Studies on the Biosynthesis of Cholesterol

10. MEVALONIC KINASE AND PHOSPHOMEVALONIC KINASE FROM LIVER*

By H. R. LEVY† AND G. POPJÁK

Medical Research Council, Experimental Radiopathology Research Unit,
Hammersmith Hospital, London, W. 12

(Received 6 October 1959)

After the discovery that mevalonic acid was a key intermediate in the biosynthesis of cholesterol (Tavormina, Gibbs & Huff, 1956), it was shown that liver preparations (Popják, Gosselin, Gore & Gould, 1958) as well as yeast extracts (Amdur, Rilling & Bloch, 1957; Lynen, 1959) required adenosine triphosphate, Mg^{2+} or Mn^{2+} and reduced pyridine nucleotides to convert mevalonate into squalene. The need for adenosine triphosphate suggested the formation of phosphorylated intermediates from mevalonate; the first ones to be identified were 5-phosphomevalonate and 5-diphosphomevalonate in yeast (Tchen, 1957; Chaykin, Law, Phillips, Tchen & Bloch, 1958; Lynen, 1959) as well as in rat-liver preparations (de Waard, 1958; de Waard & Popják, 1959; cf. Popják, 1959).

We have isolated from pig liver the enzyme catalysing the first of these phosphorylations and studied some of its properties. Shortly after this work was begun Tchen (1958) reported the partial purification of the same enzyme from yeast and named it 'mevalonic kinase'. During our purification procedures we encountered protein fractions which formed not only the 5-phosphomevalonate (5-P-MVA), but also the 5-diphosphomevalonate (mevalonic acid 5-pyrophosphate; 5-PP-MVA) from mevalonate and adenosine triphosphate. When 5-phosphomevalonate was added to such enzyme fractions, together with adenosine triphosphate and Mg^{2+} , 5-PP-MVA was obtained according to the reaction:

This second enzyme, which we propose to call 'phosphomevalonic kinase', was completely removed from mevalonic kinase. A brief account of this work has already been published (Levy & Popják, 1959). A large-scale preparation of 5-phospho[2- ^{14}C]mevalonate with mevalonic kinase is now described also.

* Part 9: de Waard & Popják (1959).

† Postdoctoral Fellow of the National Cancer Institute, National Institutes of Health, United States Public Health Service.

MATERIALS AND METHODS

Biochemical reagents. The following compounds and enzymes were obtained from the Sigma Chemical Co., St Louis, Missouri, U.S.A.: adenosine triphosphate (ATP), cytidine triphosphate, guanosine triphosphate and inosine triphosphate; reduced diphosphopyridine nucleotide (DPNH), about 65% pure; sodium phosphoenolpyruvate (PEP); crystalline phosphoenolpyruvic kinase from muscle (PEP-kinase); lactic dehydrogenase from rabbit muscle and 2-amino-2-hydroxymethylpropane-1:3-diol (tris). Adenosine diphosphate (ADP) was the preparation of Pabst Laboratories, Milwaukee, Wisconsin, U.S.A. Protamine sulphate (salmine sulphate) was obtained from British Drug Houses Ltd., Poole, Dorset, and crystalline plasma albumin from Armour and Co., Chicago, U.S.A.

DL-Mevalonic acid lactone and other substrates. DL-Mevalonic acid lactone, synthesized according to the method of Cornforth, Cornforth, Popják & Gore (1958), was the gift of Dr J. W. Cornforth and Dr Rita H. Cornforth. It was converted into the potassium salt by dissolving in water, adding a small excess of KOH and keeping for 30 min. at 37°. DL-[2- ^{14}C]Mevalonolactone was obtained from the Radiochemical Centre, Amersham, Bucks, and was also converted into the potassium salt; its specific activity was adjusted to 0.2 $\mu C/\mu\text{mole}$ by dilution with unlabelled material. The concentration of the potassium DL-mevalonate (whether labelled or not) solutions was 0.1 M.

The specimens of the anhydro analogues of mevalonate [*cis*-5-hydroxy-3-methylpent-2-enoic acid and 5-hydroxy-3-methylpent-3-enoic acid (mixture of *cis* and *trans*)] were those used in previous work (Cornforth *et al.* 1958). 3-Hydroxy-3-methylglutaric acid, 3-oxobutan-1-ol and farnesoic (farnesinic) acid were the generous gifts of Dr J. W. Cornforth and Dr Rita H. Cornforth. The farnesoic acid (3:7:11-trimethyldodeca-2:6:10-trienoic acid, mixture of *trans-trans* and *cis-trans* acids) was prepared by the slow hydrolysis (at 4°) with KOH of ethyl farnesoate synthesized from *trans*-geranylacetone. Gas-liquid chromatography of the ethyl farnesoate showed the presence, in equal amounts, of two components only, corresponding to the esters of the *trans-trans* and *cis-trans* acids. 3:7:11-Trimethyldodecanoic acid was prepared by the catalytic hydrogenation of the ethyl farnesoate preparation, followed by saponification and extraction of the free acid; it was used as the potassium salt. Gas-liquid chromatography of the methyl ester of this acid showed a single component. A specimen of this same substance was also generously provided by Dr Karl Folkers of the Merck, Sharp and Dohme Research Laboratories, Rahway, New

Jersey, U.S.A. The method of preparation of this specimen is not known to us, but it was identical with our preparation and was of the same purity as determined by gas-liquid chromatography. A specimen of farnesol, prepared by the allylic rearrangement of nerolidol, was kindly provided by Dr M. Stoll of Firmenich et Cie, Geneva, Switzerland; it contained 56.8% of *trans-trans*-farnesol, 31.8% of *cis-trans*-farnesol, and 11.4% of nerolidol as revealed by gas-liquid chromatography.

Chromatographic procedures. The methods of chromatographic separation of mevalonic acid, mevalonic acid lactone, 5-phosphomevalonate and of 5-diphosphomevalonate on paper and on Dowex-2 formate ion-exchange resins were those previously described (de Waard, 1958; de Waard & Popják, 1959). The principal solvent system in paper chromatography used by us was the *isobutyric acid*-aq. NH_3 soln. (sp.gr. 0.88)-water system (66:3:30, by vol.). The paper-chromatographic strips were analysed for radioactive spots with an automatic scanning device as described previously (Hele, Popják & Laurysse, 1957); the areas under the curves obtained in such records are proportional to the total radioactivity contained in the spots.

Assay of ^{14}C . The measurements of ^{14}C were made with thin mica-window Geiger counters on samples of 2 cm.² area and negligible thickness. The efficiency of counting was about 6%.

Crude enzyme preparations. The dialysed soluble protein fraction of rat-liver homogenates [after precipitation with $(\text{NH}_4)_2\text{SO}_4$] described previously (Popják *et al.* 1958), was used in the early experiments, but purification of mevalonic kinase from such extracts was not attempted.

For the purification and large-scale preparation of mevalonic kinase, pig liver, obtained immediately after slaughter and brought to the laboratory on ice, was extracted according to the method of Finch & Hele (1959) with a sucrose medium of the following composition: sucrose, 0.35M; KHCO_3 , 0.035M; ethylenediaminetetraacetate, 0.001M; and KCl, 0.025M. In this method the liver, in 500 g. batches, is finely chopped with a sharp knife and is then extracted with 2 vol. of the sucrose medium by gentle stirring for 90 sec., and the large particles are removed from the extract by filtering through two layers of cheese-cloth. The volume of this filtrate ('the initial extract') was about the same as the volume of the sucrose medium used and contained 60–80 mg. of protein/ml. as determined by the biuret reaction (Gornall, Bardawill & David, 1949); it served as the starting material for the purification. Its further treatment is described under Results. All procedures of extraction and fractionation were done at 0–5°.

