Terpene Metabolism in the Rat Testis. II. Metabolism of Mevalonic Acid by Cell-free Preparations*

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ABSTRACT: Mevalonic acid has been found to be rapidly converted to 5-phosphomevalonic acid by 700 × *g* supernatants of rat testicular homogenates. No appreciable amounts of other metabolites of mevalonic acid are formed by this preparation. In contrast, a 110,000 × *g* supernatant from similar homogenates readily converts mevalonic acid to 5-phosphomevalonic acid, 5-pyrophosphomevalonic acid, isopentenyl pyrophosphate, and the allylpyrophosphates. If the 700 × *g* supernatant is then added the further conversion to squalene and sterols takes place.

It is postulated that the rapid destruction of adenosine triphosphate by a microsomal adenosine triphosphatase is responsible for the inability of the 700 × *g* supernatant to further metabolize 5-phosphomevalonic acid. Intact testicular interstitial cells appear to be relatively impermeable to mevalonic acid.

The cells of endocrine tissues which form steroid hormones readily use acetate in hormone synthesis. Yet, while there has been some success in converting mevalonic acid to sterols and steroids with preparations of these tissues (Rabinowitz, 1959; Tsai *et al.*, 1964; Billiar *et al.*, 1965), the extent of this conversion has been relatively low and, coupled with the failure of others (Bryson and Sweat, 1962; Savard *et al.*, 1960), has raised the question of possible alternate routes of biosynthesis of steroids in endocrine tissues. In a previous paper we demonstrated the conversion of a metabolite of mevalonic acid, isopentenyl pyrophosphate, to squalene and sterols by homogenates of rat testis (Salokangas *et al.*, 1964), but at that time we were unable to obtain conversion of mevalonic acid to squalene. In this paper we report the synthesis of squalene and allylpyrophosphates from mevalonic acid by a preparation from a homogenate of rat testicular tissue. We also present evidence that the destruction of ATP[1] by testicular microsomes was responsible for the inefficient metabolism of mevalonic acid previously observed by us. Also the failure to obtain significant conversions of mevalonic acid with preparations involving unbroken cells appears to be owing to failure of mevalonate to penetrate normal intact interstitial cells

Experimental

Materials. DL-[5-^{3}H]Mevalonic acid (*N,N*-dibenzylethylenediamine salt) was purchased from New England Nuclear Corp. Anion exchange resin Dowex 1 (chloride), analytical grade, was a product of Bio-Rad Laboratories.

Tissue Preparations and Incubation. For the experiments with slices, testes of 2-month-old rats were removed, decapsulated, and cut into 0.5-mm-thick slices with a tissue slicer. The tissue was immediately placed in cold incubation flasks with appropriate amounts of cofactors and 0.2 M Tris buffer, *p*H 7.1, containing 0.04 M nicotinamide. For teased preparations, testes were suspended with the help of forceps in cold buffer, 2 ml/g tissue. Homogenates were prepared in the buffer described for slices (2 ml/g of tissue) using a Ten-Broeck glass tissue grinder, and centrifuged at 700 × *g* for 10 minutes at 2° in a Servall centrifuge to remove nuclei and cell debris. A soluble enzyme preparation was obtained from testis homogenate by centrifugation at 110,000 × *g* for 2 hours at 2° in a Beckman Model L-2 preparative ultracentrifuge. After addition of the appropriate substrates and cofactors, incubations were conducted at 37° for the indicated time intervals in a shaking incubator under air.

Fractionation of Phosphate-containing Metabolites of Mevalonic Acid. IPP and the phosphate esters of mevalonic acid were fractionated on a 1- × 2-cm column of Dowex 1 (formate) which was prepared from Dowex 1 (chloride) (Bloch *et al.*, 1959). Mevalonic acid was eluted with 0.2 N formic acid, MVAP with 4 N formic acid, and MVAPP and IPP (incompletely separated) with 0.8 M ammonium formate in 4 N formic acid.

