Occurrence of the enzymes effecting the conversion of acetyl CoA to squalene in homogenates of hog aorta

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Abstract Homogenates and subcellular fractions of the intima–media of hog aorta have been prepared and examined for the presence of the enzymes catalyzing the conversion of acetyl CoA to squalene. Enzyme activities effecting the conversion of acetyl CoA to 3-hydroxy-3-methylglutarate (HMG); HMG CoA to mevalonic acid; mevalonic acid to 5-phosphomevalonic acid, 5-pyrophosphomevalonic acid, and isopentenyl pyrophosphate; isopentenyl pyrophosphate to farnesyl pyrophosphate; and farnesyl pyrophosphate to squalene have been demonstrated in these homogenates. The overall conversion of mevalonate to squalene has also been demonstrated with recombined fractions of hog aorta homogenates. Data are also presented that suggest that phosphatases present in the crude homogenates act to cleave farnesyl pyrophosphate to farnesol, and phospho- and pyrophosphomevalonate to mevalonate.

Supplementary key words HMG CoA reductase · mevalonate kinase · isopentenyl pyrophosphate isomerase · prenyl transferase · squalene synthetase · aorta cell-free systems

Studies from many laboratories have shown that intact segments of arterial wall can synthesize sterols from acetate and mevalonate (2–8). Chicken, rabbit, and guinea pig aorta were shown by Azarnoff (4) to incorporate [^{14}C]acetate into cholesterol. He also found that segments of human aorta incorporated [^{14}C]-acetate into a nonsaponifiable lipid that precipitated as the digitonide but was not converted to cholesterol dibromide. Daly et al. (5) found that the aorta of rats will synthesize cholesterol from acetate in vitro. Subjecting the animals to renal hypertension was found to increase both the concentration of cholesterol in aorta and its rate of synthesis in vitro (5). Schwenk and Werthessen (6) showed that intact hog aorta will convert acetate to cholesterol. More recently, Chobanian (7) has shown clearly that human aorta and other arteries incorporate [^{14}C]acetate and [^{14}C]mevalonate into cholesterol and cholestanol. St. Clair et al. (8) found that slices and minces of pigeon aorta incorporated [^{14}C]acetate and mevalonate into cholesterol and that incorporation was greater with atherosclerotic than with normal aorta. They reported that no detectable incorporation of [^{14}C]mevalonate into squalene or sterol occurred with homogenates of these tissues. Walsh, Teal, and Gamble (9) reported the conversion of [^{14}C]mevalonate to some of the phosphorylated intermediates of cholesterol synthesis, using a 60,000 *g* supernatant fraction of a bovine aorta homogenate as an enzyme source.

It is clear that the aorta contains the enzymatic capabilities to convert acetate and mevalonate to sterols. Therefore, it seems likely that difficulty in earlier studies in demonstrating these conversions in homogenates was due either to the denaturation of one or more of the enzymes of the pathway during homogenization or to the liberation of inhibitors or degradative enzymes that would not have access to the intermediates or enzymes of the pathway in the intact tissue. We therefore examined homogenates and subcellular fractions of hog aorta for each of the reactions involved in the synthesis of squalene from acetyl CoA. This paper presents evidence that all of the enzymes necessary to convert acetyl

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Abbreviations: HMG, 3-hydroxy-3-methylglutaric acid.

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CoA to squalene are active in hog aorta homogenates. A procedure is also described for demonstrating the conversion of mevalonate to squalene, using partially purified subcellular fractions from these homogenates.

MATERIALS AND METHODS

Materials

Glucose-6-phosphate, purified glucose-6-phosphate dehydrogenase from torula, NADP, ATP, and fatty acid-poor serum albumin were purchased from the Sigma Chemical Co., St. Louis, Mo. Alkaline phosphatase from *Escherichia coli* was purchased from Worthington Biochemical Corp., Freehold, N.J. Dithiothreitol and 2-(*N*-morpholino)-ethanesulfonic acid were obtained from Calbiochem, La Jolla, Calif. Other chemicals were obtained as follows: 2-mercaptoethanol from Eastman Organic Chemicals, Rochester, N.Y.; isopentenol, farnesol, and dimethylacrylic acid from Aldrich Chemical Co., Milwaukee, Wis.; D,L-nerolidol from Koch-Light Laboratories, Buckinghamshire, England; and D,L-mevalonolactone from Mann Research Laboratories, Orangeburg, N.Y. [2-^{14}C]Mevalonic acid, [2-^{14}C]acetyl CoA, and [1-^{14}C]isopentenyl pyrophosphate were purchased from New England Nuclear Corp., Boston, Mass. The [2-^{14}C]mevalonic acid and [1-^{14}C]isopentenyl pyrophosphate were purified before use as substrates by chromatography on Whatman no. 1 paper in a solvent system composed of 1-propanol–ammonia–water 60:20:20. Iodoacetamide was purchased from K & K Laboratories, Plainview, N.Y., and was recrystallized from ethanol–diethyl ether before use. All other reagents were of analytical grade. Hog aortas were a gift of the Oscar Mayer Co., Madison, Wis.

