

# Studies on the biosynthesis of cholesterol: XIII. phosphomevalonic kinase from liver

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## SUMMARY

The enzyme, phosphomevalonic kinase, which catalyzes the formation of 5-pyrophosphomevalonate from 5-phosphomevalonate and ATP has been purified from pig liver. The reaction is reversible, the position of equilibrium lying on the side of the forward reaction, and goes at optimum rate at pH 7.3 in the presence of 5 mM Mg<sup>++</sup>, 3.6 mM ATP, and 1 mM 5-phosphomevalonate. The effect of various metal ions and inhibitors on the enzyme is described. The K<sub>m</sub> for the enzyme is 0.3 mM. The preparation and some of the properties of 5-pyrophosphomevalonic acid are described.

**P**hosphomevalonic kinase catalyzes the reaction (-)5-phosphomevalonate + ATP ⇌ 5-pyrophosphomevalonate + ADP, the second biosynthetic step in the conversion of mevalonic acid into cholesterol. The enzyme has been partially purified from yeast (1, 2), and was also found in extracts of pig liver (3) during the purification of liver mevalonic kinase. This enzyme has now been purified further from pig liver and its properties studied in detail.

## MATERIALS AND METHODS<sup>1</sup>

**Biochemical Reagents.** The sources of all biochemical reagents (ADP, ATP, ITP, DPNH, PEP, pyruvic kinase from muscle, lactic dehydrogenase, unlabeled DL-mevalonic acid lactone, and DL-mevalonic acid lactone-2-C<sup>14</sup> and -1-C<sup>14</sup>) were those given in another communication (3). N-ethylmaleimide, PCMB, and *o*-iodosobenzoate were supplied by L. Light and Co., Colnbrook, England; iodoacetamide was obtained from British Drug Houses, Ltd., Poole, Dorset.

**Substrate.** Optically active (-) 5-P-MVA-2-C<sup>14</sup> and -1-C<sup>14</sup> (4) were made with purified liver mevalonic kinase according to Levy and Popják (3); the preparations were free from ATP, ADP, and from mevalonate.

<sup>1</sup>The following abbreviations are used: ADP and ATP, adenosine di- and triphosphate; ITP, inosine triphosphate; PEP, phosphoenolpyruvate; DPN and DPNH, diphosphopyridine nucleotide and its reduced form; BAL, 2,3-dimercaptopropanol; tris, tris(hydroxymethyl)aminomethane-HCl buffer; EDTA, ethylenediamine-tetraacetate; PCMB, *p*-chloromercuribenzoate; MVA, mevalonic acid; 5-P-MVA, 5-phosphomevalonic acid; 5-PP-MVA, 5-pyrophosphomevalonic acid.

The labeled 5-P-MVA preparations were made from C<sup>14</sup>-mevalonate with specific activities of either 0.2, 0.1, or 0.002 μc per μmole. The specific activities of the 5-P-MVA preparations were, of course, the same as those of the mevalonates. The 5-P-MVA with the very low specific activity was used as substrate in the spectrophotometric assays of phosphomevalonic kinase, and the preparations with the higher specific activities in the "radiochromatographic" method.

**Preparation of ATP<sup>32</sup>.** ATP, labeled with P<sup>32</sup> terminally, was made by the oxidative phosphorylation of ADP with P<sup>32</sup>-orthophosphate and rat liver mitochondria, which were prepared essentially according to the method of Schneider (5), and which showed strict respiratory control. Oxygen was bubbled through 30 ml of a 5 mM solution of K-phosphate buffer (pH 7.4, specific activity 13.3 μc P<sup>32</sup> per μmole) containing 5 mM potassium glutamate; the osmolarity of the solution was adjusted to 0.26 with KCl. After 2 minutes, 2 ml of the mitochondrial suspension in isotonic sucrose was added to the mixture and then 9 μmoles of ADP were introduced. After 1 minute incubation at 25°, the reaction mixture was deproteinized with ice-cold perchloric acid (5% final concentration). It was ascertained in preliminary small-scale experiments that all the ADP became phosphorylated in 1 minute under the above conditions. The ATP<sup>32</sup> was isolated from the deproteinized incubation mixture (after removal of perchloric acid as potassium perchlorate) by adsorption onto acid-washed charcoal (Nuchar C 190). Inorganic P<sup>32</sup> was removed by extensive washing of the charcoal with water, and

the ATP<sup>32</sup> eluted with 50% aqueous pyridine. The pyridine was extracted with ether and the preparation lyophilized; 6.5  $\mu$ moles of ATP<sup>32</sup> were obtained. The specimen was free from ADP and was contaminated with only 0.5% of inorganic P<sup>32</sup>. The ATP<sup>32</sup> was diluted for use with unlabeled nucleotide to a specific activity of 0.2  $\mu$ c per  $\mu$ mole.

*Calcium Phosphate Gel.* The calcium phosphate gel used for the adsorption of protein was made by the method of Tsuboi and Hudson (6). The suspensions contained 27 to 30 mg of dry matter per ml and were used 3 months after preparation.

