The Activity and Kinetic Properties of Mevalonate Kinase in Superovulated Rat Ovary

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Methods are described for the assay and partial purification of mevalonate kinase from superovulated rat ovary. The total activity of mevalonate kinase in superovulated rat ovary was 1.6 ± 0.14 units/g wet wt.; it was unchanged by the administration of luteinizing hormone *in vivo*. The K_m of a partially purified preparation of mevalonate kinase for DL-Mevalonate was $3.6 \pm 0.5 \mu\text{M}$; its K_m for MgATP^{2-} was $120 \pm 7.7 \mu\text{M}$. The enzyme was inhibited by geranyl pyrophosphate and farnesyl pyrophosphate, but not by isopentenyl pyrophosphate or 3,3'-dimethylallyl pyrophosphate. DL-mevalonate 5-phosphate inhibited at high concentrations. With both geranyl pyrophosphate and farnesyl pyrophosphate the inhibition was competitive with respect to MgATP^{2-} . The K_i for inhibition by geranyl pyrophosphate was $1.3 \pm 0.2 \mu\text{M}$; the K_i for inhibition by farnesyl pyrophosphate was $1.0 \pm 0.3 \mu\text{M}$. These findings are discussed with reference to the control by luteinizing hormone of steroidogenesis from acetate.

Mevalonate kinase (ATP-mevalonate 5-phosphotransferase, EC 2.7.1.36) is known to be present in yeast (Tchen, 1958) and in the livers of the rat (Levy & Popják, 1959), pig (Levy & Popják, 1960) and rabbit (Markely & Smallman, 1961). The pig liver enzyme has been extensively purified and has been shown to be inhibited by geranyl pyrophosphate and farnesyl pyrophosphate, which are intermediates in the sterol synthesis pathway beyond the mevalonate kinase reaction (Dorsey & Porter, 1968). There are, however, no reports of the presence of mevalonate kinase in steroidogenic endocrine tissues, although sterol synthesis occurs in these tissues by a route apparently identical with that in liver (Caspi, Dorfman, Kahn, Rosenfeld & Schmid, 1962; Billiar, Oriol-Bosch & Eik-Nes, 1965; Salokangas, Rilling & Samuels, 1965). It was considered of interest therefore to examine mevalonate kinase from such a tissue and to compare its properties with those of the liver enzyme.

MATERIALS

Animals. Superovulated ovaries were obtained from immature female Wistar rats treated with pregnant-mare serum gonadotrophin and human chorionic gonadotrophin as described by Stansfield & Flint (1967). The reasons for using this preparation have been given previously (Flint & Denton, 1970). The rats were fed with stock laboratory diet 41B (Short & Parkes, 1949) and given water *ad libitum*.

Luteinizing hormone was administered intraperitoneally in doses of $20 \mu\text{g}/100 \text{g}$ body wt. in 0.5 ml of 0.9% NaCl 2 h before death. Control animals received the saline.

Chemicals. DL-[2- ^{14}C]Mevalonolactone was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. and was converted into potassium mevalonate by incubation with a 2% molar excess of KOH at 40°C for 1 h; the solution was thereafter stored at -20°C . DL-Mevalonolactone, obtained from either Sigma Chemical Co., St Louis, Mo., U.S.A., or Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K., was also converted into potassium mevalonate, and stored, in this way. Triscyclohexylammonium DL-mevalonate 5-phosphate and triscyclohexylammonium isopentenyl pyrophosphate were obtained from Mann Research Laboratories Inc., New York, N.Y., U.S.A. and were used without prior purification. Geraniol (technical grade) supplied by British Drug Houses Ltd., Poole, Dorset, U.K. was purified by distillation (collecting the fraction with b.p. $228\text{--}230^\circ\text{C}$) followed by clathrate formation with anhydrous CaCl_2 as described by Guenther (1949). 3,3'-Dimethylacrylic acid and trichloroacetonitrile were obtained from R. N. Emanuel Ltd., Alpertown, Middx., U.K.

Human chorionic gonadotrophin and pregnant-mare serum gonadotrophin (Pregnyl and Gestyl) were obtained from Organon Laboratories Ltd., Morden, Surrey, U.K.; luteinizing hormone (NIH-LH-S13, ovine) was a kind gift from the National Institutes of Health, Bethesda, Md., U.S.A.

