 50 (H^+ form). Determination of the $^{32}P/^{14}C$ ratio of the suspected 5-[β-^{32}P]pyrophospho[2-^{14}C]mevalonate showed that the sample contained 0.185μg-atom of ^{32}P and 0.170μg-atom of ^{14}C, corresponding to a $^{14}C/^{32}P$ ratio of 1:1.085; this was consistent with the

phosphorylation of each molecule of 5-phosphomevalonate with one γ-phosphate group from ATP. Hydrolysis of the 5-pyrophospho[2-^{14}C]-mevalonate in 1M-hydrochloric acid at 100°C for 30min produced a substance chromatographically identical with the material shown by Williamson & Kekwick (1965) to be 5-phosphomevalonate, suggesting that the original material was a pyrophosphate. The suspected 5-pyrophosphomevalonate was unchanged by boiling in 1M-potassium hydroxide for 1h.

Chesterton & Kekwick (1968) have reported a full identification of isopentenyl pyrophosphate formed in latex serum from mevalonate, and the radioactive material formed by the purified 5-pyrophosphomevalonate decarboxylase from 5-pyrophospho[2-^{14}C]mevalonate was chromatographically indistinguishable from this material and from chemically synthesized isopentenyl pyrophosphate. Further, when 5-pyrophospho[1-^{14}C]-mevalonate was the substrate for the decarboxylase no radioactivity could be detected in those chromatographically separated fractions that would be expected to contain isopentenyl pyrophosphate. Hydrolysis of the suspected isopentenyl pyrophosphate in 1M-hydrochloric acid for 20min at 100°C produced material with the same chromatographic characteristics as the substance identified by Chesterton & Kekwick (1968) as isopentenyl phosphate.

Stoicheiometry of the phosphomevalonate kinase reaction. The proportion in which 5-pyrophosphomevalonate and ADP were formed from 5-phosphomevalonate and ATP was investigated by incubating the enzyme with ATP (2.5mM) and 0.2mM-5-phospho[2-^{14}C]mevalonate (0.08μCi/μmol), 8mM-magnesium chloride and enzyme (0.5ml; 80μg of protein) in a total volume of 10ml at 30°C. Duplicate samples were withdrawn at various time-intervals, one sample being assayed for ADP spectrophotometrically and the other for 5-pyrophosphomevalonate by the radiochromatographic procedure described above. The results in Table 4(*a*) show that 5-pyrophosphomevalonate and ADP were formed in equimolar amounts.

Stoicheiometry of the 5-pyrophosphomevalonate decarboxylase reaction. To investigate the proportions in which isopentenyl pyrophosphate, CO_2 and ADP were formed from 5-pyrophosphomevalonate and ATP, two separate incubation mixtures were analysed. The relation between CO_2 formation and ADP production was studied by incubating 0.5mM-5-pyrophospho[1-^{14}C]mevalonate (0.1μCi/μmol) with 2.5mM-ATP and the enzyme (10μg of protein) in a total volume of 1.0ml under standard conditions, in stoppered Warburg flasks containing Hyamine in the centre well. The $^{14}CO_2$ trapped from incubations carried out for different times was assayed by scintillation counting, and the ADP produced was measured spectrophotometrically. To assess the relationship between isopentenyl pyrophosphate formation and ADP production 0.3mM-5-pyrophospho[2-^{14}C]mevalonate (0.1mCi/μmol) was incubated with 2.5mM-ATP and the enzyme (40μg of protein) under standard conditions in a total volume of 5ml; the isopentenyl pyrophosphate produced was assayed by fractionation of the radioactive products on a DEAE-cellulose column by the procedure of Skilleter & Kekwick (1967), and the material corresponding to isopentenyl pyrophosphate was assayed by scintillation counting; the ADP was assayed spectrophotometrically. The results in Table 4(*b*) show that for every mol of isopentenyl pyrophosphate produced there was a concomitant formation of 1mol of ADP and 1mol of CO_2.

Equilibrium of the phosphomevalonate kinase reaction. The concentration of ADP produced by incubating the enzyme (40μg of protein) with various concentrations of 5-pyrophosphomevalonate (0.08–0.3mM) with ATP (0.8–5.0mM) under otherwise standard conditions reached a constant value after 2h, indicating that equilibrium had been reached. Such equilibrium concentrations of ADP were used to calculate the concentrations of 5-pyrophosphomevalonate, ATP and 5-phospho-

Table 4. *Stoicheiometry of the action of the two enzymes*

Details of the incubation mixtures, conditions and methods of assay are given in the text.