The calcium phosphate gel used for adsorption of protein was made according to the method of Tsuboi & Hudson (1957). All chemicals not specifically mentioned were of analytical grade.

Enzyme assays. Two methods were used. In the first method ('radiochemical assay') the amount of the phosphorylated derivative(s) formed from ^{14}C -labelled substrates (DL-[2- ^{14}C]mevalonate, 5-phospho[2- ^{14}C]mevalon-

ate) was measured by paper chromatography of samples of the incubation mixtures deproteinized with HClO_4 as described previously (de Waard & Popják, 1959). Since mevalonic acid, mevalonic acid lactone, 5-phospho- and 5-diphospho-mevalonate are clearly separated from one another on the chromatograms, the percentage conversion of added ^{14}C -labelled substrate into new products could be readily obtained from the areas under the curves obtained by our scanning procedure with an accuracy of about $\pm 5\%$ (cf. under chromatographic procedures). When enzymic activity was measured by the radiochromatographic assay alone, the reactants contained in the incubation mixtures were the same and of the same final concentration as described for the spectrophotometric assay (see below) except that phosphoenolpyruvate, pyruvic kinase, lactic dehydrogenase and DPNH were omitted.

The spectrophotometric assay of liver mevalonic kinase was made by the same procedure as described by Tchen (1958) and by Lynen (1959) for the yeast enzyme. This assay measures the amount of ADP formed in the kinase reaction (1) by coupling with PEP-kinase (2) and lactic dehydrogenase reactions (3) as shown below.

Each mole of mevalonate phosphorylated with ATP yields 1 mole of ADP (Tchen, 1958); this is rephosphorylated by phosphoenolpyruvate and PEP-kinase (reaction 2) and the pyruvate formed is used to oxidize DPNH in the presence of lactic dehydrogenase (reaction 3). The last reaction was followed at 340 m μ in a Beckman DK-2 recording spectrophotometer fitted with a time drive. The spectrophotometer cuvettes were held at 37° in a thermostatically-heated cell carrier constructed in the Unit's workshop (unpublished work).

The reaction mixture (5.9 ml.) for the assays was made up in a tube kept at 37° and contained the following reactants ($\mu\text{moles/ml.}$ shown in parentheses): cysteine hydrochloride (10); KOH, for the neutralization of cysteine hydrochloride (16); 2-amino-2-hydroxymethylpropane-1:3-diol-HCl (tris buffer), pH 7.4 (100); MgCl_2 (5); ATP (7); DPNH (0.2); sodium phosphoenolpyruvate (0.5). Lactic dehydrogenase and phosphoenolpyruvic kinase were combined and diluted with 1% (w/v) solution of bovine albumin; 0.2 ml. of this solution, containing 52 and 200 $\mu\text{g.}$ of the two enzymes respectively, was used in the reaction mixture. The mevalonic kinase (0.1 ml.) was added last. After mixing, 2.9 ml. of the reaction mixture were transferred to the test cuvette and the remainder to the control cuvette. After recording the light-extinction at 340 m μ for 2 or 3 min. 0.1 ml. of mevalonate solution (usually 10 μmoles of the DL-substrate) was added to the test cuvette to initiate the reaction. The amount of DPNH used in the assay was less than the total amount of utilizable substrate; this circumstance in no way invalidated the assay since during the purification procedures we were interested only in rates of reaction in order to determine the specific activity of our preparations, there being no necessity to carry the reaction to completion. In order to ensure that the rate of DPNH oxidation was actually a measure of the rate of mevalonate phosphorylation, and

Table 1. *Purification of mevalonic kinase from extract of pig liver*

The enzymic activity was measured by the spectrophotometric assay.

Stage of purification	Amount of protein (mg.)	Enzyme units*	Specific activity of protein†
Initial extract	247 000	2180	0.0088
Supernatant after addition of protamine	75 000	2370	0.0315
Enzyme after heat treatment	37 000	1540	0.0415
After ammonium sulphate fractionation (30–45% saturation)	11 300	1200	0.106
Enzyme after calcium phosphate-gel treatment:			
1st eluate	1 021	668	0.654
2nd eluate	557	541	0.972

* μ moles of DPNH oxidized (= μ moles of mevalonate phosphorylated)/min. at 37°.† μ moles of DPNH oxidized/min./mg. of protein at 37°.

was not limited by the lactic dehydrogenase and pyruvic kinase-catalysed reactions, the system was tested when necessary by the addition of about 1 μ mole of ADP to the test cuvette; this caused an almost instantaneous oxidation of DPNH. It was particularly important to test the assay system in this way in those experiments in which the properties of mevalonic kinase were being studied since some of the conditions used could, conceivably, have affected the test reactions. Whenever the spectrophotometric assay could not be used, e.g. during the study of metal-ion and nucleotide requirements of mevalonic kinase, the 'radiochromatographic assay' was employed.

RESULTS

As the formation of 5-phosphomevalonate with liver enzymes was demonstrated in our Laboratory first in extracts of rat liver (de Waard, 1958, de Waard & Popják, 1959), supernatants of rat-liver homogenates centrifuged at 105 000 *g* were first used as possible sources of mevalonic kinase. Although it was possible to demonstrate spectrophotometrically the presence of mevalonic kinase in such preparations, on account of rapid utilization of 5-phosphomevalonate in further reactions (cf. de Waard & Popják, 1959; Popják, 1959) and because of other interfering reactions in the rat-liver extracts the assay was difficult. In the course of experiments on the preparation of amino acid-activating enzymes (Finch & Hele, 1959) Dr P. Hele of this Unit noted the presence of mevalonic kinase in extracts of pig liver; the enzyme could be assayed spectrophotometrically even in the crudest of extracts (the 'initial extract' described under Methods). As these assays could be readily reproduced and the 'radiochromatographic assay' also (see under Methods) showed an excellent conversion of [2-¹⁴C]mevalonate into 5-phospho[2-¹⁴C]mevalonate, extracts of pig liver were used for purification.

Purification of mevalonic kinase

Table 1 shows the results of purification of mevalonic kinase from 3.46 l. of pig-liver extract.

The 'initial extract' was treated in 1–1.2 l. batches with a solution of protamine sulphate (10 mg./ml.) to give a final concentration of 1.5 mg. of protamine/ml. of extract. This concentration of protamine was found in separate small-scale experiments to lead to a maximum increase in specific activity without loss of enzyme. The mixture was stirred slowly for 2 min. and then centrifuged to give the almost clear 'protamine supernatant' (3.16 l.). As is shown in Table 1 the protamine treatment resulted in a 3.5-fold increase in the specific activity of the preparation.

Solid ammonium sulphate (1.76 kg.) was added to 3.16 l. of 'protamine supernatant' to give a concentration of 85% saturation at 0°; the precipitate was allowed to settle overnight and collected by centrifuging. The reddish brown precipitate was dissolved in 1365 ml. of a 0.02M-potassium hydrogen phosphate buffer, pH 7.6, containing mM-ethylenediaminetetra-acetate (EDTA); a small insoluble residue was centrifuged off.