Nucleotides, sugar phosphates and enzymes were obtained from the Boehringer Corp. (London) Ltd., London W.5, U.K.; Dowex 1 (formate form) (AG-X8) was from Bio-Rad Laboratories, Richmond, Calif., U.S.A.; DEAE-cellulose (Whatman DE-11) was supplied

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by W. and R. Balston Ltd., Maidstone, Kent, U.K., and 5-(4-biphenyl)-2-(4-*tert.*-butylphenyl)-1-oxa-3,4-diazole (butyl-PBD) was obtained from CIBA (A.R.L.) Ltd., Duxford, Cambs., U.K.

PROCEDURES AND METHODS

Preparation of prenyl pyrophosphates

Geranyl pyrophosphate. Geranyl pyrophosphate was prepared by the method of Cramer & Böhm (1959) as modified by Popják, Cornforth, Cornforth, Ryhage & Goodman (1962). The trilithium geranyl pyrophosphate obtained on phosphorylation of purified geraniol with diethylamine phosphate in trichloroacetonitrile gave three spots on descending paper chromatography on Whatman 3MM paper developed in propan-2-ol-butan-2-ol-NH₃-water (40:20:1:39, by vol.), *R_F* values 0.0, 0.1 and 0.8 (the latter spot appearing blue on exposing the paper to u.v. light) corresponding to phosphate, pyrophosphate and geranyl pyrophosphate respectively (Dorsey, Dorsey & Porter, 1966). Phosphates were stained on the chromatogram with the ammonium molybdate reagent of Bandurski & Axelrod (1951). Phosphate determination by the method of Chen, Toribara & Warner (1956) after boiling with 2.5M-H₂SO₄ for 3 min gave 5.6 μmol of acid-labile phosphate/mg of geranyl pyrophosphate (i.e. 92% of the theoretical yield expected from trilithium geranyl pyrophosphate).

Dimethylallyl pyrophosphate. 3,3'-Dimethylallyl pyrophosphate (trilithium salt) was synthesized from 3,3'-dimethylallyl alcohol by the method used to phosphorylate geraniol. 3,3'-Dimethylallyl alcohol was prepared from 3,3'-dimethylacrylic acid by reduction with LiAlH₄ in anhydrous ether by the method of Yuan & Bloch (1959). The trilithium 3,3'-dimethylallyl pyrophosphate obtained gave three spots on descending paper chromatography on Whatman no. 1 paper developed in propan-1-ol-NH₃-water (6:2:3, by vol.), *R_F* values 0.0, 0.1 and 0.4-0.5 corresponding to phosphate, pyrophosphate and 3,3'-dimethylallyl pyrophosphate respectively (Dugan, Rasson & Porter, 1968). Phosphate determination, carried out as for geranyl pyrophosphate, gave 7.0 μmol of acid-labile phosphate/mg (i.e. 93% of the theoretical yield expected for trilithium 3,3'-dimethylallyl pyrophosphate).

Farnesyl pyrophosphate. Farnesyl pyrophosphate was prepared biosynthetically from DL-[2-¹⁴C]mevalonate by the method outlined by Dugan *et al.* (1968). The supernatant obtained from a homogenate of rat liver (56 g) in a solution containing sucrose (0.25M), ethanedioxybis(ethylamine)tetra-acetate (EGTA) (1mM) and tris (3.4mM), pH 7.4, after centrifugation at 90 000g for 45 min at 4°C, was fractionated with solid (NH₄)₂SO₄. The fraction precipitating between 30 and 60% saturation was dissolved in 20mM-potassium phosphate buffer (15ml), pH 7.1, and was dialysed against this buffer overnight before use. This preparation (2ml) was incubated in 20mM-potassium phosphate buffer (pH 7.1) with DL-[2-¹⁴C]mevalonate (5mM; 6.25 μCi/mmol), glutathione (10mM), MgATP²⁻ (5mM), phosphoenolpyruvate (10mM) and pyruvate kinase (0.5 unit) in a total volume of 5ml at 37°C for 4 h. The resulting mixture was applied to a column (2 cm × 20 cm) of DEAE-cellulose (acid- and base-washed as described by Dugan *et al.* 1968) equilibrated with 10mM-(NH₄)₂CO₃, at room temperature, and un-

changed mevalonate, isopentenyl pyrophosphate, 3,3'-dimethylallyl pyrophosphate, mevalonate 5-phosphate and farnesyl pyrophosphate eluted with stepwise increments in (NH₄)₂CO₃ concentration as described by Dugan *et al.* (1968). Farnesyl pyrophosphate was eluted immediately after mevalonate 5-phosphate and was concentrated by freeze-drying.