(*a*) Phosphomevalonate kinase

Time of incubation (min)	0	20	30	60	120
ADP produced (nmol)	24	42	60	82	104
Mevalonate 5-pyrophosphate (nmol)	21.5	40	59.4	84	111

(*b*) 5-Pyrophosphomevalonate decarboxylase

Time of incubation (min)	5	10	15	30	40
ADP produced (nmol)	41	89	115	216	170
CO_2 (nmol)	39	—	110	207	—
Isopentenyl pyrophosphate (nmol)	—	84	—	—	160

mevalonate from which an estimate of the equilibrium constant could be made. The values of the equilibrium constant thus obtained varied from 0.26 to 0.59, with a mean value of 0.34.

Properties of the enzymes

Thiol requirement of the two enzymes. As preliminary experiments had shown that the specific activity of phosphomevalonate kinase in reconstituted freeze-dried latex serum could be enhanced by the addition of thiol compounds, samples of both this reconstituted latex serum and the purified phosphomevalonate kinase were dialysed against 50mM-potassium phosphate buffer, pH7.5, alone and fortified with 5mM-magnesium chloride and 4mM-*N*-acetylcysteine. When either preparation was dialysed against buffers not containing *N*-acetylcysteine or magnesium chloride, activity was very much less than when these compounds were included. The activity lost was only partially restored by addition of *N*-acetylcysteine to the incubation mixture.

The 5-pyrophosphomevalonate decarboxylase of both reconstituted serum and the purified preparations was unaffected by thiol compounds.

Metal ion activation of the enzymes. As shown above enhanced enzyme activity was obtained when 5mM-magnesium chloride was included in the dialysis buffers for the phosphomevalonate kinase. The results of a detailed investigation of the activating effect of a range of bivalent metal ions is shown in Fig. 3(*a*). The activating effect of Mn^{2+} was investigated by using the spectrophotometric assay, that of Fe^{2+} and Zn^{2+} by the radiochromatographic procedure, and that of Mg^{2+} was measured by both techniques. All the ions studied activated the enzyme to some extent; the metal ion/ATP molar ratios giving maximum activity were Mg^{2+} 3:1, Mn^{2+} 1:1, Zn^{2+} 2:1, Fe^{2+} 4:1.

When the effect of metal ions on 5-pyrophosphomevalonate decarboxylase was investigated it was found that all the bivalent metal ions studied produced some activation (Fig. 3*b*), Mn^{2+} and Mg^{2+} being the two most effective ions. The molar ratio of metal ion to ATP giving maximal activation was approximately 1:2 for every ion studied.

Nucleotide specificity. Investigation of the capacity of phosphomevalonate kinase to utilize CTP, UTP, GTP, or ITP instead of ATP as substrates at concentrations in the range 1–10mM under otherwise standard incubation conditions showed that ATP was really the only effective substrate, although about 13% of the activity obtained with 5mM-ATP was obtained with 5mM-ITP and about 6% of the activity was obtained with 5mM-UTP; the enzyme was unable to utilize CTP or GTP. No activity at all was found when the above nucleotides were used to replace ATP in the standard incubation mixture of the 5-pyrophosphomevalonate decarboxylase.

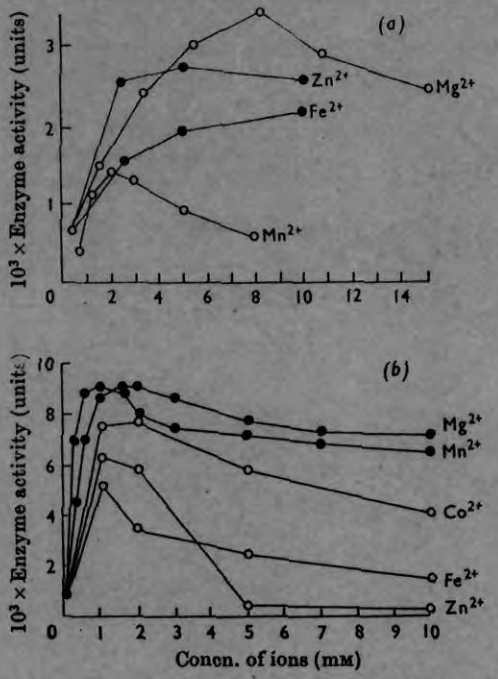

Fig. 3. Metal ion activation of (*a*) phosphomevalonate kinase (●, spectrophotometric assay; ○, radiochemical assay of 5-pyrophospho[2-^{14}C]mevalonate) and (*b*) pyrophosphomevalonate decarboxylase (●, spectrophotometric assay; ○, radiochemical assay of $^{14}CO_2$). Experimental details are given in the text.