Identification of Phosphate Esters of Mevalonic Acid. MVAP, MVAPP, and IPP recovered from ion-exchange columns were further characterized by paper chroma-

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[1] Abbreviations used in this work: ATP, adenosine-5′-triphosphate; MVAP, 5-phosphomevalonic acid; MVAPP, 5-pyrophosphomevalonic acid; IPP, isopentenyl pyrophosphate; ATPase, adenosine triphosphatase; DPN, diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

tography in the systems of *t*-butyl alcohol–formic acid–water (40:10:16) and 1-butanol–formic acid–water (77:10:13) (Bloch *et al.*, 1959). On incubation with snake venom (Naja Naja) phosphatase, MVAP and MVAPP were dephosphorylated to mevalonic acid which was identified by comparison with authentic [^{3}H]mevalonate on paper chromatograms, and [^{3}H]IPP was converted to a hexane-soluble, volatile material, presumably [^{3}H]isopentenyl alcohol.

Saponification and Hydrolysis of Terpene Pyrophosphates. Saponification and extraction of nonsaponifiable material was performed as described earlier (Salokangas *et al.*, 1964). The aqueous residue remaining after ether-chloroform extraction, containing the alkali-stable terpene pyrophosphates, was made 2 N in acid with concentrated hydrochloric acid and allowed to stand at room temperature for 2 hours under a protective layer of Skellysolve B to hydrolyze these compounds. The aqueous phase was made alkaline with solid KOH using phenolphthalein as indicator, and the terpene alcohols were extracted twice with 5 ml of Skellysolve B. The organic solvent was washed twice with 0.5 ml of water and dried over anhydrous Na_2SO_4. Half of the extract was transferred to counting vials for radioactive estimation.

The nonsaponifiable material was fractionated on alumina as described earlier (Salokangas *et al.*, 1964) and aliquots from column fractions were taken to dryness in counting vials for determination of radioactivity, which was performed as described in the previous publication. Aqueous solutions up to 0.2 ml were counted in absolute ethanol–scintillation fluid (3:10).

Estimation of Adenosine-5'-triphosphate in Incubations. Aliquots from incubations were applied on a 1- × 2-cm Dowex 1 (chloride) column (Cohn, 1957) previously washed to neutrality with water. After loading, the columns were washed with 10 ml of 0.01 M ammonium chloride. Nucleotide monophosphates were eluted with 100 ml of 0.005 N hydrochloric acid and diphosphates with 100 ml of 0.01 N hydrochloric acid containing 0.04 N sodium chloride. ATP was eluted with 50 ml of 0.01 N hydrochloric acid 0.2 N in sodium chloride. Quantitative estimation of ATP was performed with a Zeiss PMQ II spectrophotometer by measuring the absorbancy at 260 mμ. Parallel incubations with no added ATP were included in these experiments as controls.

Results

Experiments with the 110,000 × g Supernatant. Since the 700 × *g* supernatant from rat testicular homogenates was found to convert IPP to squalene and sterols while mevalonic acid was not metabolized (Salokangas *et al.*, 1964), it is apparent that the failure of this homogenate to make sterols from mevalonate lay in the steps between mevalonic acid and IPP. The enzymes in other tissues necessary for the conversion of mevalonic acid to IPP are known to be soluble (Tchen, 1962). We decided, therefore, to examine a high-speed supernatant preparation from rat testis for its ability to metabolize mevalonic acid. With this preparation as a source of enzymes, mevalonic acid was rapidly converted to a number of intermediates on the biosynthetic pathway to squalene (Table I). In this experiment MVAPP and IPP were determined together as a single column fraction; in other similar experiments, however, paper chromatography of this fraction revealed the presence of both MVAPP and IPP. In this experiment as well as in others in this paper the radioactive materials rendered soluble in organic solvent by mild acid hydrolysis were assumed to be the allylpyrophosphates: dimethylallylpyrophosphate, geranylpyrophosphate, and farnesylpyrophosphate (Goodman and Popjak, 1960). No radioactive squalene was detected. Considering that the mevalonic acid used was a mixture of the D and L isomers, about 60–80% of the natural isomer was converted to the phosphate-containing intermediates.

TABLE I: Metabolism of Mevalonic Acid by a 110,000 × *g* Supernatant of Rat Testis Homogenate.[a]

Time of Incubation (min)	mμMoles [^{3}H]Mevalonate Incorporated into: MVAP	MVAPP + IPP	Allyl-pyrophos-phates	Total
10	1.14	2.37	0.78	4.29
20	1.82	1.37	2.10	5.29

[a] Average of three determinations. Each incubation flask contained: Tris buffer, *p*H 7.1, 206 μmoles; nicotinamide, 55 μmoles; sodium EDTA, 3 μmoles; $MgCl_2$, 20 μmoles; reduced glutathione, 40 μmoles; ATP, 5 μmoles; sodium fluoride, 10 μmoles; DL-[^{3}H]mevalonic acid, 13 mμmoles (specific activity 103 mc/mmole); homogenate, 0.5 ml in a total volume of 2 ml.