*Paper Chromatography.* Two main systems were used for descending chromatography on Whatman No. 1 paper at 25° for about 16 hours. The solvent system A, isobutyric acid-aqueous NH<sub>3</sub> (sp gr 0.88)-water (66/3/30, v/v), gave the most reproducible R<sub>f</sub> values. Solvent B, *tert*-butanol-formic acid-water (40/10/16, v/v) (2), was used mainly on a semimicro preparative scale to separate the adenine nucleotides from 5-PP-MVA. The 1 $\frac{1}{2}$ -inch strips were scanned for radioactivity with an automatic recording device described previously (7). Chromatograms containing substances labeled with P<sup>32</sup> and C<sup>14</sup> were scanned twice, both with and without aluminum foil, 25 mg per cm<sup>2</sup>, placed between the strip and the G-M counters. With the foil, C<sup>14</sup> was not detectable and the efficiency of counting for the P<sup>32</sup> was reduced by only 7% of that obtained without foil. Alternatively, autoradiograms were made after exposing Ilfex X-ray films to the chromatograms for 70 hours. Nucleotides were detected by photographic printing of the strips with ultraviolet light (8).

*Measurement of C<sup>14</sup> and P<sup>32</sup>.* C<sup>14</sup> was measured with thin mica-window Geiger counters on samples of 2 cm<sup>2</sup> area and negligible thickness. The efficiency of counting was about 6%. P<sup>32</sup> was counted in a M.6 liquid counter<sup>2</sup> of 9 ml capacity, with an efficiency of 10.9%. The P<sup>32</sup> counts were corrected for the decay of the isotope.

*Crude Enzyme Preparations.* The initial extract from pig liver was prepared according to Hele and Finch (9) in the same way as for the preparation of mevalonic kinase (3). The crude extract, filtered through cheesecloth, was treated with protamine sulfate (1.5 mg per ml of extract), and the precipitate removed by centrifuging. The clear "protamine supernatant" served as starting material for the purification of the enzyme described under Results. All steps in the purification were carried out at 0° to 5°.

<sup>2</sup> The M.6 liquid counter was made by Twentieth Century Electronics, Ltd., King Henry's Drive, New Addington, Surrey, England.

*Enzyme Assays.* Two methods were used, either singly or together. In the radiochromatographic assay (3) the incubation mixture was deproteinized by heating it at 100° for 1 minute and chromatographing a sample in system A. The product, 5-PP-MVA, was identified by its R<sub>f</sub> of 0.21–0.27 (de Waard and Popják [10]). This was clearly distinguishable from 5-P-MVA (R<sub>f</sub> 0.36), mevalonic acid (R<sub>f</sub> 0.66), and mevalonolactone (R<sub>f</sub> 0.75). For quantitative work, the peaks recorded with the automatic scanner were copied onto typewriting paper and cut out and weighed.

The spectrophotometric assay of phosphomevalonic kinase measures in the presence of pyruvic kinase and lactic dehydrogenase the ADP generated during the reaction (2). The oxidation of DPNH at 37° was followed at 340 m $\mu$  in a Beckman DK-2 recording spectrophotometer using 3-ml cells with a 1-cm light path.

After determination of the optimum conditions for the action of the enzyme, the assay mixture was made to contain in a volume of 5.9 ml 0.1 M tris or K-phosphate buffer pH 7.35, NaF 10 mM, MgCl<sub>2</sub> 5 mM, 10 mM cysteine (neutralized with KOH), 0.6 mM PEP, 0.2 mM DPNH, 3.6 mM ATP (neutralized with KOH), 52  $\mu$ g lactic dehydrogenase, 200  $\mu$ g pyruvic kinase, and 2 mg bovine serum albumin. When very crude preparations were assayed, 30 mM nicotinamide was added to the reaction mixture. The phosphomevalonic kinase was added last. After incubation at 37° for 2 minutes, 2.9 ml of the reaction mixture was transferred to the test cuvette and the remainder to the control. One-tenth milliliter of substrate (containing 2.2 or 3.4  $\mu$ moles of 5-P-MVA) was then added to the test cuvette to initiate the reaction. The crude enzyme fractions still contained the 5-PP-MVA anhydrodecarboxylase which also utilizes ATP in the formation of isopentenylpyrophosphate (2). This did not invalidate the assay, as only initial rates were used to determine enzyme activity. When preparations were assayed for their mevalonic kinase content, the spectrophotometric method of Levy and Popják (3) was used. In the stoichiometric and equilibrium studies, ADP was determined spectrophotometrically by adding aliquots of the incubation mixture to the test cuvette containing 2.9 ml of the pyruvic kinase-lactic dehydrogenase assay system (see above) and excess DPNH.

*Standard Incubation Mixtures.* Unless stated otherwise, all incubation mixtures in the experiments on the properties of the enzyme were as described above when the spectrophotometric assay was used. When the radiochromatographic technique was employed, the incubation mixture in a volume of 1 ml contained 100 mM tris pH 7.35, 10 mM NaF, 5 mM MgCl<sub>2</sub>, 5

mM cysteine (neutralized with KOH), 3.6 mM ATP, and 1 mM 5-P-MVA (specific activity either 0.1  $\mu$ c per  $\mu$ mole, i.e., 16,000 cpm per  $\mu$ mole, or 0.2  $\mu$ c per  $\mu$ mole, i.e., 32,000 cpm per  $\mu$ mole). The reaction was started by the addition of phosphomevalonic kinase. These incubations were carried out at 37° for 1 hour, except when stated otherwise.