pH-dependence of enzyme activity. Measurement of the rate of phosphomevalonate kinase action at different pH values under otherwise standard conditions by the spectrophotometric procedure showed (Fig. 4*a*) that the enzyme had a sharp optimum of activity at pH7.0, the overall pH–activity curve being similar to that previously found for mevalonate kinase (Williamson & Kekwick, 1965). The pH-stability of the enzyme was investigated by adjusting the pH of the standard incubation mixture appropriately, maintaining these values for 1h at 4°C and finally assaying the enzyme activity at pH7.0. It was found that the enzyme was particularly unstable to pH values below 6.0 and that activity was also lost at pH values above 7.5.

When the pH-dependence of the decarboxylase was investigated by the spectrophotometric assay of ADP a different relationship was obtained. This enzyme showed only a slight variation of activity with pH, having a broad optimum at about pH6.4.

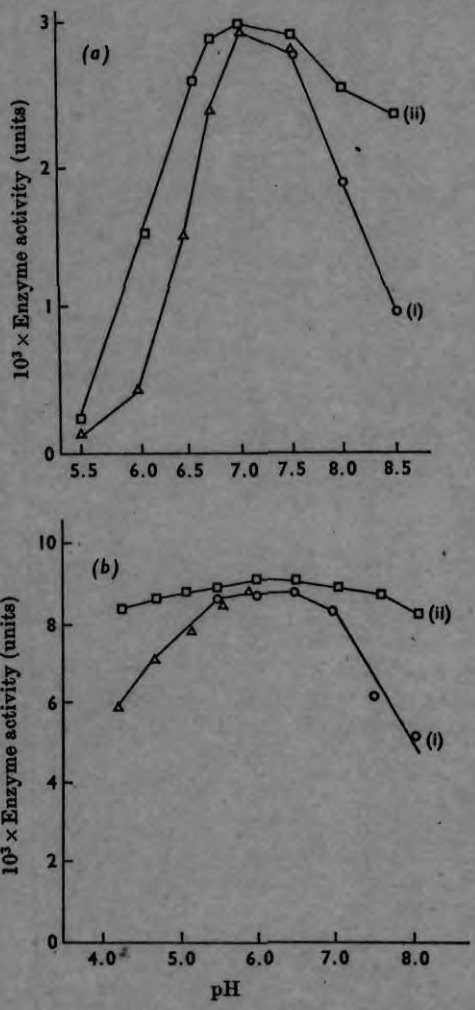

Fig. 4. Effect of pH on (*a*) phosphomevalonate kinase and (*b*) pyrophosphomevalonate decarboxylase. In (*a*) and (*b*) curve (i) represents the activity at the pH given and curve (ii) is the activity at pH 7.0 after exposure to the given pH for 1 h at 4°C. △, Potassium citrate–phosphate buffer; ○, potassium phosphate buffer.

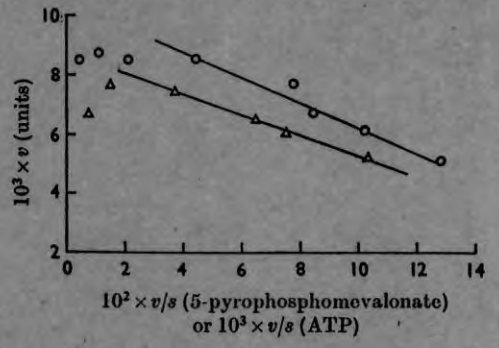

Fig. 5. Effect of substrate concentration on the activity of phosphomevalonate kinase. v is the initial reaction rate in μmol of ADP formed/min and s is substrate concentration (μM). ○, 5-Phosphomevalonate; △, ATP.

The enzyme activity at pH 6.4 was not greatly affected by prior adjustment to pH 4.0 or 8.0 (Fig. 4*b*).