Synthesis of substrates and standard compounds

[3-^{14}C]HMG anhydride was synthesized by the procedure of Goldfarb and Pitot (10). [3-^{14}C]HMG CoA was synthesized from the anhydride by the procedure of Hilz et al. (11) and purified by paper chromatography (12). [2-^{14}C]Mevalonolactone was prepared from the (*N*,*N*′-dibenzylethylene diammonium salt) of [2-^{14}C]-mevalonic acid by acidifying an aqueous solution to pH 2 with HCl and then extracting the product with diethyl ether as described by Lynen and Grassl (13). Dimethylallyl alcohol was prepared by $LiAlH_4$ reduction of dimethylacrylic acid as described by Yuan and Bloch (14). Nonradioactive dimethylallyl pyrophosphate and farnesyl pyrophosphate were prepared from the corresponding alcohols as described by Popják et al. (15). [4,8,12-^{14}C$_3$]Farnesyl pyrophosphate was synthesized as described by Dugan, Rasson, and Porter (16) with the following modification: 12 μmoles of KF were added per milliliter of incubation mixture to inhibit the phosphatases present in the hog liver soluble protein fraction. The product was isolated and purified by DEAE column and paper chromatography as described by Dugan et al. (16).

Preparation of aorta subcellular fractions

Hog aortas were obtained at the time of slaughter and held on ice until homogenized. The tissue was homogenized within 90 min of the time it was obtained from the slaughterhouse. Fat and connective tissue were trimmed from the outside of the aorta. The whole aorta was opened longitudinally and the inner surface was rinsed with buffer. A layer consisting of the intima and part of the media was stripped from the remaining media plus adventitia. Buffer, 4.0 ml, was added to 2-g portions of the intima–media and the tissue was finely minced with scissors. (The buffer used contained 0.1 M potassium phosphate, pH 7.4, 4 mM $MgCl_2$, 1 mM EDTA, and 10 mM 2-mercaptoethanol [17]. For some of the experiments in which squalene synthetase was measured, the buffer contained 30 mM nicotinamide and no EDTA or mercaptoethanol.) The mince was then homogenized in a Kontes Duall ground glass, motor-driven homogenizer. The pestle was driven once to the bottom of the tube. The homogenate was centrifuged at 16,000 *g* for 15 min and the pellet was discarded. The supernatant fraction was centrifuged at 105,000 *g* for 1 hr. The microsomal pellet was then resuspended in a small volume of the homogenizing buffer.

For the synthesis of HMG, either the 16,000 *g* supernatant fraction, the microsomes, or the 105,000 *g* supernatant fraction was used as the source of enzyme protein. HMG CoA reductase activity was measured with microsomes. For the study of the conversion of mevalonic acid to isopentenyl pyrophosphate, the supernatant fraction was fractionated with solid ammonium sulfate, and the fraction precipitating between 15 and 60% of saturation was used. The 105,000 *g* supernatant fraction was used without further treatment for the study of isopentenyl pyrophosphate isomerase and prenyl transferase activities. For the study of squalene synthesis, microsomes were washed twice with the above buffer. The pellet was resuspended in half the original volume of buffer, centrifuged for 45 min at 105,000 *g*, and then this process was repeated.

Protein concentrations were determined by precipitating protein from aliquots of suspensions with 5% trichloroacetic acid, redissolving the precipitated protein in 3% NaOH, and then measuring the redissolved protein by the biuret procedure (18).

Conversion of acetyl CoA to HMG

The incubation mixture contained potassium phosphate buffer, pH 7.0, 50 μmoles; EDTA, 2 μmoles; nicotinamide, 0.6 μmole; [2-^{14}C]acetyl CoA, 100 nmoles