## RESULTS

*Purification of Phosphomevalonic Kinase.* Table 1 shows the purification of phosphomevalonic kinase in a typical experiment. To 3,140 ml of protamine super-

TABLE 1. PURIFICATION OF PHOSPHOMEVALONIC KINASE\*

Fraction	Protein	Specific Activity	Total Enzyme Units
	<i>g</i>		
Protamine supernatant	78.5	0.0053	415
Fraction II	18.9	0.0158	305
Gel supernatant	4.17	0.0366	146
Gel supernatant after acid treatment	1.67	0.078	130

\* Preparation from 1,600 g pig liver. The enzyme was assayed by the spectrophotometric method.

natant (see Methods), obtained from 1.6 kg of pig liver, there was added 720 g of solid  $(\text{NH}_4)_2\text{SO}_4$  to give 30% saturation. After standing for 1 hour, the precipitate (fraction I) was removed by centrifuging at  $2,000 \times g$ . To 3,350 ml of the supernatant there was added 355 g of  $(\text{NH}_4)_2\text{SO}_4$  (48% saturation). The precipitate (fraction II) was collected by centrifuging, and served as the main source of phosphomevalonic kinase. By the addition of 306 g of  $(\text{NH}_4)_2\text{SO}_4$  to the supernatant from fraction II, a further precipitate containing phosphomevalonic kinase was obtained, but the specific activity of the preparation was only one-half of that of fraction II, and was therefore discarded. The precipitates I and II were dissolved in 70 and 300 ml, respectively, of 20 mM K-phosphate buffer pH 7.6 containing 1 mM EDTA, and dialyzed for 4 hours against two changes of 10 mM K-phosphate buffer pH 7.6 containing 1 mM EDTA. Fraction I contained 390 units<sup>3</sup> of mevalonic kinase of relatively high specific

<sup>3</sup> One enzyme unit of mevalonic and phosphomevalonic kinase is defined as that amount of enzyme which phosphorylates 1  $\mu$ mole of substrate per minute at 37°, and the specific activity of the enzyme as  $\mu$ moles substrate phosphorylated per minute per milligram protein at 37°.

activity (0.098) and virtually no phosphomevalonic kinase. Fraction II contained 750 units of mevalonic kinase and 305 units of phosphomevalonic kinase in a volume of 525 ml. This fraction also contained traces of 5-pyrophosphomevalonic decarboxylase. The solution of fraction II containing 18.9 g protein was then treated with 13.32 g calcium phosphate gel which adsorbed very nearly all the mevalonic kinase, leaving the second kinase in the supernatant (720 ml), which was acidified in 30-ml batches to pH 5.2 with 2 N acetic acid. The inactive precipitate was centrifuged off and the pH of the supernatant adjusted to 7.4 with 1 N KOH. This preparation had a specific activity of 0.078 and was completely free of mevalonic kinase (which is partly denatured at low pH), but contained traces of 5-PP-MVA decarboxylase. This latter enzyme could be completely removed by a second  $(\text{NH}_4)_2\text{SO}_4$  fractionation, the kinase being recovered in the fraction precipitating between 30% and 45% saturation. This was done only for the preparations used for the equilibrium studies of the phosphomevalonic kinase reaction as it resulted in some loss of enzyme.

*Decarboxylase Assay.* The preparations were assayed for the 5-pyrophosphomevalonic decarboxylase by incubating in two Warburg flasks 1 ml of the standard incubation mixture, but using 5 mM ATP, plus 3 mM PEP, 35  $\mu$ g pyruvic kinase, and 1.05 mg of phosphomevalonic kinase, No. 12-2. To one flask, 1  $\mu$ mole of 5-P-MVA-2-C<sup>14</sup>, and to the other, 1  $\mu$ mole (32,000 cpm) of the 1-C<sup>14</sup> substrate was added. The incubations were carried out for the usual time under N<sub>2</sub>, then 2 N acetic acid was tipped in from the side bulb of the flask to liberate CO<sub>2</sub> which was trapped in 40% KOH and precipitated subsequently as BaCO<sub>3</sub> and counted. Both incubation mixtures were deproteinized and chromatographed. The BaCO<sub>3</sub> from the incubation with 5-P-MVA-1-C<sup>14</sup> contained a total of 1,120 cpm. This amount of decarboxylase did not affect the determination of optimum conditions for the activity of phosphomevalonic kinase.

*Enzyme Preparations.* In the various experiments to be described, four preparations of phosphomevalonic kinase were used: No. 10-5, specific activity 0.041; Nos. 10-6, specific activity 0.069, and 9-6, specific activity 0.051, both free of decarboxylase; and No. 12-2, specific activity 0.078.

*Properties of Phosphomevalonic Kinase.* Although the spectrophotometric assay can be used with crude preparations, it was not entirely satisfactory with such preparations because a rereduction of DPN occurred. With the purified enzyme the initial rates were always linear, the assays were reproducible, and the rate of reaction was proportional to the protein

concentration up to about 1.7 mg per ml, which is in excess of the amounts commonly used in the assays (Fig. 1). The enzyme lost only 20% of its activity during storage of its solution (10 mg protein per ml) at  $-15^{\circ}$  for 6 months.

**Effect of pH.** Figure 2 shows the dependence of reaction rate on pH in K-phosphate and tris-HCl buffers as determined by the spectrophotometric assay. The pH optimum lies between 7.2 and 7.3, the rate being reduced by half at pH's 5.8 and 7.8.

**Coenzyme Requirements.** Figure 3 shows the requirement for ATP. The highest rate of the reaction was observed with 3.6 mM ATP in the presence of 5 mM  $Mg^{++}$ ; concentrations of ATP up to 9.3 mM were not inhibitory. ITP could not replace ATP as a phosphate donor, as determined by the radiochromatographic assay.