At this stage of purification several small samples of the preparation were heated to various temperatures (up to 60°) in the absence or presence of mM-mevalonate. The results encouraged us to heat the bulk of the enzyme with added mevalonate (1 mM), in 200 ml. lots, at 55° for 2 min.; the preparation was cooled immediately afterwards in ice. A large precipitate was centrifuged off and washed with the dilute phosphate-EDTA buffer, and the washings were added to the supernatant. In contrast with the good recovery and an increased specific activity of the enzyme in small-scale experiments, heating the enzyme in bulk caused relatively large losses with only a little gain in specific activity (cf. Table 1; enzyme after heat treatment).

After heat treatment the protein solution (1850 ml.) was subjected to ammonium sulphate fractionation; about 80% of the enzyme precipitated between 30 and 45% ammonium sulphate saturation with an over twofold increase in specific activity. The precipitate, containing the mevalonic

kinase, was dissolved in 288 ml. of the dilute phosphate-EDTA buffer and was dialysed for 3½ hr. against two changes of 0.01M-potassium hydrogen phosphate buffer, pH 7.5. The dialysed protein solution (335 ml.) was stirred with 35 ml. of a suspension of calcium phosphate gel (35 mg. dry weight/ml. of suspension) for a few minutes; the gel was centrifuged off and the supernatant solution assayed for protein concentration and enzyme activity. This first gel treatment removed much inert protein from solution and no enzyme. The supernatant from the first gel treatment was stirred with further portions of gel, which began to adsorb the enzyme; after the addition of 175 ml. of gel suspension 95 % of the mevalonic kinase was adsorbed; the supernatant after centrifuging was almost completely inactive and was discarded.

The calcium phosphate gel, containing the adsorbed mevalonic kinase, was washed successively with approximately equal volumes of potassium hydrogen phosphate buffers, pH 7.5, and once with 0.01M- and twice with 0.02M-buffer. These washings removed inert protein only. Four washes with 0.05M-potassium hydrogen phosphate buffer, pH 7.5, eluted mevalonic kinase quantitatively from the gel; these washes were combined in two fractions to give the '1st eluate' and the '2nd eluate' shown in Table 1. The enzyme was precipitated with ammonium sulphate (80 % saturation), the precipitate collected by centrifuging and the supernatant decanted. The precipitated enzyme was stored at -15° . When needed, solutions were made in 0.02M-phosphate buffer, pH 7.5, and dialysed before use against the same buffer for 4 hr. Solutions of the enzyme may be kept at -15° for several weeks with relatively little deterioration.

The over-all yield of the two calcium phosphate gel eluates was 56 %, based on the enzyme units contained in the initial extract; the '1st eluate' contained mevalonic kinase purified 74-fold and the '2nd eluate' 110-fold. In another preparation (nos. 8-29), similar to this one, the final product phosphorylated 1.2 μ moles of (+)-mevalonate/min./mg. of protein at 37° ; that preparation was used for the study of the properties of the enzyme which follow.

Properties of mevalonic kinase

The final spectrophotometric assay system described under Methods was arrived at after the properties of mevalonic kinase in the most highly purified preparation (specific activity 1.2 μ moles of mevalonate phosphorylated/min./mg. of protein at 37°) were studied. The accuracy of this assay on 'initial extracts' is probably not very great. We have observed during the use of this assay on crude enzyme preparations a fairly rapid 'back-reaction' after the DPNH has been partially

oxidized. The product of this reaction had an absorption maximum at 340 m μ and was presumably DPNH. This back-reaction did not depend on mevalonic acid; it also occurred when ADP was used as substrate. This phenomenon, which disappeared during further purification of the enzyme, may account for the apparent rise in total enzyme units after protamine treatment (cf. Table 1). After this step of purification, however, the assays became reproducible and the rates of reaction proportional to protein concentration. This is shown in Fig. 1b for a partially purified preparation with a specific activity of about 0.15 μ mole/min./mg. of protein; in Fig. 1a the actual spectrophotometric records, from which the points on Fig. 1b were calculated, are reproduced. Similar data were obtained also with the more highly purified preparations.

Activation of mevalonic kinase by cysteine. The effect of cysteine on the activity of the enzyme is shown in Fig. 2. In the absence of cysteine the rate of reaction was only 17 % of the rate found in the presence of 0.66 μ mole of cysteine/ml., the lowest concentration tested. A 20-fold increase in cysteine concentration resulted in a further 25-30 % rise in activity. Glutathione was almost as effective as cysteine, both at a concentration of 10 μ moles/ml.,

Fig. 1. Proportionality between protein concentration and phosphorylation of mevalonate as measured spectrophotometrically by the phosphoenolpyruvic kinase-lactic dehydrogenase-coupled assay system. In (a) are shown copies of the spectrophotometric records from which the data of (b) were calculated. The spectrophotometric assays were set up exactly as described under Methods, the amounts of enzyme protein (preparation no. 5-36) being varied from 23 to 1140 μ g. per assay as indicated on graph (a). The upward stroke after the initial recording of light-extinction indicates the addition of mevalonate to the test cuvettes. The pen of the recording spectrophotometer was brought to the same starting point for each assay and the mevalonate was added also at the same point in each test.

but ascorbic acid was without effect. Cysteine or glutathione was needed, however, only when air was not excluded from the incubations.

Addition of mM-*p*-chloromercuribenzoate produced an 80% inhibition of mevalonic kinase in the presence of mM-cysteine as measured by the radiochromatographic assay. This inhibition was completely reversed by raising the concentration of cysteine to 0.02M. Iodoacetamide did not inhibit the enzyme at either mM or 10 mM concentration.

Metal-ion requirements of mevalonic kinase. The enzyme needs a bivalent metal ion for activation, as was shown by both spectrophotometric and radiochromatographic assays. Fig. 3 shows the effect of varying Mg^{2+} and Mn^{2+} ion concentrations on enzyme activity as determined by the spectrophotometric assay. The validity of these assays was established by the fact that even at the lowest concentrations of Mn^{2+} and Mg^{2+} ion used the pyruvic kinase-lactic dehydrogenase assay system was fully responsive, i.e. addition of ADP caused an almost instantaneous oxidation of DPNH. A correlation was established between the concentration of Mg^{2+} ion and ATP and enzyme activity (see under Nucleotide requirements). The data of Fig. 3 were obtained with an ATP concentration of 4.2 μ moles/ml.; when the ATP concentration was raised the curve showing enzyme activity against

Mg^{2+} concentration was shifted upward and to the right.