The material so obtained was contaminated with mevalonate 5-phosphate and was further purified by descending paper chromatography on Whatman no. 1 paper developed in propan-2-ol-butan-2-ol-NH₃-water (40:20:1:39, by vol.); the material with *R_F* 0.8-0.9 was eluted with propan-2-ol-butan-2-ol-NH₃-water (40:20:1:39, by vol.), extracted twice with 2 vol. of diethyl ether and concentrated by rotary evaporation under reduced pressure at 40°C. The farnesyl pyrophosphate obtained was chromatographically pure in the propan-2-ol-butan-2-ol-NH₃-water system described above. The concentration of the resulting solution was determined from its ¹⁴C content and a knowledge of the specific radioactivity of the [2-¹⁴C]mevalonate used in the preparation.

Assays for mevalonate kinase

Assay with DL-[2-¹⁴C]mevalonate. Mevalonate kinase was assayed in crude homogenates of superovulated rat ovary (where there is an approximate fivefold excess of adenosine triphosphatase activity over that of mevalonate kinase) by a radioactivity assay as follows. About 200 mg of tissue was homogenized in 5 ml of 100mM-triethanolamine-HCl buffer, pH 7.4, containing MgCl₂ (6mM) in a Kontes grinder (Kontes Glass Co., Vineland, N.J., U.S.A.) at 4°C. Up to 0.1 ml of this homogenate was then incubated for 10 min at 37°C in 100mM-triethanolamine-HCl buffer, pH 7.4, containing MgCl₂ (6mM), ATP (2mM), phosphoenolpyruvate (0.5mM), pyruvate kinase (1 unit) and DL-[2-¹⁴C]mevalonate (1mM; 6.25 μCi/mmol) in a total volume of 1 ml. The reaction was stopped by the addition of 4M-formic acid (1 ml) and the reaction mixture was applied to a column (0.5 cm × 10 cm) of Dowex 1 (formate form) equilibrated with 2M-formic acid. After washing the column with 4M-formic acid (10 ml), the material eluted with 6 ml of 8M-formic acid containing 0.8M-ammonium formate was taken for radioactivity assay (0.5-1.0 ml in 15 ml of scintillation fluid containing 7 g of butyl-PBD and 100 g of naphthalene/l of dioxan). Counting efficiencies (determined by using an external standard of ¹³³Ba) were always at least 40%, and more than 10⁴ counts were always recorded. More than 95% of the radioactive material left on the column after washing with 10 ml of 4M-formic acid was eluted by this procedure; all ¹⁴C present as mevalonate 5-phosphate, mevalonate 5-pyrophosphate or isopentenyl pyrophosphate, was, therefore, included in the material counted for radioactivity. The activity of mevalonate kinase was calculated from a knowledge of the specific radioactivity of the DL-[2-¹⁴C]mevalonate used as substrate.

Under these conditions the ¹⁴C content of the eluant increased linearly with time of incubation (Fig. 1*a*), but not with the concentration (above 0.1 ml) of ovary homogenate (Fig. 1*b*). This indicates the presence of an inhibitor of mevalonate kinase in the homogenate; as a result this assay could not be used with volumes of homogenate in excess of 0.1 ml.

Fig. 1. Radioactivity assay for mevalonate kinase. (a) Time-course of the incorporation of ^{14}C from DL-[2- ^{14}C]mevalonate into mevalonate 5-phosphate, measured as described in the Procedures and Methods section, with various volumes of tissue homogenate as follows: $\blacktriangle$, 0.05 ml; $\circ$, 0.1 ml; $\triangle$, 0.2 ml; $\bullet$, 0.5 ml. (b) Dependence of reaction rate on concentration of homogenate; data of (a) replotted as slopes versus volume of homogenate.