**Metal Ion Requirements.** The enzyme needs a divalent metal ion for activation. Figure 4 demonstrates the superiority of  $Mg^{++}$  over  $Mn^{++}$  as the activating ion, the optimum concentration being 5 mM. Higher concentrations were inhibitory.  $Mn^{++}$  could not effectively replace  $Mg^{++}$ , as at the optimum  $Mn^{++}$  concentration of 3 mM the rate was only about 20% of that obtained with 5 mM  $Mg^{++}$ . The effectiveness

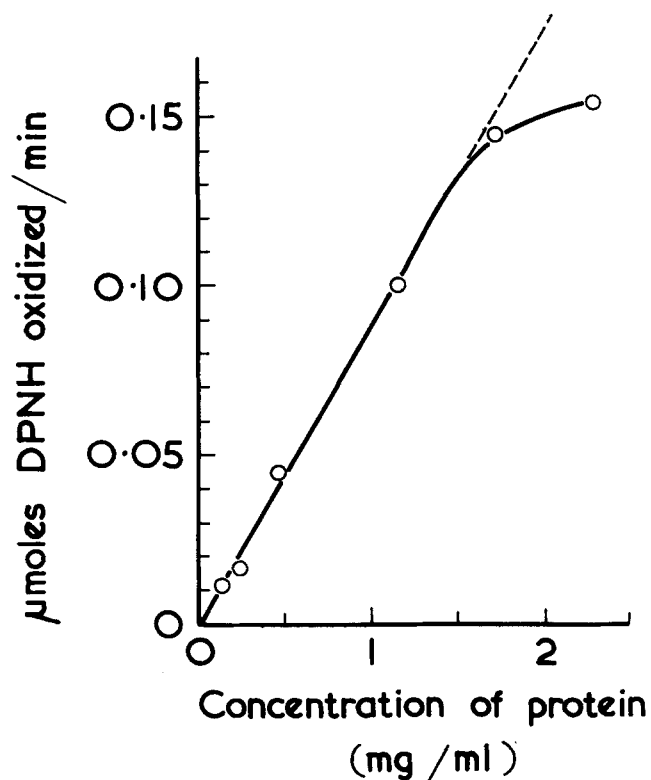


FIG. 1. Proportionality between protein concentration and rate of phosphorylation of 5-P-MVA as measured by the spectrophotometric assay. Concentration of ATP, 2 mM and of 5-P-MVA, 0.73 mM. Enzyme preparation No. 10-5.

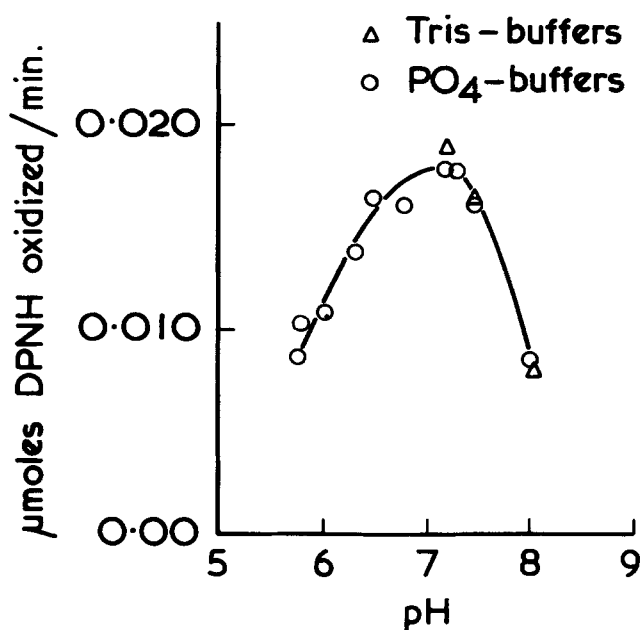


FIG. 2. Effect of pH on phosphomevalonic kinase activity. Standard spectrophotometric assays were used except that the concentration of ATP was 7.5 mM; and of 5-P-MVA, 0.73 mM. Enzyme preparation No. 12-2, 0.6 mg per assay.

of  $Mn^{++}$  was measured by the spectrophotometric assay in the presence of 0.13 mM  $Mg^{++}$  needed for the activation of the assay system. The "blank" value for this  $Mg^{++}$  concentration was subtracted from the final rates before plotting the graph. Cysteine was not used in the assay because it forms an insoluble complex with  $Mn^{++}$ .

The effects of various other metal ions on the phosphomevalonic kinase were tested in the presence of 5 mM  $Mg^{++}$  by the radiochromatographic assay.  $Ag^{+}$ ,  $Cu^{++}$ , and  $Zn^{++}$  in 5 mM concentration completely inhibited the reaction, and  $Co^{++}$  was without effect.

**Effect of Inhibitors.** Standard 1-ml incubations were set up for the radiochromatographic assay without cysteine, using 1 mg of enzyme preparation 10-5 and 1  $\mu$ mole of 5-P-MVA-2- $C^{14}$ , specific activity 0.1  $\mu$ c per  $\mu$ mole. The results are shown in Table 2. PCMB was the most potent inhibitor, its effect being partially reversed with 10 mM BAL or glutathione. Ten millimolar EDTA produced 61% inhibition, but KCN was without effect. It is of interest that although 5 mM N-ethylmaleimide and 5 mM *o*-iodosobenzoate caused 88% inhibition, 5 mM iodoacetamide gave only slight (9%) inhibition.

**Effect of Substrate Concentration.** The effect of changing the substrate concentration was determined by using the spectrophotometric assay. Figure 5 shows the increase in rate of reaction with increased concen-

trations of (-)5-P-MVA up to about 0.85 mM. The apparent Michaelis constant is approximately 0.3 mM.