Besides Mg^{2+} and Mn^{2+} other bivalent metal ions, Ca^{2+} (as the chloride), Ba^{2+} , Zn^{2+} (as the acetates), Co^{2+} (as the nitrate), Fe^{2+} and Cu^{2+} (as the sulphates), were also tested by the radiochromatographic assay for their ability to activate mevalonic kinase. In a representative experiment eight 1 ml. incubations, each containing one of the above metal ions in a concentration of 5 mM (5 μ moles/ml.), were set up with 1 μ mole of DL-[2- ^{14}C]mevalonate, 7.7 μ moles of ATP, 10 μ moles of cysteine (neutralized with potassium hydroxide), 100 μ moles of tris-HCl buffer, pH 7.4, and 62 μ g. of purified mevalonic kinase. After 5 min. of incubation at 37° the contents of each incubation were deproteinized and samples of each chromatographed on Whatman no. 1 paper. The records obtained from scanning the paper chromatograms for radioactivity showed that with Mg^{2+} 0.368, with Mn^{2+} 0.116 and with Ca^{2+} 0.214 μ mole of 5-phosphomevalonate was formed; a trace of 5-phosphomevalonate formed in the presence of Fe^{2+} and Co^{2+} , and none in the presence of Ba^{2+} , Cu^{2+} and Zn^{2+} ions. The activation of mevalonic kinase by Ca^{2+} ions was checked and confirmed in further experiments. The calcium chloride solution used was analysed in a flame spectrophotometer and was found to be essentially free of Mg^{2+} ions. Ca^{2+} in a concentration of 5 mM did not inhibit mevalonic kinase in the presence of 5 mM- Mg^{2+} ions.

Fig. 2. Effect of cysteine on mevalonic-kinase activity as measured by spectrophotometric assay. The spectrophotometric assays for each point on the graph were set up as described under Methods, except that the concentration of cysteine was varied from 0–13 μ moles/ml. Enzyme preparation no. 8–29 was used (93 μ g. of protein per assay).

Fig. 3. Effect of Mg^{2+} and Mn^{2+} ions on mevalonic-kinase activity as determined spectrophotometrically. The spectrophotometric assays for each point on the graphs were set up as described under Methods, except that the concentration of ATP was 4.2 μ moles/ml., instead of the usual 7.5 μ moles/ml., and the concentration of Mg^{2+} and Mn^{2+} ions varied from 0 to 9 μ moles/ml. Enzyme preparation no. 8–29 was used (93 μ g. of protein per assay).

Nucleotide requirements of mevalonic kinase. The effects of varying concentrations of ATP on mevalonic-kinase activity was first measured by the spectrophotometric assay. The optimum concentration of ATP depended on the concentration of Mg^{2+} ions in the incubations. Thus with 3.3 mM- Mg^{2+} the apparent optimum concentration of ATP was 5.5 mM, and with 5 mM- Mg^{2+} the optimum concentration of ATP was 7–8 mM. With an ATP concentration higher than 8 mM the coupled spectrophotometric assay could not be used because of an almost complete inhibition of pyruvic kinase. Mevalonic kinase, on the other hand, was not inhibited by higher concentrations of ATP as judged by the results of the radiochromatographic assays. The optimum ratio of ATP to Mg^{2+} ($\mu\text{mole}/\mu\text{mole}$) for maximum rates of mevalonic-kinase activity was found by the spectrophotometric assay to be 1.5–2.

A number of nucleotides, adenosine diphosphate, cytidine triphosphate, guanosine triphosphate and inosine triphosphate were tested, in a concentration of 7.4 mM in the presence of 5 mM- Mg^{2+} , for their ability to replace ATP in the phosphorylation of mevalonate. The experiments were set up essentially in the same way as for the tests with the various metal ions, except that the incubations lasted for 15 min. The radiochromatographic assay showed that only inosine triphosphate could replace ATP; the amount of 5-phosphomevalonate formed with inosine triphosphate was very nearly identical with that formed in the presence of ATP, 41 and 50% conversion respectively of the added DL-[2- ^{14}C]mevalonate. The inosine triphosphate was not contaminated with ATP, neither was it converted by the enzyme into ATP, for paper chromatograms of the deproteinized incubation mixture revealed neither ATP nor ADP. These nucleotides are well recognizable on the paper chromatograms developed with the *isobutyric acid*-aq. NH_3 solvent system.

In crude enzyme preparations sodium fluoride (0.012M) was added during assays of mevalonic kinase in order to inhibit adenosine triphosphatase activity. After purification, the enzyme preparations were tested for enzymes which could hydrolyse ATP to ADP and these were found to be absent. The purified enzyme was not affected by the addition of 0.012M-sodium fluoride.

Effects of substrate concentrations; substrate specificity. When DL-mevalonic acid lactone was used as substrate with the most highly purified mevalonic kinase the reaction rate, as measured spectrophotometrically, was at first very slow, but it gradually increased. If, instead of lactone, the potassium salt of mevalonic acid was used as substrate, a rapid reaction set in immediately after its addition to the incubation mixture without any

subsequent change in rate. Clearly the enzyme cannot utilize mevalonic acid lactone; the slow reaction rates observed when the lactone was used as substrate obviously depended on the rate of hydrolysis of the lactone to the acid at the slightly alkaline pH of the incubations.

The effect of the concentration of substrate (potassium DL-mevalonate) on the rate of its phosphorylation is shown in Fig. 4; these data were derived from spectrophotometric assays. A maximum rate was observed when the concentration of DL-mevalonate was about 2 $\mu\text{moles/ml}$. As is shown below (cf. Fig. 5), liver mevalonic kinase, like the yeast enzyme (Tchen, 1958), acts on only one of the optical enantiomorphs of mevalonate. If we assume that the binding of the active enantiomorph by the enzyme was not affected by the presence of the unnatural enantiomorph, the apparent Michaelis constant (K_m) for the natural isomer is approximately 50 μM .

In another experiment the spectrophotometric assay was set up in such a way that the amounts of phosphoenolpyruvate (1.5 μmoles) and of DPNH (1.3 μmoles) were in excess of the amount of DL-mevalonate (0.4 and 0.1 μmole) added to the reaction mixture. As can be seen from Fig. 5, after the addition of 0.4 μmole of DL-mevalonate the reaction came to a standstill when 0.21 μmole of DPNH was oxidized; after the addition of a further 0.1 μmole of DL-mevalonate 0.05 μmole of DPNH was oxidized. Thus it appears that mevalonic kinase is specific for one optical enantiomorph of mevalonate.

In addition to mevalonate we have tested related substances whether they can serve as substrate for mevalonic kinase. Neither the *cis*-5-hydroxy-3-methylpent-2-enoic acid (as the potassium salt), nor the 5-hydroxy-3-methylpent-3-enoic acid (mixture of the *cis* and *trans* isomers) (cf.

Fig. 4. Effects of substrate concentration on phosphorylation of mevalonate as measured by the spectrophotometric assay. The assays for each point on the graph were set up as described under Methods, except that the concentration of DL-mevalonate was varied from 0.035 to 6 $\mu\text{moles/ml}$. Enzyme preparation no. 8-29 (93 $\mu\text{g.}$ of protein per assay) was used.

Cornforth *et al.* 1958), nor 3-oxobutan-1-ol nor farnesol could be phosphorylated by mevalonic kinase; none of these substances inhibited the phosphorylation of mevalonate.

pH optimum for mevalonic kinase. Fig. 6 shows the dependence of rate of reaction on pH in phosphate and tris-HCl buffers as determined by the spectrophotometric assay. The pH optimum lies close to 7.3; at pH 6 and 8.7 the rate of phosphorylation of mevalonate was approximately one-half of that found at pH 7.3. In the pH range studied, 6–9.1, the rates of the pyruvic kinase–lactic dehydrogenase assay system did not limit the assay of ADP formed in the mevalonic kinase reaction.