Parameters of Incubation Conditions Using the 110,000 × g Supernatant. The effect of various concentrations of Mg^{2+}, ATP, and F^- on the ability of the 110,000 × *g* supernatant to metabolize mevalonic acid was determined in a number of experiments. ATP, F^-, and Mg^{2+} were all necessary for the efficient metabolism of mevalonic acid. When F^- was omitted some MVAP was formed but no conversion of mevalonic acid either to pyrophosphate or to the acid-labile material was observed. On the other hand, increasing fluoride concentration in steps from 5 to 60 mM had little effect on the formation of MVAP and MVAPP + IPP fraction. Fluoride at 60 mM inhibited the formation of allylpyrophosphates. Increasing ATP concentration from 2.5 to 10 mM did not enhance the yield of the phosphorylated compounds. A concentration of 10–15 mM Mg^{2+} was optimal for the synthesis of the allylpyrophosphates.

The effect of *p*H on the formation of the various phosphorylated intermediates is shown in Figure 1. There was a rather sharp optimum at *p*H 7.1 for the

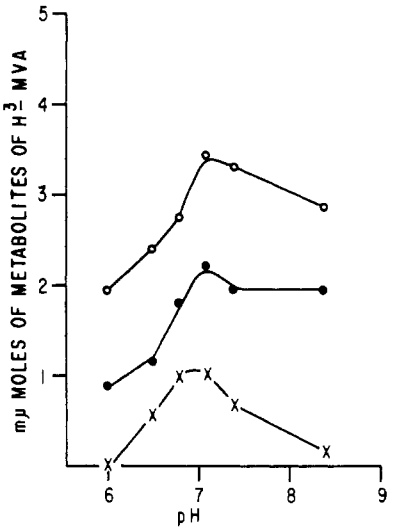

FIGURE 1: The *p*H dependence of formation of phosphorylated metabolites of mevalonic acid (MVA). In the incubations performed at *p*H 6.0 and 6.5, histidine-HCl buffer (205 μmoles) was used. In incubations at *p*H 6.8–8.4, Tris-HCl buffer (205 μmoles) was used. In all incubations sodium EDTA, 3 μmoles; $MgCl_2$, 20 μmoles; sodium fluoride, 10 μmoles; ATP, 10 μmoles reduced glutathione, 20 μmoles; [^{3}H]mevalonic acid, 11.9 mμmoles (specific activity of 103 μc/μmole), were added. Supernatant of testis homogenate 110,000 × *g* (0.5 ml) was utilized as a source of enzymes in total volume of 2 ml. Time of incubation was 20 minutes. O—O, MVAP + MVAPP + IPP + acid-labile compounds; ●—●, MVAPP + IPP + acid-labile compounds; ×--×, acid-labile compounds.

synthesis of the allylpyrophosphates. The *p*H optimum for the formation of the other phosphorylated intermediates was considerably broader and had a maximum at a slightly more alkaline *p*H. In view of the rather sharp optimum for the synthesis of the allylpyrophosphates, great care was taken to adjust incubation mixtures to *p*H 7.1.

To test the possibility that the products of incubation with the 110,000 × *g* supernatant could be converted to squalene and sterols by the particulate enzymes, mevalonic acid was first incubated with the soluble fraction and then with the 700 × *g* supernatant. The enzyme activity of each fraction alone was tested at the same time so that direct comparison with the sequential incubations was possible.

The incubations with each of the two fractions alone confirmed previous results: no significant amount of radioactive nonsaponifiable material was formed. When, however, mevalonate was first incubated with the high-speed supernatant for various times and then with the 700 × *g* preparation for a constant time (20 minutes) the yield of squalene and sterols increased with the length of the first incubation (Table II). It is apparent, therefore, that all of the enzymes necessary for the conversion of mevalonic acid to squalene and sterols are present in testicular homogenates. Why then does the whole homogenate fail to convert mevalonic acid to squalene?