**Equilibrium and Reversibility of Reaction.** The phosphomevalonic kinase reaction goes to completion only when the ADP formed during the reaction is rephosphorylated to ATP with phosphoenolpyruvate and pyruvic kinase. This was demonstrated both by spectrophotometric and radiochromatographic assays. When the ATP is not being regenerated, only a part of the 5-phosphomevalonate is phosphorylated to 5-pyrophosphomevalonate. In order to establish the equilibrium of the reaction, standard incubations for the radiochromatographic assay were set up with two phosphomevalonic kinase preparations, which were free from 5-pyrophosphomevalonic decarboxylase. The concentration of substrate (5-P-MVA-2-C<sup>14</sup>) was 1 mM, and that of ATP was varied from 3 to 7.5 mM. The incubations were continued beyond the time needed for complete phosphorylation of the substrate when ATP was regenerated with PEP and pyruvic kinase; the reaction was therefore considered to have reached equilibrium. Table 3A and Figure 6 show the equilibrium to be well on the side of the forward reaction.

When in experiments, similar to those described above, 1  $\mu$ mole of 5-PP-MVA-2-C<sup>14</sup> (see preparation of 5-PP-MVA-2-C<sup>14</sup>) and ADP were incubated with phosphomevalonic kinase without ATP, the reaction was reversed and 5-P-MVA and ATP were formed (Table 3B and Fig. 7). The reversibility of the phosphomevalonic kinase reaction was reported previously by both Lynen's and Bloch's groups working with the yeast enzyme (1, 2).

**Stoichiometry of the Phosphomevalonic Kinase Reaction.** A 3-ml standard incubation for radiochromato-

TABLE 2. EFFECT OF VARIOUS SUBSTANCES ON PHOSPHOMEVALONIC KINASE\*

Reagent	Concentration	Inhibition
	mM	per cent
EDTA	10	61
KCN	10	0
Iodoacetamide	5	9
N-ethylmaleimide	5	88
Na arsenite	5	0
BAL	10	0
Glutathione	10	0
Cysteine	10	0
PCMB	0.1	100
PCMB	1.0	100
PCMB + BAL	1.0 + 10.0	32
PCMB + glutathione	1.0 + 10.0	10
<i>o</i> -Iodosobenzoate	5	88

\* Standard radiochromatographic assays were used with 1 mg enzyme No. 10-5 and 1 mM 5-P-MVA-2-C<sup>14</sup>.

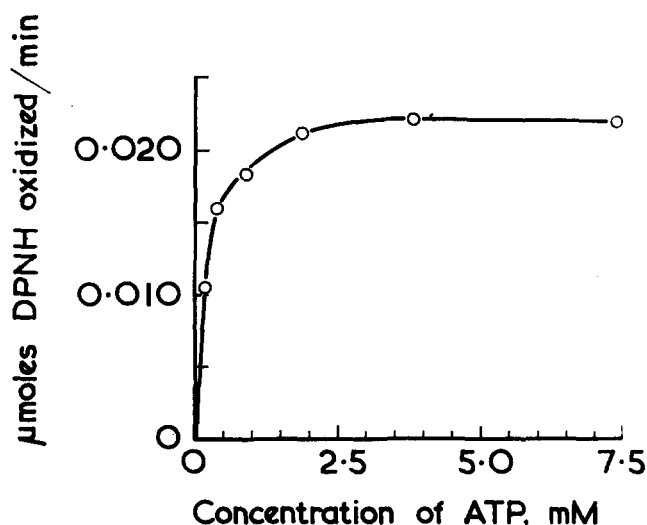


FIG. 3. Effect of ATP concentration on phosphomevalonic kinase reaction. Standard spectrophotometric assays were used with varying ATP concentration, 5-P-MVA, 0.73 mM; enzyme preparation No. 12-2, 0.6 mg per assay.

graphic assay was set up containing 1 mM 5-P-MVA-2-C<sup>14</sup>, 3 mM ATP, and 1 mg of enzyme preparation No. 10-5. At intervals aliquots of the incubation mixture were assayed for ADP spectrophotometrically and for extent of reaction by radiochromatography. As is shown in Table 3C, the amount of ADP produced was equal to the amount of 5-PP-MVA formed.

In another experiment 1 mM 5-P-MVA-2-C<sup>14</sup> (specific activity 0.2  $\mu$ c per  $\mu$ mole) was incubated with 5 mM P<sup>32</sup>-ATP (specific activity 0.2  $\mu$ c per  $\mu$ mole) and with 1 mg of preparation No. 9-6 in a 1-ml standard

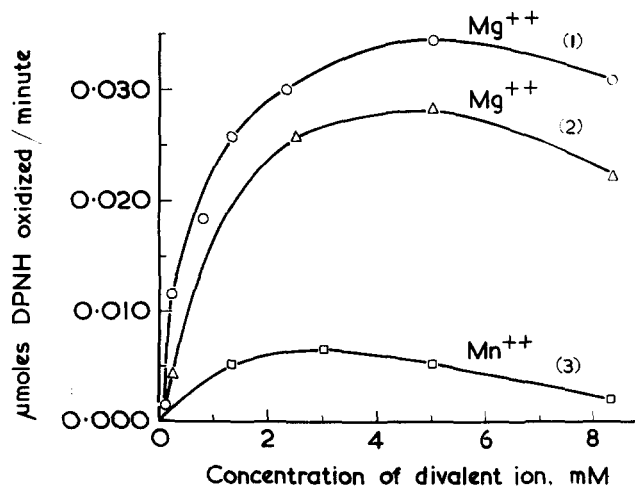


FIG. 4. Effect of Mg<sup>++</sup> and Mn<sup>++</sup> ions on phosphomevalonic kinase reaction. Standard spectrophotometric assays were used without cysteine, 5-P-MVA, 1.1 mM; enzyme preparation No. 12-2, 1.0 mg per assay. The data of curve (1) were obtained with the fresh preparation and those for curves (2) and (3) with the same preparation after 160 days of storage in solution at -15°.