Action of compounds related to mevalonic acid and to farnesoic acid on mevalonic kinase. In addition to the two anhydro-analogues of mevalonate, 3-oxobutan-1-ol and farnesol, we have tested the following substances (in a final concentration of 1 mM) for their effect on mevalonic kinase: 3-hydroxy-3-methylglutarate, isovalerate, farnesoate (mixture of the *cis-trans* and *trans-trans* acids) and 3:7:11-trimethyldodecanoate (each as the potassium salt). Of these only the farnesoate and the 3:7:11-trimethyldodecanoate had an effect on mevalonic kinase: the former caused a 25% and the latter a

95% inhibition of the enzyme. These two acids were without effect on the pyruvic kinase–lactic dehydrogenase system.

5-Phosphomevalonic kinase

In addition to 5-phosphomevalonate the formation of another phosphorylated derivative of mevalonic acid was observed both in yeast extract (Chaykin *et al.* 1958) and in rat-liver extracts (de Waard, 1958; Popják, 1959; de Waard & Popják, 1959). Chaykin *et al.* (1958) showed by the use of [32 P]ATP and de Waard & Popják (1959) by the direct chemical analysis of the substance isolated that it contained 2 atoms of P/molecule of mevalonic acid. Although this substance still requires further chemical characterization, Chaykin *et al.* (1958) deduced from data of isotopic labelling with [32 P]ATP that it is most probably the 5-pyrophosphoryl derivative of mevalonic acid. We refer below to this substance as the 5-diphosphomevalonate. 5-Diphosphomevalonate is clearly separated from 5-phosphomevalonate both on paper chromatograms with the *isobutyric acid*–aq. ammonia solvent system and on Dowex-2 or Amberlite IRA-400 ion-exchange resins (cf. Popják, 1959; de Waard & Popják, 1959).

Fig. 5. Experiment illustrating specificity of mevalonic kinase for one optical enantiomorph of DL-mevalonate. The graph is a copy of the spectrophotometric recording. Spectrophotometric assay was set up with enzyme preparation no. 8-29 A (specific activity 0.5 μ mole/min./mg. of protein). The test cuvette contained (in 3 ml.) 0.58 mg. of protein and the usual reactants of the spectrophotometric assay (see under Methods) except that 1.2 μ moles of phosphoenolpyruvate and 1.2 μ moles of DPNH were used in the assay. At the first arrow (at 1 min.) 0.4 μ mole and at the second arrow (after 6 min.) 0.10 μ mole of DL-mevalonate was added. The slight lag in the starting of the reaction after the two additions of mevalonate is due to the sub-optimum concentration of substrate.

Fig. 6. Dependence of mevalonic-kinase activity on pH of reaction mixture as determined in spectrophotometric assays. The assays for each point have been set up as described under Methods. $\circ$, Phosphate buffers, 0.1M; $\bullet$, tris-HCl buffers, 0.1M. Enzyme preparation no. 8-29 was used.

We have observed in the radiochromatographic assays of some of the cruder mevalonic-kinase preparations that in addition to 5-phosphomevalonate some 5-diphosphomevalonate was also formed. In the preparations obtained after calcium-gel fractionation 5-diphosphomevalonate was never formed. We have therefore assumed the existence of a second phosphorylating enzyme and in the following brief account we wish to present experimental evidence on this point, although this second enzyme has not been obtained free from mevalonic kinase.

A partially purified mevalonic-kinase preparation [no. 10-8; specific activity $0.22 \mu\text{mole of (+)-mevalonate phosphorylated/min./mg. of protein}$] obtained after protamine treatment and ammonium sulphate fractionation of the 'initial extract' of pig liver, when incubated with $[2-^{14}\text{C}]$ mevalonate and the usual components of the mevalonic-kinase assay system, was found to catalyse the formation not only of 5-phosphomevalonate but also of 5-diphosphomevalonate as determined by the radiochromatographic assay. A large-scale incubation (final volume 50 ml.) was set up with this enzyme preparation by using 75.6 mg. of protein, $100 \mu\text{moles of DL-}[2-^{14}\text{C}]$ mevalonate, $370 \mu\text{moles of ATP}$, $250 \mu\text{moles of MgCl}_2$, 5 m-moles of tris-HCl buffer, pH 7.4, $500 \mu\text{moles of cysteine}$ (neutralized with potassium hydroxide), $23 \mu\text{moles of phosphoenolpyruvate}$ and 1 mg. of phosphoenolpyruvic kinase. After 33 min. of incubation at 37° the mixture was deproteinized with an equal volume of ice-cold 10% (w/v) perchloric acid solution and the acid removed as the insoluble potassium perchlorate. Radiochromatographic assays on 0.1 ml. samples of the deproteinized mixture showed that 49% of the added DL-mevalonate was converted into 5-phosphomevalonate + 5-diphosphomevalonate. Of the phosphorylated derivatives 70% was accounted for as 5-phosphomevalonate and 30% as 5-diphosphomevalonate. The bulk of the extract was used for the purification of the two phosphorylated derivatives according to de Waard & Popják (1959) by using their method of ion-exchange chromatography followed by adsorption on and elution from charcoal. Two radiochemically pure specimens, uncontaminated by nucleotides or inorganic phosphorus, were obtained, the chromatographic properties and phosphorus contents of which were identical with those described by de Waard & Popják (1959) for 5-phospho $[2-^{14}\text{C}]$ mevalonate and 5-diphospho $[2-^{14}\text{C}]$ mevalonate. These were used as substrates in further experiments.

Addition to enzyme preparation no. 10-8 of 5-phospho $[2-^{14}\text{C}]$ mevalonate, together with ATP and Mg^{2+} ions, resulted in the formation of 5-diphospho $[2-^{14}\text{C}]$ mevalonate. Both the spectropho-

metric assay, as used for mevalonic kinase, and the radiochromatographic assay were satisfactory for measuring the activity of the second phosphorylating enzyme. Since the substrate for this enzyme is 5-phosphomevalonate we propose to name it '5-phosphomevalonic kinase' (or phosphomevalonic kinase).

In a spectrophotometric assay set up with 5-phospho $[2-^{14}\text{C}]$ mevalonate as substrate ($0.81 \mu\text{mole/3 ml.}$) and 1 mg. of enzyme preparation no. 10-8, DPNH was oxidized at an initial rate of $0.018 \mu\text{mole/min.}$, but this rate fell off gradually. After 25 min. at 37° radiochromatographic assay was carried out on the deproteinized incubation mixture; this showed that 30% ($0.243 \mu\text{mole}$) of the added 5-phospho $[2-^{14}\text{C}]$ mevalonate was converted into the diphosphate. No hydrolysis of the 5-phospho $[2-^{14}\text{C}]$ mevalonate into $[2-^{14}\text{C}]$ mevalonate could be observed. Photographic prints of the paper chromatograms, made with ultraviolet light, revealed only ADP and ATP, but no adenosine monophosphate. Therefore we conclude that the reaction catalysed by phosphomevalonic kinase may be represented by:

In the absence of Mg^{2+} ions 5-diphosphomevalonate was not formed from 5-phosphomevalonate; Ca^{2+} could not replace Mg^{2+} in the reaction, but neither did Ca^{2+} antagonize Mg^{2+} . In an experiment run parallel with the above spectrophotometric assay, in the presence of 5 mM- Ca^{2+} plus 5 mM- Mg^{2+} , 32% ($0.26 \mu\text{mole}$) of the added 5-phospho $[2-^{14}\text{C}]$ mevalonate was converted into 5-diphospho $[2-^{14}\text{C}]$ mevalonate. In a further experiment we examined the possibility that the phosphomevalonic kinase reaction might be reversible; however, we could not demonstrate the formation of 5-phosphomevalonate and of ATP from 5-diphosphomevalonate and ADP in the presence of Mg^{2+} ions.