Spectrophotometric assay. Partially pure preparations of mevalonate kinase were assayed by following the rate of oxidation of NADH at 340 nm in 100 mM-triethanolamine-HCl buffer, pH 7.4, containing MgCl_2 (6 mM), ATP (2 mM), phosphoenolpyruvate (0.2 mM), NADH (0.1 mM), DL-mevalonate (1 mM), pyruvate kinase (1 unit) and lactate dehydrogenase (1 unit), total volume 2.5 ml, at 25°C, with a Hilger spectrophotometer with a Gilford kinetic recording attachment. All components of the assay system (including inhibitors, when present) were mixed before the addition of DL-mevalonate (which was used to start the reaction). The reaction rate was linear for up to 10 min after an initial lag period of 1–2 min; it was linear with mevalonate kinase concentration up to 0.4 ml (0.1–0.2 mg of protein) of the material eluted from DEAE-cellulose.

Partial purification of mevalonate kinase

Extraction of mevalonate kinase. Ovaries (2–3 g) from 15–20 rats were washed in 10 mM-potassium phosphate buffer, pH 7.4, and homogenized in 4 vol. of the same buffer at 4°C in a Kontes grinder. The homogenate was centrifuged at 25000 g_{av} for 30 min at 4°C.

DEAE-cellulose fractionation. The supernatant obtained from the above extraction procedure was layered on to a fast-flowing (4–6 ml/min) column (2.5 cm $\times$ 15 cm) of DEAE-cellulose equilibrated with 10 mM-potassium phosphate buffer, pH 7.4. After washing with 20 ml of the same buffer, material on the column was eluted with a continuous linear gradient of potassium phosphate buffer, pH 7.4, generated by the addition of 150 mM-potassium phosphate buffer, pH 7.4 (150 ml), to 10 mM-potassium phosphate buffer, pH 7.4 (150 ml), in an open vessel. Mevalonate kinase, which was eluted as a single peak after 90–110 ml of this gradient, was contaminated with <10% of adenosine triphosphatase plus NADH-oxidase

activity and <1% of phosphomevalonate kinase activity; this therefore represents an approximate 50-fold purification of mevalonate kinase with respect to adenine triphosphatase activity. The specific activity of mevalonate kinase in the eluate varied between preparations in the range 60–120 mU/mg of protein; the recovery of activity in the eluate was never less than 60%.

Protein assay. Protein in homogenates and in eluates from DEAE-cellulose was assayed by the method of Lowry, Rosebrough, Farr & Randall (1951), with a solution of bovine serum albumin as a standard.

Analysis of kinetic results

Reaction rates determined at various inhibitor and substrate concentrations were fitted to the Michaelis-Menten equation, and to the equations for competitive, mixed, non-competitive and uncompetitive types of inhibition by using a computer program (written in Algol-60) kindly lent by Dr P. J. England, Department of Biochemistry, University of Bristol. This program fits by the method of least squares (assuming an equal variance on all velocities) and computes values for V_{max} , K_m and K_i with standard errors, as described by Haarhoff (1969).

RESULTS

Total activity of mevalonate kinase in superovulated rat ovary and effect of administration of luteinizing hormone in vivo. The total activity of mevalonate kinase in unfractionated homogenates of superovulated rat ovary (assayed by the radioactive method, with DL-[2- ^{14}C]mevalonate, and carried out at 37°C) was 1.6 ± 0.14 U/g wet wt. (mean $\pm$ S.E.M. of six values); the total activity in

the ovaries of rats treated with luteinizing hormone was 1.6 ± 0.29 U/g wet wt. (mean $\pm$ S.E.M. of five values). [One unit converts one μ mol of substrate/min.] Expressed in terms of the protein content of the homogenates the values were 15.0 ± 1.20 U/g of protein in ovaries from control animals and 12.9 ± 1.20 U/g of protein in ovaries from animals treated with luteinizing hormone.

Kinetic properties of mevalonate kinase. The results described in this section were obtained by using the partially purified preparation of mevalonate kinase and the spectrophotometric assay.