Products of Incubation with a 700 × g Supernatant. Our approach to this question was to compare the synthesis of phosphorylated intermediates between mevalonic acid and IPP by the 700 × *g* homogenate with that using the high-speed supernatant. Table III gives the results of an experiment typical of many carried out

TABLE II: Effect of Particulate Fraction on Formation of Nonsaponifiable Material.[a]

Expt	Type of Preparation	Volume of Homogenate (ml)	1st Incub. (min)	2nd Incub. (min)	mμMoles [^{3}H]Mevalonate Incorporated into: Acid Labile	Total Nonsaponifiable	Squalene	Lanosterol	Cholesterol
1	700 × *g* × 10′	0.5	10		0.003	0.031			
			20		0.004	0.026			
			30		0.006	0.027			
2	110,000 × *g* × 120′	0.5	10		0.043	0.022			
			20		0.193	0.025			
			30		0.390	0.027			
3	110,000 × *g* × 120′	0.5	10	20	0.260	0.089	0.054	0.005	0.007
	followed by		20	20	0.438	0.327	0.272	0.017	0.026
	700 × *g* × 10′	0.5	30	20	0.562	0.513	0.440	0.027	0.046

[a] Each flask contained: Tris buffer, *p*H 7.1, 171 μmoles; nicotinamide, 34 μmoles; sodium EDTA, 3 μmoles; $MgCl_2$, 20 μmoles; reduced glutathione, 40 μmoles; ATP, 10 μmoles; sodium fluoride, 10 μmoles; DPN, 1.5 μmoles; TPN, 3 μmoles; glucose-6-phosphate, 10 μmoles; about 50 μg of glucose-6-phosphate dehydrogenase; and DL-[^{3}H]-mevalonic acid, 12 mμmoles (specific activity 92 mc/mmole). The final volume of incubations in experiments 1 and 2 was 2 ml, in experiment 3, 2.5 ml.

TABLE III: Metabolism of Mevalonic Acid by 700 × *g* Supernatant.[a]

Time of Incubation (min)	Homogenate (ml)	mμMoles of [³H]Mevalonate Incorporated into: MVAP	MVAPP + IPP
30	0.3	3.15	0.03
30	0.5	3.32	0.05
30	0.7	3.23	0.025

[a] Each incubation flask contained: Tris buffer, *p*H 7.1, 171 μmoles; nicotinamide, 34 μmoles; sodium EDTA, 3 μmoles; $MgCl_2$, 25 μmoles; reduced glutathione, 40 μmoles; ATP, 10 μmoles; sodium fluoride, 10 μmoles; DPN, 1.5 μmoles; TPN, 3 μmoles; glucose-6-phosphate, 10 μmoles; about 50 μg of glucose-6-phosphate dehydrogenase; and DL-[³H]mevalonic acid, 12.3 mμmoles. Total volume was 2 ml.

with 700 × *g* supernatant. A substantial conversion of mevalonic acid to MVAP was observed but only traces of radioactivity were found in the MVAPP + IPP fraction. The data given in Table IV show clearly that, when MVAP was used as a substrate, the conversion of MVAP to other intermediates in the squalene pathway was much poorer with the 700 × *g* preparation than with the high-speed supernatant.

Destruction of ATP by Testicular Homogenates. A reasonable explanation for the inability of the 700 × *g* supernatant to convert MVAP to MVAPP would be that a microsomal ATPase was destroying ATP before an appreciable amount of the MVAPP was synthesized. The microsomal ATPase would be removed by high-speed centrifugation.

To test this hypothesis, the concentration of ATP was checked at various time intervals in incubations using either the 700 × *g* supernatant or the 110,000 × *g* supernatant as a source of enzymes. The amount of ATP recovered from the 700 × *g* supernatant incubation progressively decreased with increasing time of incubation (Table V). In contrast to this the recovery

TABLE IV: Metabolism of Mevalonic Acid Phosphate.[a]

Source of Enzymes	[³H]MVAP Added[b] (cpm)	Recovered Radioactivity (cpm) MVAP	MVAPP	Allylpyrophosphates
700 × *g* supernatant	39,000	32,300	4,800	500
110,000 × *g* supernatant	39,000	6,600	14,400	4000

[a] Each incubation flask contained the cofactors indicated in Table III. In addition to these cofactors, 20 μmoles of creatine phosphate and 2 mg of phosphocreatine kinase were present. Tissue preparation (0.5 ml) was added to each flask. Time of incubation was 30 minutes. [b] Since specific activity of the [³H]MVAP was not accurately known, values cannot be given in mμmoles.