Preparation of 5-phospho $[2-^{14}\text{C}]$ mevalonate

Although the method of de Waard & Popják (1959) is satisfactory for the preparation of the two phosphorylated derivatives of mevalonic acid in a pure form, the yields are rather poor on account of losses encountered during absorption on and elution from charcoal. We have developed a simple procedure, not involving chromatography, which is suitable for the large-scale preparation of 5-phosphomevalonic acid made enzymically from DL-mevalonate.

The method depends on the properties of 5-phosphomevalonic acid and of mevalonic acid. These are: (i) 5-phosphomevalonic acid is readily soluble in 85% ethanol, but it forms a barium salt

sparingly soluble in water and insoluble in 80–85 % ethanol; (ii) although mevalonic acid also forms a barium salt insoluble in ethanol, mevalonic acid lactone does not; (iii) free mevalonic acid may be converted into the lactone under dehydrating conditions and the lactone is fairly stable even at slightly alkaline pH at low temperatures. These characteristics of 5-phosphomevalonic acid and of mevalonic acid enable one to separate these two substances (a) from proteins, nucleotides and salts present in the enzymic incubations and (b) from one another.

In a large-scale experiment (final volume 500 ml.) we have used 40 mg. of our most highly purified enzyme preparation (no. 8–29, specific activity 1.2 μ moles of mevalonate phosphorylated/min./mg. of protein at 37°) and 2700 μ moles of DL-[2- 14 C]-mevalonate (specific activity 0.2 μ C/ μ mole) for the preparation of 5-phospho[2- 14 C]mevalonate. The final concentrations of the reactants were: ATP, 7.5 mM; MgCl₂, 5 mM; cysteine (neutralized with potassium hydroxide), 1.8 mM; tris-HCl buffer, pH 7.3, 100 mM; DL-[2- 14 C]mevalonate, 5.4 mM; enzyme 80 μ g./ml. The reaction mixture, without mevalonate, was warmed to 37° and then the mevalonate added; the incubation was continued (without shaking) for 117 min. Radiochromatographic assay carried out at this time on a small sample of the incubation mixture showed that 42.5 % of the DL-mevalonate [or 85 % of the natural (+)-isomer] was converted into 5-phospho[2- 14 C]-mevalonate. There was no trace of the 5-diphosphomevalonate.

The whole reaction mixture was frozen and dried from the frozen state. The dry residue was taken up in water, the pH adjusted to 4.0 with HCl and the volume made up to 45 ml. with water; ethanol (255 ml.) was then added to give a concentration of 85 %. A large white precipitate was allowed to settle overnight and was then removed by centrifuging. The precipitate was washed repeatedly with 85 % ethanol and the washings were added to the first ethanol supernatant (total volume 440 ml.). The ethanol was first removed from this solution on a rotary evaporator under vacuum at about 50–60°, then the aqueous solution was concentrated to a small volume by sublimation from the frozen state. The aqueous solution (41.5 ml.) was adjusted with potassium hydroxide to about pH 8.5 (phenolphthalein to pale pink), then 10 ml. of m-BaCl₂ solution and ethanol to a final concentration of 85 % were added. A bulky white precipitate formed, which was allowed to accumulate at 0° overnight and was separated by centrifuging; the supernatant was drawn off and the precipitate washed with 85 % ethanol. The washing was combined with the previous supernatant. The precipitate was dissolved in 25 ml. of N-HCl and the solu-

tion diluted with 25 ml. of water to give a volume of 53 ml. Radiochromatographic assay showed that the ethanol supernatant contained only unused mevalonic acid. Similar assays and 14 C-counting made on the solution of the barium salts revealed that about 91 % of the 14 C counts in this solution were associated with 5-phosphomevalonate and 9 % with mevalonate and that the 5-phosphomevalonate formed in the incubation was recovered quantitatively in this specimen. The chromatograms also showed that only a trace of ATP was present in the solution of the barium salts, most of the nucleotides having been left behind after the first ethanol extraction.

In order to remove the small amount of nucleotide left with the 5-phosphomevalonate, the solution of the barium salts (after removal of barium with sodium sulphate) was dried from the frozen state and the dry residue was extracted with 85 % ethanol as in the first step and the barium precipitation repeated once again. Although all traces of nucleotide disappeared after this treatment, there still remained about 5 % of mevalonate contaminating the 5-phosphomevalonate. The recovery of 5-phosphomevalonate was quantitative even after this second barium precipitation.

We now realized that in the further handling of the specimen we made mistakes, as at this stage of the work we did not appreciate fully the basis of the remarkably good separation of 5-phosphomevalonate from mevalonate in the first barium-precipitation step, i.e. the formation of a fairly stable lactone of free mevalonic acid during freeze-drying of a slightly acid solution. In the light of further experience, however, we now recommend that after the second barium precipitation the barium salts should be dissolved in dilute hydrochloric acid and the barium removed with sodium sulphate, followed by drying the specimen from the frozen state *in vacuo*. After this dried specimen, containing mostly free 5-phosphomevalonic acid and a little mevalonic acid, is heated *in vacuo* to about 60° the mevalonic acid lactonizes; the mixture is then dissolved in ice-cold water and the solution adjusted to pH 8.5 with a saturated solution of barium hydroxide while the specimen is kept packed in ice. Immediate addition of ethanol to 85 % concentration then completes the precipitation of the barium salt of 5-phosphomevalonate, leaving mevalonic acid lactone in solution.

In order that others may avoid our mistakes after the second barium precipitation, we describe briefly the various procedures that we found ineffective in removing the small amounts of mevalonic acid that remained with the 5-phosphomevalonate. Extraction of an acid solution with ether did not remove mevalonic acid without some 5-phosphomevalonate as well. An attempt to sublime off the mevalonic acid from the freeze-dried specimen failed also;

by this time only 87% of the ^{14}C -counts contained in the specimen after the first barium precipitate were left, the 5-phosphomevalonate still being contaminated with about 5% of mevalonic acid. This specimen was chromatographed on Dowex-2 (formate) as described previously (de Waard & Popják, 1959) by using gradient elution with formic acid and ammonium formate. After collection of the 5-phosphomevalonate peak the fractions were combined and acidified with hydrochloric acid and most of the formic acid removed by extensive extraction with ether, the ammonium chloride being left behind. The aqueous solution was now made slightly alkaline and barium chloride added once more, followed by ethanol to 85%. The precipitate formed was collected by centrifuging and then treated in the usual way to remove the barium; the solution was then dried again from the frozen state. The dry residue was dissolved in a minimum of water, ethanol added to 85% and a large precipitate (ammonium chloride mostly) removed. Radiochromatographic analysis showed that the specimen now contained not even a trace of mevalonic acid, but only 750 μmoles of 5-phosphomevalonate out of the 1147 formed originally were recovered. After removal of the ethanol by evaporation the solution of 5-phosphomevalonic acid was kept at -15° , where it showed no signs of deterioration after some months. Phosphorus analyses on the specimens carried out according to a modification of the method of Allen (1940) showed that it contained 1 $\mu\text{g. atom}$ of organic phosphorus per 31 500 counts/min. of ^{14}C ; 1 μmole of the mevalonate used in the enzyme incubation contained 32 000 counts/min. The specimen contained no inorganic phosphorus. The losses encountered during these last procedures were due partly to the large number of manipulations and possibly to some hydrolysis of 5-phosphomevalonate to mevalonate; all this can now be avoided. The 5-phospho[2- ^{14}C]mevalonate thus prepared was tested in enzymic reactions leading to the formation of further intermediates (notably of farnesyl pyrophosphate) in squalene biosynthesis and was utilized almost quantitatively in such reactions.