TABLE 3. THE EQUILIBRIUM, REVERSIBILITY, AND STOICHIOMETRY OF THE PHOSPHOMEVALONIC KINASE REACTION

A. Equilibrium of Reaction\*

Expt.	Time of Incubation	ATP Concentration	Conversion of 5-P-MVA to 5-PP-MVA
	<i>minutes</i>	<i>mM</i>	<i>per cent</i>
1	60	3.63	76
2	120	3.00	76
3	120	5.00	79
4	120	7.50	79

B. Reversibility of Reaction\*

Expt.	Time of Incubation	ADP Concentration	Conversion of 5-PP-MVA to 5-P-MVA
	<i>minutes</i>	<i>mM</i>	<i>per cent</i>
1	60	5	29
2	120	4	39
3	120	7.5	44
4	120	0.0	0

C. Stoichiometry of Reaction\*

Expt.	Time of Incubation	5-PP-MVA Formed	ADP Formed
	<i>minutes</i>	<i>μmoles</i>	<i>μmoles</i>
1	16.5	0.32	0.30
2	28.0	0.45	0.46

\* Experimental conditions are described in the text.

incubation mixture. The products were then assayed by the radiochromatographic method after development of the chromatograms in solvent system B which separates 5-P-MVA, 5-PP-MVA, and the adenine nucleotides satisfactorily. The chromatograms were scanned for  $C^{14}$  and for  $P^{32}$ ; the 5-PP-MVA formed contained  $P^{32}$ , the ratio of  $C^{14}$  to  $P^{32}$  in it being 0.93.

All these experiments fully establish the reaction catalyzed by phosphomevalonic kinase as:

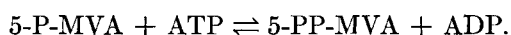


TABLE 4. PROGRESS OF HYDROLYSIS OF 5-PP-MVA AND OF 5-P-MVA TO MVA BY 0.5 N HCL AT 100°

Time of Hydrolysis	5-PP-MVA	5-P-MVA	MVA
<i>minutes</i>	<i>μmoles</i>	<i>μmoles</i>	<i>μmoles</i>
0	0.90	0.10	0.0
4	0.60	0.40	0.0
8	0.30	0.70	0.0
23	0.00	0.00	1.0

*Preparation of 5-Pyrophosphomevalonate.* A 300-ml incubation was set up containing reactants in the following final concentrations: tris, pH 7.35, 0.1 M; cysteine (neutralized with KOH), 10 mM; NaF, 10 mM;  $MgCl_2$ , 5 mM; ATP, 3.6 mM; PEP, 1.5 mM; pyruvic kinase, 35  $\mu g$  per ml; 5-P-MVA-2- $C^{14}$  (specific activity 0.1  $\mu c$  per  $\mu mole$ ), 1 mM; and a total of 280 mg phosphomevalonic kinase preparation No. 10-5. After 1 hour incubation at 37° the reaction mixture was deproteinized by heating in a boiling water bath for 90 seconds. According to radiochromatographic assay, all the 5-P-MVA-2- $C^{14}$  was used up and the products of the reaction were 5-PP-MVA-2- $C^{14}$  and a trace of isopentenylpyrophosphate and of ADP (ATP was regenerated with PEP and pyruvic kinase). The main problem in the purification of 5-PP-MVA was therefore its separation from relatively large amounts of ATP. For this purpose the deproteinized incubation was lyophilized, and the residue taken up in a small volume of water; insoluble material was removed by centrifugation. The clear solution was cooled in ice and its pH adjusted to 6.0. Then ethanol was added slowly to a concentration of 75%. The precipitate formed (mostly ATP and salts) was washed repeatedly with 75% ethanol, and the washings were combined with the main ethanol supernatant. It is important to adjust the pH of the solution to 6.0 before the ethanol precipitation, and also not to use concentrations of ethanol higher than 75%, otherwise a substantial proportion of the 5-PP-MVA may be precipitated with the ATP and salts. After removal of ethanol on a rotary evaporator at room temperature the aqueous solution was made alkaline (pH 8.5) with KOH, and 550  $\mu moles$  of  $BaCl_2$  was added, which removed further amounts of ATP and only a little of 5-PP-MVA. After separation of the Ba salt of ATP by centrifugation, a further 500  $\mu moles$  of  $BaCl_2$  and ethanol to a concentration of 50% were added to the solution in order to precipitate 5-PP-MVA as the Ba salt, which was collected by centrifugation and washed with 50% ethanol and absolute ethanol. The Ba salt of 5-PP-MVA was then dissolved in a minimum amount of dilute HCl and the Ba precipitated by the addition of a  $Na_2SO_4$  solution. After removal of the  $BaSO_4$  precipitate, the pH of the solution was adjusted to 6.9 with KOH. Assay of the preparation showed it to contain 170  $\mu moles$  of 5-PP-MVA-2- $C^{14}$  free of any other radioactive substance, but contaminated with 85  $\mu moles$  of ATP. The residual ATP was removed by differential adsorption onto charcoal. The solution of the 5-PP-MVA was diluted to a concentration of 1.38 mM. Acid-washed charcoal (Nuchar C 190), 70 mg for each