Variation of reaction rate with substrate concentration. Measurement of the rate of action of the two enzymes at different concentrations of ATP and constant concentration of 5-phosphomevalonate or 5-pyrophosphomevalonate, and at various concentrations of 5-phosphomevalonate or 5-pyrophosphomevalonate and constant ATP concentrations, showed a linear relation between v and v/s (Fig. 5). This indicated that each reaction was probably being catalysed by only one enzyme. The K_m value for the phosphomevalonate kinase in the presence of ATP (5mM) was determined by the spectrophotometric procedure to be 0.042mM-5-phosphomevalonate and the maximum velocity was obtained at 2mM-5-phosphomevalonate with no inhibition by excess of substrate. Variation of the ATP concentration at constant concentration (0.2mM) of 5-phosphomevalonate produced a maximum rate at a concentration greater than 2mM-ATP; the K_m for ATP was 0.2mM, there being slight substrate inhibition at 10mM-ATP.

Measurement of the K_m values for 5-pyrophosphomevalonate decarboxylase proved somewhat difficult; preliminary experiments showed that the K_m value for 5-pyrophosphomevalonate was too low to be measured with adequate accuracy by the spectrophotometric assay of ADP produced. When, however, 5-pyrophospho[1-^{14}C]mevalonate was used as substrate and the $^{14}CO_2$ produced was measured by scintillation counting a maximum rate was obtained at 0.1mM-5-pyrophosphomevalonate in the presence of 2.5mM-ATP, there was no substrate inhibition up to 2.5mM-5-pyrophosphomevalonate, and the K_m was 0.004mM-5-pyrophosphomevalonate (Fig. 6).

When the effect of varying the ATP concentration on the rate of enzyme action was measured by both the radiochemical and the spectrophotometric procedures similar results were obtained. The activity was a maximum in 1.0mM-ATP, there was no inhibition by excess of substrate and K_m was 0.12mM-ATP (Fig. 6).

The effect of the reaction products on the enzyme was investigated by using 5-pyrophospho[1-^{14}C]-mevalonate as substrate and the radiochemical assay procedure; Table 5 shows that both ADP and isopentenyl pyrophosphate inhibit the action of the decarboxylase to some extent; 5mM-isopentenyl

pyrophosphate inhibits the enzyme almost completely, and the same concentration of ADP decreases its activity by about half. Although phosphomevalonate was slightly inhibitory, isopentenyl phosphates had no effect on the enzyme.

Temperature-dependence of enzyme activity. (*a*) Phosphomevalonate kinase. Preliminary experiments suggested that phosphomevalonate kinase was a heat-labile enzyme having increased K_m values at high temperatures. When the standard enzyme incubation mixture was incubated at different temperatures for different periods before assay at 30°C, nearly 70% of the activity at 30°C was lost after incubation for 30min at 40°C, and 30min at 45°C destroyed all the activity. The apparent energy of activation of the enzyme was measured by assaying the enzyme in a standard incubation mixture in which the concentration of 5-pyrophosphomevalonate had been increased to 0.5mM and that of ATP to 3.0mM to ensure saturation of the enzyme with substrate at all temperatures. The ADP produced was assayed spectrophotometrically after 30min incubation at 12 and 20°C and after 20min incubation at higher temperatures. The $V_{max.}$ values obtained at temperatures at which no denaturation had occurred gave an activation energy of 14.83kcal/mol when substituted in the Arrhenius equation. The optimum temperature for the preparation used was 40°C (Fig. 7).

(*b*) 5-Pyrophosphomevalonate decarboxylase. The thermal stability of 5-pyrophosphomevalonate decarboxylase was greater than that of the phosphomevalonate kinase. By using the spectrophotometric assay of ADP it was found that standard incubation mixtures lost only about one-third of their activity at 30°C after prior incubation for 30min at 40°C, and even after 30min at 60°C one-fifth of the initial activity remained. Measurement of $V_{max.}$ of standard incubation mixtures fortified with excess of 5-pyrophosphomevalonate (0.25mM) and ATP (2.5mM) at temperatures from 6 to 40°C showed that the apparent activation energy of the

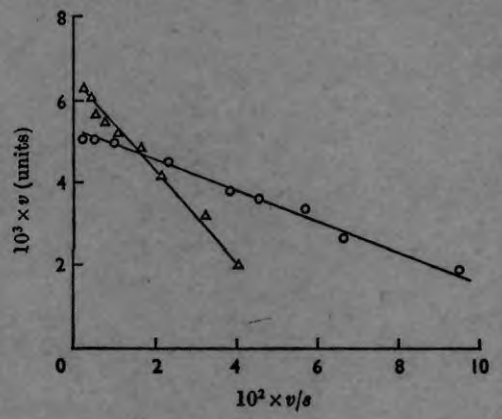

Fig. 6. Effect of substrate concentration on the activity of 5-pyrophosphomevalonate decarboxylase, v is the initial reaction rate in μmol of $^{14}CO_2$ formed/min and s is the substrate concentration (μM). ○, 5-Pyrophosphomevalonate; △, ATP.