TABLE V: Hydrolysis of ATP by Testicular Preparations.[a]

Expt	Type of Preparation	Volume of Homogenate (ml)	Time of Incubation (min)	ATP Added (μmoles)	ATP Recovered (μmoles)
1	700 × *g* × 10′	0.5	10	10	2.1
	700 × *g* × 10′	0.5	20	10	0.6
	700 × *g* × 10′	0.5	37	10	0.2
2	110,000 × *g* × 120′	0.1	30	15	8.6
	110,000 × *g* × 120′	0.3	30	15	8.9
	110,000 × *g* × 120′	0.5	30	15	8.9
3	Buffer		30	10	8.3

[a] Cofactors for incubations as indicated in Table III. The final volume of incubations was 2 ml, and 0.4 ml was taken for each estimation of ATP.

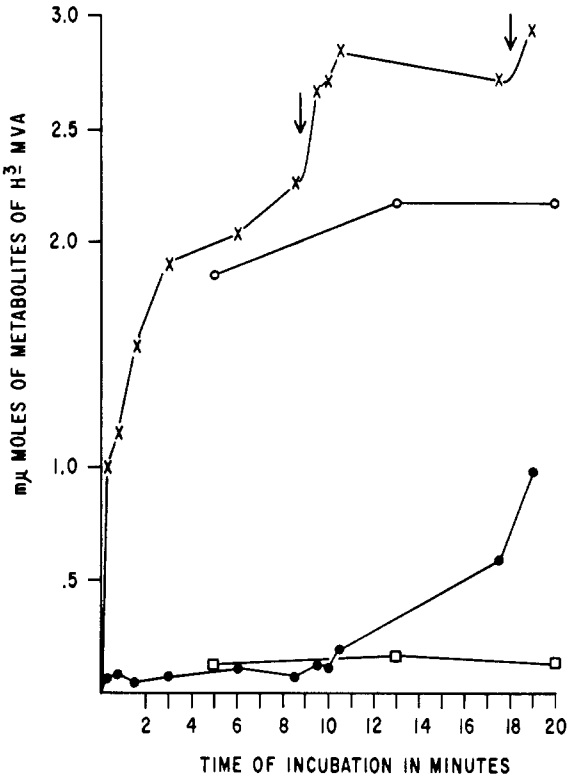

FIGURE 2: Time course of conversion of mevalonic acid (MVA) and MVAP and pyrophosphates. Tris buffer, *p*H 7.1, 1062 μmoles; nicotinamide, 212 μmoles; sodium EDTA, 27 μmoles; $MgCl_2$, 225 μmoles; reduced glutathione, 360 μmoles; ATP, 45 μmoles; sodium fluoride, 90 μmoles; DPN, 13.5 μmoles; TPN, 27 μmoles; glucose-6-phosphate, 90 μmoles; about 50 μg of glucose-6-phosphate dehydrogenase; [^{3}H]mevalonic acid, 256 mμmoles, specific activity 92 μc/μmole; added to 9 ml of 700 × *g* supernatant in total volume of 18 ml. Aliquots (1 ml) were taken at time intervals indicated in graph. At 9 and 18 minutes, as indicated by arrows, 2.5 μmoles of ATP per ml of incubation volume was added. ×—×, MVAP; ●—●, total pyrophosphates derived from mevalonic acid. Results of a similar incubation with no ATP at the 9- and 18-minute time intervals: O—O, MVAP; □—□, total pyrophosphates.

of ATP from the incubation with the high-speed supernatant was in all instances nearly the same as from the enzyme-free control. If the reason for the poor conversion of MVAP to MVAPP was this rapid destruction of ATP, one would expect to be able to increase the yields of the phosphorylated intermediates by adding increments of ATP during the incubation. In the experiment shown in Figure 2 there is a definite increase in both MVAP and MVAPP upon each addition of ATP. Another similar experiment showed only a minor increase in the MVAPP + IPP fraction, but a more dramatic jump in the level of MVAP was observed on each addition of ATP.