DISCUSSION

Extracts of pig liver made according to the method of Finch & Hele (1959) are much richer sources of mevalonic kinase than yeast extracts used by Tchen (1958); furthermore, even in the crudest pig-liver extracts, the 5-phosphomevalonate formed from mevalonate and ATP is metabolized only to the 5-diphosphomevalonate stage. This is in contrast with the soluble supernatants of rat-liver homogenates, in which after an incubation lasting longer than about 10 min. most of the 5-phosphomevalonate and 5-diphosphomevalonate formed during the first 5 min. may be completely dissipated into other products (cf. de Waard & Popják, 1959; Popják, Horning, Bucher & Cornforth, 1959). It seems probable that the advantages of the pig-liver extract derive from the gentle method of extraction, which does not release too many interfering enzymes. Possibly this initial advantage is reflected also in gaining preparations of mevalonic kinase of much higher specific activity than those reported by Tchen (1958) for yeast

mevalonic kinase. The best preparation from yeast catalysed the phosphorylation of about 0.08 μmole of mevalonate/min./mg. of protein at 30° , as compared with 1.2 $\mu\text{moles/min./mg.}$ of protein at 37° for our best preparation. The recovery of mevalonic kinase from the yeast extracts was only about 7%; from the pig-liver extracts it was 56%.

Although yeast mevalonic kinase and liver mevalonic kinase are broadly similar, their properties differ in detail. Both enzymes appear to be SH-enzymes, but the liver enzyme is readily inactivated by molecular oxygen and needs cysteine or glutathione for reactivation under aerobic conditions; the yeast enzyme, according to Tchen (1958), was not affected by air or oxygen and did not need cysteine for activation. Although the complete squalene- and sterol-synthesizing system of rat liver (Popják *et al.* 1958) could be activated by cysteine, glutathione or ascorbic acid, the last substance was totally ineffective with mevalonic kinase; this observation suggests a further reaction in squalene synthesis, requiring a reducing agent. This has now been identified in the microsomes (as originally deduced; cf. Popják *et al.* 1958) and is known to be involved in the coupling of two sesquiterpenoid intermediates in the last stage of squalene synthesis (Popják, unpublished work).

Since more commonly Ca^{2+} antagonizes Mg^{2+} in enzyme reactions dependent on ATP, the activation of liver mevalonic kinase not only by Mg^{2+} but also by Ca^{2+} is unusual. The metal-ion and nucleotide requirements of mevalonic kinase are similar to those of hexokinase. However, we found that purified yeast hexokinase could not phosphorylate mevalonate and mevalonic kinase could not phosphorylate glucose. Calcium ions were ineffective with the yeast mevalonic kinase. While the liver enzyme can function only with ATP and inosine triphosphate, the yeast enzyme can use ATP and the triphosphates of guanosine, cytidine and uridine almost equally well. The pH optimum of liver mevalonic kinase is about 7.3 and that of the yeast enzyme 6.9. Both the yeast enzyme and the liver enzyme are specific for one optical enantiomorph of mevalonate; this is assumed to be the natural (+)-isomer. de Waard & Popják (1959) reported that the mevalonic acid, isolated after about 20 min. of incubating DL-[2- ^{14}C]mevalonate with ATP, Mg^{2+} ions and liver-soluble enzymes, was biologically inactive. Animals injected with DL-[2- ^{14}C]mevalonate excrete in their urine one-half of the radioactive dose (Gould & Popják, 1957); the mevalonate excreted in the urine is the unnatural optical enantiomorph (R. H. Cornforth, K. Fletcher & G. Popják, unpublished work).

The relative ease with which highly potent preparations of mevalonic kinase may be obtained from pig liver enabled us to prepare reasonable

quantities of 5-phospho[2-¹⁴C]mevalonate. Although there can be very little doubt as to the identity of this product, nevertheless its chemical characterization has been meagre up to now.

The second phosphorylating enzyme, which uses 5-phosphomevalonate as substrate, is clearly distinct from mevalonic kinase. The enzyme preparation in which we made the preliminary studies reported here had a specific activity for the phosphorylation of mevalonate of 0.22 μ mole/min./mg. of protein and for that of 5-phosphomevalonate of 0.018 μ mole/min./mg. This second enzyme was activated by Mg^{2+} and not at all by Ca^{2+} ions, and could be completely removed from mevalonic kinase. The product of the action of 5-phosphomevalonic kinase on 5-phosphomevalonic acid with ATP is undoubtedly identical with compound II of Chaykin *et al.* (1958) and with the diphosphomevalonate of de Waard & Popják (1959). Chaykin *et al.* (1958) determined by isotopic labelling that this substance must be the 5-pyrophosphate of mevalonic acid; nevertheless chemical characterization of this substance is much needed. The presence of phosphomevalonic kinase in cruder preparations of mevalonic kinase does not influence seriously the spectrophotometric assays of the latter enzyme as long as the assays are based on initial reaction rates, as the concentrations of 5-phosphomevalonate are very low at first and the activity of 5-phosphomevalonic kinase is much lower than that of mevalonic kinase.

The inhibition of mevalonic kinase by farnesoate and its saturated analogue, 3:7:11-trimethyldodecanoate, is of some interest. Inhibition of cholesterol synthesis in liver homogenates from mevalonate by farnesoate was first reported by Wright & Cleland (1957) and confirmed in this Laboratory (unpublished work). 3:7:11-Trimethyldodecanoate is an even more powerful inhibitor of cholesterol synthesis *in vitro* than is farnesoate (G. Popják & K. Clifford, in preparation) and it is somewhat surprising that it acts at such an early step in the biosynthesis. *Trans-trans*-farnesoate is formed, among other branched-chain acids, enzymically from mevalonate in liver homogenates (Popják *et al.* 1959) by the metabolism of farnesylpyrophosphate. This substance represents an alternative pathway for the metabolism of intermediates in squalene biosynthesis and may be involved in a physiological controlling mechanism of cholesterol biosynthesis (G. Popják & M. Horning, unpublished work).

Since this paper was submitted for publication, two articles appeared (Henning, Möslin & Lynen, 1959; Bloch, Chaykin, Phillips & de Waard, 1959) in which the partial purification of phosphomevalonic kinase from yeast extracts is described. Both groups of workers established beyond doubt

that the diphosphomevalonate is in fact the 5-diphospho(pyrophospho)mevalonate. They also showed that 5-diphosphomevalonate yielded 5-phosphomevalonate in the presence of ADP and phosphomevalonic kinase, thereby implying the reversibility of the phosphomevalonic-kinase reaction. We did not succeed in demonstrating this.

SUMMARY

1. The enzyme which phosphorylates mevalonic acid in the 5-position, mevalonic kinase, has been partially purified from extracts of pig liver and its properties have been studied.