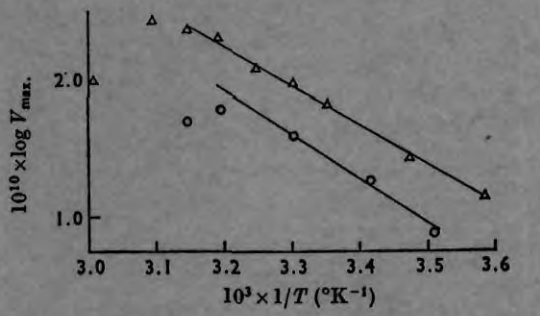

Fig. 7. Arrhenius plot showing the effect of temperature on phosphomevalonate kinase (○) and 5-pyrophosphomevalonate decarboxylase (△).

Table 5. *Effect of the reaction products, isopentenyl phosphate and 5-phosphomevalonate, on 5-pyrophosphomevalonate decarboxylase*

Enzyme activity was measured by assaying $^{14}CO_2$ formed. Incubations were for 5min at 30°C and contained: 5-pyrophosphomevalonate decarboxylase (0.05ml), 0.25mM-5-pyrophospho[1-^{14}C]mevalonate (0.1Ci/mol), 5mM-$MgCl_2$, 50mM-tris–maleate buffer, pH7.2, in a total volume of 1ml. The P_i was potassium phosphate buffer, pH7.2. Isopentenyl pyrophosphate and isopentenyl monophosphate were prepared by the procedure of Skilleter & Kekwick (1967).

	$10^3 \times$ Enzyme activity (units)				
Concn. of metabolite (mM) ...	0	1	2	5	10
Isopentenyl pyrophosphate	7.8	4.6	3.0	0.2	0.0
ADP	7.8	—	5.5	3.7	2.9
P_i	7.8	—	—	7.9	7.8
5-Phosphomevalonate	7.5	7.0	6.8	6.4	5.6
Isopentenyl phosphate	7.5	7.5	7.3	6.9	6.3

enzyme-catalysed reaction was similar to that of the phosphomevalonate kinase at 13.7 kcal/mol. The optimum temperature of the enzyme preparation was 50°C (Fig. 7).

DISCUSSION

Table 6 shows a comparison of the specific activities of the three enzymes concerned in the conversion of mevalonate into isopentenyl pyrophosphate in *Hevea* latex with the reported activities of these enzymes in yeast and pig liver. The activities of all three enzymes in latex are higher than in the other two tissues and latex is an unusually active source of 5-pyrophosphomevalonate decarboxylase; this must surely reflect the bias of latex metabolism toward polyisoprenoid formation.

The properties of *Hevea* latex phosphomevalonate kinase described above are quite similar to those reported by Hellig & Popják (1961*b*) for the pig liver enzyme. This is the only other preparation on which a number of kinetic properties have been studied. The K_m of *Hevea* phosphomevalonate kinase for 5-phosphomevalonate (0.04 mM) is somewhat lower than that of the pig liver enzyme (0.3 mM). The two enzymes have a similar restricted nucleotide specificity and are similarly activated by bivalent Mg^{2+} and Mn^{2+}, but unlike the *Hevea* enzyme pig liver phosphomevalonate kinase was completely inhibited by 5 mM-Zn^{2+}. The pH optima were similar.

Two markedly different characteristics of the *Hevea* enzyme from the pig liver enzyme were the lability at acid pH values and the requirement of thiol compounds for both activation and maintenance of activity. Although the pig liver enzyme was reported by Hellig & Popják (1961*b*) to be inhibited by *N*-ethylmaleimide and by *p*-chloromercuribenzoate, the enzyme was not greatly affected by iodoacetamide, and these workers did not report activation by thiols.

Little has been reported about the properties of 5-pyrophosphomevalonate decarboxylase from other sources. The bivalent ion requirement is similar to that of the pig liver enzyme (Hellig & Popják, 1961*a*) but the K_m value of 5-pyrophosphomevalonate is somewhat higher (4 μM) than that of pig liver (0.5 μM); such properties of the yeast enzyme as have been reported (Bloch *et al.* 1959) are similar to those of the *Hevea* enzyme.