and 5.0 × 10^5 dpm; 2–4 mg of protein suspended in 0.40 ml of the homogenizing buffer; and water to make a final volume of 1.0 ml. The reaction mixture was incubated for 1 hr at 37°C with shaking. When CoA esters were to be recovered, the reaction was stopped by adjustment of the pH to 4–4.5 with 0.077 ml of 1 N HCl, and the mixture was frozen in acetone–dry ice. The mixture was lyophilized, and the residue was extracted with three 1-ml portions of 99% methanol. The methanol extract was concentrated to a small volume and applied to Whatman 3MM paper. The chromatogram was developed with isobutyric acid–ammonia–0.1 M EDTA, pH 4.5–water 124:4.9:2.0:75. When CoA esters were not to be recovered, the reaction was stopped by the addition of 0.025 ml of 4 N KOH (final pH = 11). The mixture was then incubated at 37°C for 1 hr to ensure cleavage of CoA esters. The mixture was acidified to pH 2, and 100 nmoles of HMG were added. The water was evaporated under a stream of nitrogen, and the dry residue was extracted with three 1-ml portions of 98% ethanol. The ethanol extract was concentrated to a small volume and applied to Whatman no. 1 paper. The chromatogram was developed in 1-propanol–ammonia–water 60:20:20. Radioactive standard compounds were spotted in lanes adjacent to the sample lanes, and 1-cm segments of both standard and sample lanes were assayed for radioactivity in dioxane–phosphor solution.

Assay for HMG CoA reductase activity

The incubation mixture contained potassium phosphate buffer, pH 7.0, 100 μmoles; dithiothreitol, 2 μmoles; EDTA, 1 μmole; NADP, 1 μmole; glucose-6-phosphate, 6 μmoles; glucose-6-phosphate dehydrogenase, 1.2 units and 5 μg of protein; [3-^{14}C]HMG CoA, 150 nmoles and 1.3 × 10^5 dpm of the biologically active isomer; and microsomal protein, 2–12 mg, in a total volume of 1 ml. Reaction mixtures were incubated at 37°C for 1–4 hr. Reactions were stopped and the product was isolated as reported by Slakey et al. (17).

Conversion of mevalonic acid to phosphorylated intermediates

Incubation mixtures contained potassium phosphate buffer, pH 7.0, 50 μmoles; $MgCl_2$, 15 μmoles; ATP, 15 μmoles; KF, 30 μmoles; iodoacetamide, 5 μmoles; D,L-[2-^{14}C]mevalonic acid, 17 nmoles and 2.2 × 10^5 dpm; and 2–8 mg of protein. The fraction of the 105,000 *g* supernatant solution that precipitated between 15 and 60% saturation with ammonium sulfate was used. Reaction mixtures were incubated at 37°C for 3 hr. Enzyme action was stopped by heating in a boiling water bath for 2 min. The reaction mixtures were lyophilized, and the residue was extracted with 80% ethanol containing 2% ammonia. The extracts were evaporated under nitrogen to a volume of approximately 0.3 ml and then chromatographed on Whatman no. 1 paper in a system composed of 1-propanol–ammonia–water 60:20:20.

Assay for conversion of isopentenyl pyrophosphate to farnesyl pyrophosphate

Incubation mixtures contained 2-(*N*-morpholino)-ethanesulfonic acid buffer, pH 6.0, 50 μmoles; $MgCl_2$, 10 μmoles; 2-mercaptoethanol, 5 μmoles; [4-^{14}C]isopentenyl pyrophosphate, 4.5 nmoles and 5.0 × 10^4 dpm; and 1–4 mg of protein (105,000 *g* supernatant fraction), in a total volume of 1.0 ml. Reaction mixtures were incubated at 37°C for 20–90 min. If incorporation of radioactivity into acid-labile compounds was to be measured, reactions were stopped by the addition of 0.5 ml of a solution of ethanol–water–HCl 8:1:1. The mixture was incubated at 37°C for 10 min to ensure cleavage of allylic pyrophosphates, and the liberated terpenols were extracted with three 3-ml portions of petroleum ether. An aliquot of the petroleum ether extract was taken for assay of radioactivity. Cold carrier farnesol and nerolidol (nerolidol is derived from farnesol by exposure to acid) were added to the extract, and the latter was concentrated to a small volume and applied to a silica gel G plate. The plate was developed with benzene–ethyl acetate 80:20. Farnesol and nerolidol were visualized with iodine, and the spots were scraped from the plate and assayed for radioactivity in dioxane–phosphor solution (see below). The quantity of radioactivity found in nerolidol plus farnesol is reported as farnesyl pyrophosphate formed in the reaction. If pyrophosphate products were to be isolated and identified, the reaction was stopped by heating in a boiling water bath for 3 min. The solution was then lyophilized, extracted, and chromatographed on Whatman no. 1 paper as described above.