The pH optimum of mevalonate kinase was between pH 7.0 and pH 7.5. As was demonstrated by Levy & Popják (1960) the activities of the ancillary enzymes used in the assay system (pyruvate kinase and lactate dehydrogenase) could be maintained in an excess over that of mevalonate kinase at all the pH values used. The buffers used were: 0.1M-citrate-potassium hydroxide, pH 5.0; 0.1M-potassium phosphate, pH 6.0 and pH 6.5; 0.1M-triethanolamine-HCl, pH 7.0 and pH 7.5; 0.1M-tris-hydrochloric acid, pH 8.0 and pH 8.5; 0.1M-glycine-potassium hydroxide, pH 9.0. As a result of this finding assays were carried out at pH 7.4.

The enzyme required Mg^{2+} for activity; with 2mM-ATP maximum activity was reached with 3mM- Mg^{2+} or greater. The activity of the ancillary enzyme system was shown to be in excess of the mevalonate kinase activity at all Mg^{2+} concentrations tested.

The partially purified preparation of mevalonate kinase had K_m values for DL-mevalonate and $MgATP^{2-}$ of $3.6 \pm 0.5 \mu M$ (10) and $120 \pm 7.7 \mu M$ (6) respectively (means $\pm$ S.E.M.; the number of rate measurements made is given in parentheses). The K_m for DL-mevalonate was obtained by using 2mM- $MgATP^{2-}$ and DL-mevalonate concentrations between 1 and 10 μM , and that for $MgATP^{2-}$ by using 1mM-DL-mevalonate and $MgATP^{2-}$ concentrations between 50 and 340 μM .

Mevalonate kinase from superovulated rat ovary was inhibited by both geranyl pyrophosphate and farnesyl pyrophosphate, but not by isopentenyl pyrophosphate or 3,3'-dimethylallyl pyrophosphate (Fig. 2); DL-mevalonate 5-phosphate was an inhibitor at high concentrations (Fig. 2). Farnesyl pyrophosphate was a more potent inhibitor than geranyl pyrophosphate; the results in Fig. 3 give a value of $1.0 \pm 0.3 \mu M$ for the K_i for farnesyl pyrophosphate inhibition versus $MgATP^{2-}$; the K_i for geranyl pyrophosphate inhibition versus $MgATP^{2-}$ calculated from the results of Fig. 4 was $1.3 \pm 0.2 \mu M$ (means $\pm$ S.E.M.). Both farnesyl pyrophosphate and geranyl pyrophosphate were competitive inhibitors with respect to $MgATP^{2-}$.

The following compounds had no effect on the

Fig. 2. Effect of steroidogenic intermediates on mevalonate kinase activity. Activities of mevalonate kinase were determined with the spectrophotometric assay in the presence of various concentrations of additives as follows: Δ , 3,3'-dimethylallyl pyrophosphate; $\blacktriangle$, isopentenyl pyrophosphate; $\circ$, DL-mevalonate 5-phosphate; $\bullet$, geranyl pyrophosphate; $\square$, farnesyl pyrophosphate. Activities are expressed in terms of the maximum activity of the enzyme, which was measured in the absence of additives.

Fig. 3. Inhibition of mevalonate kinase by farnesyl pyrophosphate. Activities (v) were determined by using the spectrophotometric assay in the presence of 1mM-DL-mevalonate at various $MgATP^{2-}$ concentrations. The concentrations of farnesyl pyrophosphate were as follows: $\circ$, 0.0 μM ; Δ , 2.0 μM ; $\bullet$, 5.8 μM ; $\blacktriangle$, 8.4 μM .

Fig. 4. Inhibition of mevalonate kinase by geranyl pyrophosphate. Activities (v) were determined by using the spectrophotometric assay in the presence of 1 mM DL-mevalonate at various MgATP^{2-} concentrations. The concentrations of geranyl pyrophosphate were as follows: $\square$, 0.0 μM ; $\triangle$, 0.6 μM ; $\circ$, 1.9 μM ; $\blacksquare$, 3.1 μM ; $\blacktriangle$, 6.2 μM ; $\bullet$, 15.4 μM .

activity of superovulated rat ovary mevalonate kinase at the concentrations indicated: phosphate (sodium salt), pyrophosphate (sodium salt), citrate (potassium salt), Li^+ (chloride), ascorbate and dehydroascorbate, all at 1 mM; glutathione and L-cysteine, at 10 mM; 3':5'-cyclic AMP, at 50 μM .