It is difficult to use the kinetic results presented above to contribute to the discussion of the mechanism of 5-pyrophosphomevalonate decarboxylase initiated by Bloch *et al.* (1959). From the inhibition of 5-pyrophosphomevalonate decarboxylase by its reaction products it may be inferred that these are bound to the enzyme. This observation, taken with the finding that isopentenyl phosphate is not inhibitory, suggests strongly that it is the pyrophosphate moiety that is responsible for binding both the substrate (5-pyrophosphomevalonate) and the product (isopentenyl pyrophosphate) to the enzyme.

Bloch *et al.* (1959) have suggested the transitory existence of an intermediate in the decarboxylation reaction, phosphorylated on the 3-hydroxyl group of mevalonate, and have demonstrated the transfer of the oxygen of this hydroxyl group to P_i as a result of the decarboxylation reaction (Lindberg, Yuan, de Waard & Bloch, 1962). We investigated the possibility of obtaining a chemical phosphorylation of the 3-hydroxyl group with concomitant decarboxylation by attempting a chemical phosphorylation of 5-pyrophosphomevalonate with cyanoethyl phosphate in the presence of dicyclohexylcarbodiimide, by the procedure of Tener (1961). When the phosphorylation of both 5-pyrophospho[1-^{14}C]-mevalonate and 5-pyrophospho[2-^{14}C]mevalonate were separately attempted there was no evidence of decarboxylation and formation of $^{14}CO_2$ from the former material nor of [4-^{14}C]isopentenyl pyrophosphate from the latter substance.

Table 6. *Activity of the enzyme catalysing the formation of isopentenyl pyrophosphate from mevalonate in three tissues*

For pig liver, enzymes were assayed at 37°C. Data for mevalonate kinase are from Levy & Popják (1960) and for phosphomevalonate kinase from Hellig & Popják (1961*b*). For yeast, enzymes were assayed at 30°C. Data for mevalonate kinase are from Tchen (1958) and for phosphomevalonate kinase and pyrophosphomevalonate decarboxylase from Bloch *et al.* (1959). For *Hevea* latex serum, enzymes were assayed at 30°C. Data for mevalonate kinase are from Williamson & Kekwick (1965).

	$10^3 \times$ Specific activity (units/mg of protein)		
	Mevalonate kinase	5-Phosphomevalonate kinase	5-Pyrophosphomevalonate decarboxylase
Pig liver	8.8	3.9	—
Yeast (autolysate)	1.12	1.15	6.0
Hevea (latex serum)	11.5	4.6	43.2

We are grateful to the Natural Rubber Producers Research Association for a maintenance grant to D.N.S. and to the Science Research Council for a grant to R.G.O.K.

REFERENCES

Archer, B. L. & Sekhar, B. C. (1955). *Biochem. J.* **61**, 503.
Bloch, K., Chaykin, S., Phillips, A. H. & de Waard, A. (1959). *J. biol. Chem.* **234**, 2595.
Bray, E. A. (1960). *Analyt. Biochem.* **1**, 279.
Cherkin, A., Martinez, F. & Dunn, M. S. (1953). *J. Am. chem. Soc.* **75**, 1244.
Chesterton, C. J. & Kekwick, R. G. O. (1968). *Archs Biochem. Biophys.* **125**, 76.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Hellig, H. & Popják, G. (1961*a*). *Biochem. J.* **80**, 47P.
Hellig, H. & Popják, G. (1961*b*). *J. Lipid Res.* **2**, 235.
Levy, H. R. & Popják, G. (1960). *Biochem. J.* **75**, 417.
Lindberg, M., Yuan, C., de Waard, A. & Bloch, K. (1962). *Biochemistry, Easton*, **1**, 182.
Loomis, W. D. & Battaile, 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.
Skilleter, D. N. & Kekwick, R. G. O. (1967). *Analyt. Biochem.* **20**, 171.
Skilleter, D. N. & Kekwick, R. G. O. (1968). *Biochem. J.* **108**, 11P.
Skilleter, D. N., Williamson, I. P. & Kekwick, R. G. O. (1965). *Biochem. J.* **92**, 27P.
Snyder, F. & Godfrey, P. (1961). *J. Lipid Res.* **2**, 195.
Tchen, T. T. (1958). *J. biol. Chem.* **233**, 1100.
Tener, G. M. (1961). *J. Am. chem. Soc.* **83**, 159.
Williamson, I. P. & Kekwick, R. G. O. (1965). *Biochem. J.* **96**, 862.