2. Liver mevalonic kinase is probably a sulphhydryl enzyme requiring cysteine for activation under aerobic conditions. Adenosine triphosphate (or inosine triphosphate) is its coenzyme, with the Mg^{2+} ion as activator. Calcium ions, or less effectively Mn^{2+} ions, also activate the enzyme; its pH optimum is 7.3.

3. Mevalonic kinase is distinct from hexokinase, and it cannot phosphorylate a number of substances related to mevalonate. It is active on only one enantiomorph of DL-mevalonate. Potassium farnesoate, and particularly the salt of its saturated analogue, 3:7:11-trimethyldodecanoate, inhibited mevalonic kinase.

4. A large-scale preparation and purification of 5-phospho[2-¹⁴C]mevalonate formed from DL-[2-¹⁴C]mevalonate with mevalonic kinase is described.

5. The properties of a second enzyme, which phosphorylates 5-phosphomevalonate to the 5-diphosphomevalonate, are briefly described. This enzyme has been named 5-phosphomevalonic kinase.

6. The properties of liver mevalonic kinase are discussed in relation to a similar enzyme partially purified from yeast extracts by Tchen (1958).

We wish to thank Dr and Mrs J. W. Cornforth of the National Institute for Medical Research, London, Dr Karl Folkers of the Merck Sharp and Dohme Research Laboratories, New Jersey, U.S.A., and Dr M. Stoll of Firmenich et Cie., Geneva, Switzerland, for their generous gifts of compounds. We are also indebted to Dr P. Hele of our Unit for providing us with extracts of pig liver used in the early parts of this investigation. Purified yeast hexokinase was provided by Dr F. A. Holton; Miss Hermione Hellig and Mr K. Clifford assisted during some of the experiments and Mr F. Gaebler carried out the phosphorus analyses on the phosphomevalonate specimens.

REFERENCES

- Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Amdur, B. H., Rilling, H. & Bloch, K. (1957). *J. Amer. chem. Soc.* **79**, 2647.
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.
- Cornforth, J. W., Cornforth, R. H., Popják, G. & Gore, I. Y. (1958). *Biochem. J.* **69**, 146.
- de Waard, A. (1958). *Doctoral Thesis: University of Utrecht, Holland; Investigations on the intermediates in the biosynthesis of fatty acids and cholesterol*. Nijmegen: Drukkerij Gebr. Janssen.
- de Waard, A. & Popják, G. (1959). *Biochem. J.* **73**, 410.
- Finch, L. & Hele, P. (1959). *Biochem. J.* **73**, 11 p.
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
- Gould, R. G. & Popják, G. (1957). *Biochem. J.* **66**, 51 p.
- Hele, P., Popják, G. & Lauryssens, M. (1957). *Biochem. J.* **65**, 348.
- Henning, U., Möslin, E. M. & Lynen, F. (1959). *Arch. Biochem. Biophys.* **83**, 259.
- Levy, H. R. & Popják, G. (1959). *Biochem. J.* **72**, 35 p.
- Lynen, F. (1959). *Ciba Foundation Symp., Biosynthesis of Terpenes and Sterols*, p. 95. London: J. and A. Churchill Ltd.
- Popják, G. (1959). *Ciba Foundation Symp., Biosynthesis of Terpenes and Sterols*, p. 148. London: J. and A. Churchill Ltd.
- Popják, G., Gosselin, L., Gore, I. Y. & Gould, R. G. (1958). *Biochem. J.* **69**, 238.
- Popják, G., Horning, M., Bucher, N. L. R. & Cornforth, R. H. (1959). *Biochem. J.* **72**, 34 p.
- Tavormina, P. A., Gibbs, M. H. & Huff, J. W. (1956). *J. Amer. chem. Soc.* **78**, 4498.
- Tchen, T. T. (1957). *J. Amer. chem. Soc.* **79**, 6345.
- Tchen, T. T. (1958). *J. biol. Chem.* **233**, 1100.
- Tsuboi, K. K. & Hudson, P. B. (1957). *J. biol. Chem.* **224**, 879.
- Wright, L. D. & Cleland, M. (1957). *Proc. Soc. exp. biol., N.Y.*, **96**, 219.

Biochem. J. (1960) **75**, 428

Guanosine Diphosphate Glucose and Guanosine Diphosphate Fructose from *Eremothecium ashbyii*

By H. G. PONTIS,* A. L. JAMES AND J. BADDILEY

Department of Chemistry, King's College, University of Durham, Newcastle upon Tyne

(Received 18 September 1959)

Guanosine diphosphate mannose was first isolated from baker's yeast (Cabib & Leloir, 1954) and has been shown to be present also in chicken oviduct (Strominger, 1955) and moulds (Ballio, Casinovi & Serlupi-Crescenzi, 1956). During the course of an investigation of the water-soluble nucleotides in the mould *Eremothecium ashbyii* we have isolated guanosine diphosphate mannose and shown that it is accompanied by two hitherto undescribed nucleotides which appear to be guanosine diphosphate glucose and guanosine diphosphate fructose. A preliminary account of this work has been published (Pontis, James & Baddiley, 1959).

EXPERIMENTAL

Materials. The guanosine and uridine phosphates were purchased from Pabst Laboratories, Wis., U.S.A. Uridine diphosphate glucose, uridine diphosphate *N*-acetylglucosamine and guanosine diphosphate mannose (GDPM) were a generous gift from Dr E. Cabib. These were supplied as barium salts and, before chromatography, the barium was removed by double decomposition with sodium sulphate.

Methods. Extinctions were measured in a Unicam SP. 500 spectrophotometer. The following analytical procedures were used: that of Fiske & Subbarow (1925) for phosphates,

that of Park & Johnson (1949) for reducing power and the Roe (1934) or the indole- H_2SO_4 method (Colowick & Kaplan, 1957) for fructose. In both methods for fructose the total volume was reduced to 0.5 ml.

Paper chromatography of sugars was carried out with butanol-pyridine-water (3.0:2.0:1.5) (Jeanes, Wise & Dimler, 1951) or phenol-water (Partridge, 1948). The chromatograms were developed with the alkaline silver (Trevelyan, Procter & Harrison, 1950) or benzidine-trichloroacetic acid (Bacon & Edelman, 1951) reagents.

For the chromatography of nucleotides the $(\text{NH}_4)_2\text{SO}_4$ -propan-2-ol-sodium acetate solvent (Markham & Smith, 1951) was used, and also ethanol-ammonium acetate mixtures (Paladini & Leloir, 1952). The latter solvent (pH 3.8) was used also for the chromatography of sugar phosphates. The position of the ultraviolet-absorbing substances was ascertained with a Hanovia lamp and that of phosphates by spraying with the Hanes & Isherwood (1949) molybdate reagent.

Ionophoresis on paper was performed with an apparatus similar to that described by Markham & Smith (1952), with Whatman no. 1 paper soaked in 0.05 M-sodium borate, pH 9.2, for sugars (Consden & Stanier, 1952) and 0.02 M-sodium acetate-HCl buffer, pH 2.0, for bases. The sugars were revealed on paper electrophoresis with the alkaline silver reagent by the modified technique described by Cabib & Leloir (1958).

In chromatographic as well as in ionophoresis experiments the position of the substances on the paper was referred to the position of an appropriate standard (ribose for

* Present address: Instituto de Investigaciones Bioquímicas, Obligado 2490, Buenos Aires, Argentina.