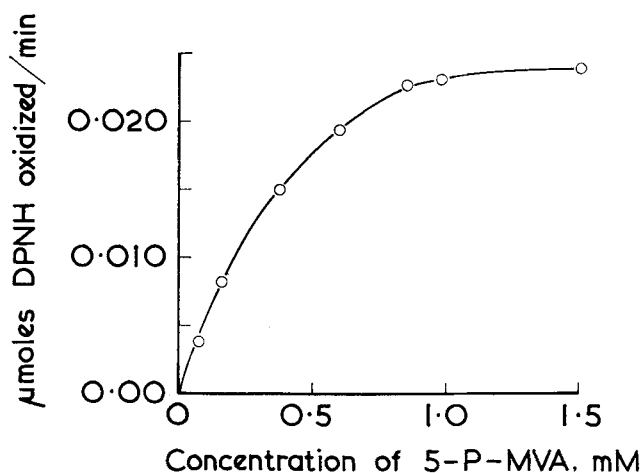


Fig. 5. Effect of substrate concentration on phosphomevalonic kinase reaction. Standard spectrophotometric assays were used with preparation No. 12-2, 0.6 mg per assay.

10 ml, was then stirred into the solution and removed by filtration after about 30 minutes. Preliminary small-scale experiments showed that the ATP was adsorbed first onto the charcoal, and that the small amounts of 5-PP-MVA that were also adsorbed could be eluted with water. After this simple treatment most of the ATP was removed, and only about 10% of the 5-PP-MVA was lost. For purposes of special experiments, e.g., for the study of the reversibility of the phosphomevalonic kinase reaction, when 5-PP-MVA completely free of ATP was needed, samples of the preparation were chromatographed on Whatman No. 1 paper in solvent system B in which 5-PP-MVA and ATP have  $R_f$  values of 0.33 and 0.10, respectively. The 5-PP-MVA was eluted from the paper with dilute aqueous ammonia. 5-PP-MVA, free from ATP, could also be prepared by the use of larger amounts of

charcoal during the differential adsorption procedure or by repeating the Ba-precipitation; both these techniques, however, caused 20% to 30% losses of 5-PP-MVA.

*Characterization of 5-Pyrophosphomevalonate.* This compound is identical with "Compound II" of Chaykin *et al.* (11) and with that described by de Waard and Popják (10) as diphosphomevalonate, and characterized by Bloch *et al.* (2) and Henning *et al.* (1) as the 5-pyrophosphomevalonate. The behavior of this substance, both on paper chromatograms and on ion

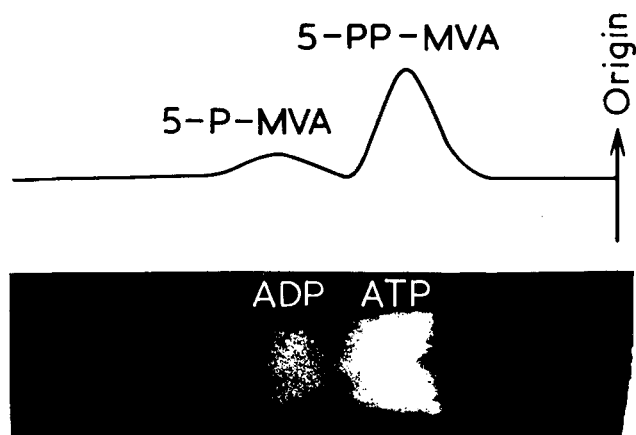


Fig. 6. Formation of ADP from ATP during phosphorylation of 5-P-MVA to 5-PP-MVA. The ATP used in the incubation contained no ADP as determined spectrophotometrically with the pyruvic kinase-lactic dehydrogenase assay system for ADP.

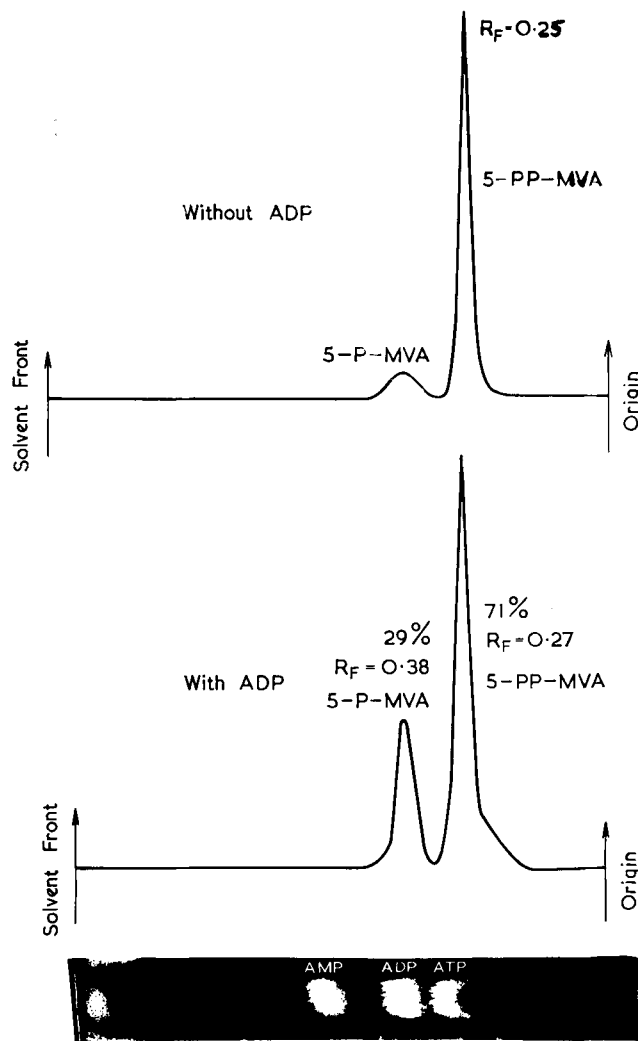


Fig. 7. Radiochromatographic evidence on the reversibility of phosphomevalonic kinase reaction. Standard 1-ml incubations were made with 1  $\mu$ mole of 5-PP-MVA-2- $C^{14}$  without and with 5  $\mu$ moles of ADP. After 1 hour incubation aliquots of the deproteinized incubations were chromatographed in solvent system A, and the paper strips scanned for radioactivity and printed with ultraviolet light. The lower graph and the ultraviolet print from the chromatogram shows that both 5-P-MVA and ATP were formed from 5-PP-MVA and ADP. The ADP used was free of ATP, but was contaminated with AMP (Expt. 1 of Table 3B).