TABLE VI: Metabolism of Mevalonic Acid in Tissue Slices and Teased Preparations.[a]

Type of Preparation	mμMoles Radioactive Substance Recovered from: Incubation Medium: Mevalonate	MVAP	MVAPP + IPP	Tissue[b]: Mevalonate
Slices[c]	17	0.65	0	0.27
Teased[d]	11	1.9	0.46	1.5

[a] The cofactors were as indicated in Table III, experiment 2, except 280 μmoles of Tris and 45 μmoles of nicotinamide were used. [b] No radioactivity was recovered in the MVAP or MVAPP + IPP fraction. [c] 350 mg of testis slices was incubated with 20 mμmoles [^{3}H]mevalonic acid for 30 minutes. [d] 150 mg of teased tissue was incubated with 15.4 mμmoles [^{3}H]mevalonic acid for 30 minutes.

Slices and Teased Preparation. Mevalonic acid has been shown to be metabolized by teased preparations of testis tissue (Tsai *et al.*, 1964). To evaluate the usefulness of such preparations and testis slices for the conversion of mevalonic acid to the phosphorylated intermediates, the experiment given in Table VI was carried out. Following incubation the flasks were chilled and the tissue was removed from the medium by centrifugation, washed once, and then analyzed for phosphorylated intermediates and nonsaponifiable material. The data indicate that only small amounts of mevalonic acid penetrate into the tissue and that most of the phosphorylated metabolites are recovered from the medium. Only a trace of squalene and sterols was detected in this experiment.

Effect of Human Chorionic Gonadotropin. In a number of experiments rats were injected subcutaneously with 400 IU of human chorionic gonadotropin 26 hours and then given the same dose intraperitoneally 2 hours before sacrifice. The rate and extent of the conversion of mevalonic acid to MVAP, MVAPP + IPP, and allylpyrophosphates by the 110,000 × *g* supernatant preparations from the testes of these animals did not differ significantly from those of control animals, nor was there a significant difference in the conversion of IPP to squalene by 700 × *g* supernatants from treated and control animals.

Discussion

We have now shown that subcellular preparations from rat testis are capable of the conversion of mevalonic acid to a variety of intermediates on the biosynthetic pathway to squalene and sterols. This, in conjunction with an earlier demonstration of the conversion of IPP to squalene and sterols, demonstrates quite

clearly that the biosynthesis of squalene and sterols in testis is by way of mevalonate just as in liver and yeast.

This study has also shown why whole-cell preparations of testes have been relatively ineffective in the conversion of mevalonic acid to sterols and steroids in spite of good conversion of acetate (Savard *et al.*, 1960). In the experiments with whole-cell preparations (slices or teased tissue) very little mevalonic acid penetrated the cells. The minor conversion observed in the medium could be explained by a leakage of enzymes from damaged cells. The conversion after prolonged incubation, observed by some workers (Billiar *et al.*, 1965), was probably due to the gradual change in permeability of the cell walls associated with cellular degeneration (Savard *et al.*, 1963).

The inability to obtain conversion of mevalonic acid to squalene and sterols in the presence of testis homogenates is also explained. While microsomal enzymes are essential for squalene synthesis and cyclization, the microsomes obviously interfered with the synthesis of the allylpyrophosphates by the cytoplasmic enzymes, although there was rapid formation of MVAP for a short time. There are three reasonable explanations: (1) There could be an inhibitor of MVAP kinase in the homogenate that is removed by centrifugation at 110,000 $\times$ *g*. (2) A particulate MVAPP phosphatase could be present in the 700 $\times$ *g* supernatant. (3) A potent microsomal ATPase could be destroying ATP before there is time for a significant conversion of MVAP to MVAPP and IPP. The data in Table V show that there is substantial ATPase activity in the 700 $\times$ *g* supernatant and none in the 110,000 $\times$ *g* supernatant. Microsomal ATPases have been reported elsewhere (Caffrey *et al.*, 1956). The enhanced formation of MVAP and MVAPP on addition of ATP during incubation strengthens the hypothesis that it is the destruction of ATP that is responsible for the poor metabolism of MVAP (Figure 1). The experiment in which synthesis of MVAPP from MVAP was observed makes it unlikely that an inhibitor of MVAP kinase is present in the 700 $\times$ *g* supernatant. We have not yet examined these preparations for the presence of an MVAPP phosphatase.

The lack of effect of prior treatment of rats with human chorionic gonadotropin on the conversion of mevalonic acid to phosphorylated intermediates or of IPP to squalene and sterols makes it unlikely that this hormone directly affects any of these biosynthetic reactions.

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