DISCUSSION

The present investigation has disclosed two major differences between the mevalonate kinase from mammalian liver and that from superovulated rat ovary. First, the liver enzyme is activated by glutathione and cysteine (Levy & Popják, 1960; Markely & Smallman, 1961), whereas that from the superovulated rat ovary is not. Secondly, there is a difference between the K_m values of the two enzymes for DL-mevalonate; the liver enzyme has a K_m of 50 μM for DL-mevalonate (Levy & Popják, 1960); that for the enzyme from superovulated rat ovary is 3.6 μM . These differences could be related to the more extensive purification of the liver enzyme used in kinetic studies. In other respects the two enzymes are alike: both are inhibited by geranyl pyrophosphate and farnesyl pyrophosphate, but not by isopentenyl pyrophosphate or 3,3'-dimethylallyl pyrophosphate. The liver enzyme has not been tested for inhibition by DL-mevalonate 5-phosphate.

The inhibition of mevalonate kinase by geranyl

pyrophosphate and farnesyl pyrophosphate offers a means by which these steroidogenic intermediates can control the rate of their own production. This is of obvious advantage to the cell, since 6 mol of ATP are required for the synthesis of 1 mol of geranyl pyrophosphate, and 9 mol of ATP are required for the synthesis of 1 mol of farnesyl pyrophosphate, from mevalonate. No data are available for the concentrations of geranyl pyrophosphate or farnesyl pyrophosphate in superovulated rat ovary; it can be calculated, however, from the rate equation for competitive inhibition and a knowledge of the intracellular MgATP^{2-} concentration (which is approx. 1 mM in tissue slices incubated in a bicarbonate-buffered medium; Flint, 1970) that 50% inhibition of mevalonate kinase would result from a geranyl pyrophosphate concentration of 9.5 μM or a farnesyl pyrophosphate concentration of 7.3 μM (assuming strictly competitive kinetics and a saturating concentration of mevalonate). It seems likely, therefore, that significant inhibition of the enzyme could occur at physiological concentrations of these prenyl pyrophosphates; the K_m for geranyl pyrophosphate in the synthesis of farnesyl pyrophosphate has been reported to be 4 μM in liver (Benedict, Kett & Porter, 1965; Holloway & Popják, 1967), and it is possible that concentrations of this order are found in other tissues.

It has been observed (Armstrong, 1968; Flint & Denton, 1969) that a stimulation of steroidogenesis from acetate follows the transient stimulation of steroidogenesis from stored sterol ester caused in the superovulated rat ovary *in vitro* by luteinizing hormone administered *in vivo*. That this effect may involve activation at a step in the steroidogenic pathway between mevalonate and squalene is indicated by the finding that trophic hormone stimulation of bovine corpus luteum slices *in vitro* results in an increased incorporation of ^{14}C from DL-[2- ^{14}C]mevalonate into squalene (Hellig & Savard, 1965). Such a mechanism is consistent with the explanation given by Kowal (1969) for the effects of adrenocorticotrophin and aminoglutethimide on steroid synthesis from acetate in functional monolayer cultures of murine adrenal cortex tumours. The present investigation has revealed that such a stimulation is unlikely to be the result of an effect of luteinizing hormone on the total activity of mevalonate kinase or of a direct activation of the enzyme by 3':5'-cyclic AMP. It is possible however that a de-inhibition of mevalonate kinase occurs as the result of a decrease in the concentrations of geranyl pyrophosphate or farnesyl pyrophosphate. Such an effect seems to be involved in the control of sterol synthesis in liver, as part of a complex system of feedback steps involving 3-hydroxy-3-methylglutaryl-CoA reductase (EC

1.1.1.34), mevalonate kinase and squalene synthase (Gould & Swyrd, 1966; Siperstein & Fagan, 1966; Dorsey & Porter, 1968). The inhibition of mevalonate kinase by geranyl pyrophosphate and farnesyl pyrophosphate may therefore play an important part in the control of steroidogenesis from acetate in steroidogenic endocrine tissues.

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