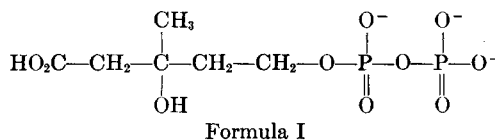
exchange columns, has been described in detail (10). 5-PP-MVA is resistant to hydrolysis by 1 N KOH at 100° for 5 minutes, but it is hydrolyzed in 0.5 N HCl at 100° to MVA with 5-P-MVA as intermediate (Table 4). The mevalonic acid formed from the hydrolysis of 1  $\mu$ mole of 5-PP-MVA-2-C<sup>14</sup> (specific activity 16,000 cpm per 1  $\mu$ mole) was mixed with 63.5 mg of unlabeled mevalonic acid and the benzhydrylamide prepared (12) from the mixture. After recrystallization, the benzhydrylamide (m.p. 96°) had a specific activity of 130 cpm per  $\mu$ mole.

*5-Pyrophosphomevalonate as Precursor of Allyl Pyrophosphates.* It has already been shown that 5-PP-MVA-2-C<sup>14</sup> is converted into cholesterol more efficiently than DL-MVA-2-C<sup>14</sup>, but that ATP was still needed in this conversion (10). Supplementing this evidence is our finding that 5-PP-MVA-2-C<sup>14</sup> is readily converted into C<sup>14</sup>-allyl pyrophosphates (mainly the pyrophosphates of geraniol and of farnesol), which were shown to be the precursors of squalene in liver (13) as well as in yeast enzyme preparations (14). A 1-ml incubation mixture was set up containing 1  $\mu$ mole of 5-PP-MVA-2-C<sup>14</sup> (specific activity 32,000 cpm per  $\mu$ mole), 100  $\mu$ moles of tris buffer, pH 7.4, 10  $\mu$ moles of NaF, 5  $\mu$ moles of MgCl<sub>2</sub>, 5  $\mu$ moles of ATP, and 14 mg of a soluble enzyme fraction (F<sub>30</sub><sup>60</sup>-enzymes) made from rat liver and which was shown to convert DL-MVA-2-C<sup>14</sup> or (-)5-P-MVA-2-C<sup>14</sup> into the allyl pyrophosphates. The mixture was incubated at 37° for 1 hour, at the end of which it was analyzed for allyl pyrophosphates according to the procedure of Goodman and Popják (13). One-half of the substrate (16,060 cpm) was converted into these substances, most of the radioactivity (85%) being associated with farnesyl pyrophosphate and a little with geranyl pyrophosphate.

#### DISCUSSION

Phosphomevalonic kinase is closely associated in liver extracts with mevalonic kinase; its separation from the latter enzyme is rendered the more difficult because usually one-quarter to one-half as much phosphomevalonic kinase as mevalonic kinase is found in the extracts. These two kinases differ from one another in that only ATP can serve as coenzyme for phosphomevalonic kinase, whereas liver mevalonic kinase functions with ATP and ITP almost equally well (3), and also in that Ca<sup>++</sup>, which can activate mevalonic kinase, cannot activate the phosphokinase. The affinity of the phosphomevalonic kinase for its substrate (5-phosphomevalonate) is appreciably lower ( $K_m = 3 \times 10^{-4}$ M) than the affinity of mevalonic

kinase for mevalonate ( $K_m = 5 \times 10^{-5}$ M). This circumstance suggests that the phosphokinase may be rate limiting in the conversion of mevalonate into squalene, although the favored equilibrium of the reaction may partially counteract this. As the phosphomevalonic kinase reaction is reversible, it is evident that the second phosphate bond in 5-PP-MVA is an energy-rich bond, probably of the same order of magnitude as that of ATP. The values for the free energy changes in the phosphomevalonic kinase reaction could not be calculated from our equilibrium data, as the "activities" of the phosphorylated derivatives of mevalonate in solutions of high ionic strength are unknown. All our data on 5-PP-MVA (the transfer of the terminal phosphate group of ATP to 5-P-MVA, reversibility of the phosphomevalonic kinase reaction, the formation of 5-P-MVA and of MVA from 5-PP-MVA by acid hydrolysis) are entirely consistent with earlier conclusions (1, 2) that in "diphosphomevalonate" (10) the second phosphate group is in an acid anhydride linkage with the phosphate in 5-P-MVA and that the product of the phosphomevalonic kinase reaction is the 5-diphospho(pyrophospho)mevalonate (Formula I).



The properties of liver phosphomevalonic kinase differ from the similar enzyme studied in yeast (1, 2). Whereas the liver enzyme showed a distinct pH optimum around 7.3, it was reported (2) for the yeast enzyme that its activity did not vary in the pH range of 5.5 to 10. Also, yeast phosphomevalonic kinase was activated by Zn<sup>++</sup> almost as well as by Mg<sup>++</sup>, whereas for liver mevalonic kinase, Zn<sup>++</sup> ions were severely inhibitory.

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