Assay for squalene synthetase activity

The conversion of farnesyl pyrophosphate to squalene was measured by a modification of the procedure of Goodman (19). Incubation mixtures contained potassium phosphate buffer, pH 7.4, 100 μmoles; $MgCl_2$, 5 μmoles; KF, 10 μmoles; nicotinamide, 30 μmoles; serum albumin, 3 mg; NADP, 2.5 μmoles; glucose-6-phosphate, 2.7 μmoles; glucose-6-phosphate dehydrogenase, 1.2 units and 5 μg of protein; [^{14}C]farnesyl pyrophosphate, 75 nmoles and 1.0 × 10^5 dpm; and washed microsomes, 1–6 mg of protein, in a final volume of 1.0 ml. Reaction mixtures were incubated under nitrogen in a shaking water bath at 37°C for 10–80 min. The reaction was stopped by the addition of an equal volume of ethanol. The products were extracted with three 2.5-ml portions of petroleum ether, and carrier farnesol and squalene were added to the combined petroleum ether extracts. The petroleum ether was evaporated under a stream of nitrogen, and the products were chromato-

graphed on silica gel G in petroleum ether–diethyl ether 98:2. The area corresponding to squalene was scraped from the plate and assayed for radioactivity in toluene–phosphor solution (see below).

Assay for conversion of mevalonate to squalene

Incubation mixtures contained potassium phosphate buffer, pH 7.0, 200 μmoles; ATP, 20 μmoles; $MgCl_2$, 20 μmoles; nicotinamide, 60 μmoles; NAD, 2 μmoles; NADP, 2 μmoles; glucose-6-phosphate, 10 μmoles; glucose-6-phosphate dehydrogenase, 1.2 units and 5 μg of protein; bovine serum albumin, 6 mg of protein; D,L-[2-^{14}C]mevalonic acid, 0.85 μmole and 1.1 × 10^6 dpm; washed microsomal protein, 8 mg; and soluble protein (the fraction of the 105,000 *g* supernatant solution that precipitated between 15 and 60% saturation with ammonium sulfate), 14 mg, in a final volume of 2.0 ml. Reaction mixtures were incubated under nitrogen at 37°C for 5 hr. Reactions were stopped by addition of 2 ml of 10% ethanolic KOH. The mixture was saponified for 1 hr at 75°C and then extracted three times with 4-ml portions of petroleum ether. The extract was concentrated to a small volume, applied to a thin-layer plate, and chromatographed in a solution of petroleum ether–benzene 96:4. The squalene area was scraped from the plate and then assayed for radioactivity in toluene–phosphor solution.

Product identification

Thin-layer chromatography was carried out on plates coated with silica gel G, 0.25 or 0.30 mm thick, and activated at 110°C for 1 hr before use. The solvent systems used for each assay are reported in the previous paragraphs. Compounds were located on the plates by spraying with iodine or by chromatographing radioactive standards in lanes adjacent to the sample lanes and then assaying 1-cm sections for the length of each lane for radioactivity. Plates were scored vertically between lanes containing radioactive samples to prevent diffusion of radioactive compounds into adjacent lanes.

Gas–liquid chromatography was carried out with Barber-Colman model 10 or model 5000 instruments. Column packings, temperatures, and flow rates used are described in the figure legends. For the measurement of radioactivity in samples analyzed on the model 10 instrument, the effluent fractions were trapped in U tubes containing glass wool and toluene scintillator fluid and held in a bath of acetone–dry ice. The contents of the traps were subsequently washed into vials with additional toluene scintillator fluid and assayed for radioactivity. The model 5000 was equipped with a gas flow counter for monitoring radioactivity.

Phosphomevalonate, pyrophosphomevalonate, isopentenyl pyrophosphate, and farnesyl pyrophosphate were each identified by comparison of R_F values with those of authentic compounds chromatographed on paper in lanes adjacent to the samples. Each compound was then eluted from the paper with a 2% solution of ammonia in water. The water was removed by lyophilization, and the phosphorylated intermediate was cleaved with alkaline phosphatase. Incubation mixtures contained Tris buffer, pH 9.2, 100 μmoles; $MgCl_2$, 10 μmoles; and alkaline phosphatase, 1 mg of protein, in a final volume of 1.0 ml. Incubations were carried out for 3 hr at 37°C. Mevalonate was recovered as the lactone as described above. Isopentenol and farnesol were recovered by extraction of the incubation mixture with three 3-ml portions of petroleum ether. The alcohols and mevalonolactone were further identified by gas–liquid chromatography.

HMG was identified by paper chromatography and by gas–liquid chromatography of the dimethyl ester derivative of the eluted sample. The segment of the paper chromatogram corresponding to authentic HMG was eluted with water and concentrated to a small volume by lyophilization. The aqueous solution was acidified to pH 2, saturated with sodium sulfate, and then extracted with ten 2-ml portions of diethyl ether. The ether extract was concentrated, and the HMG was converted to the dimethyl ester with diazomethane.

Squalene was identified by thin-layer chromatography. The identity of squalene was confirmed by gas–liquid chromatography after elution from the thin-layer plate.

Measurement of radioactivity

Radioactivity was determined in either toluene–phosphor solution (4 g of 2,5-diphenyloxazole and 0.1 g of 2,5-bis[5-(*tert*-butylbenzoxazole)]-thiophene per liter of toluene) or dioxane–phosphor solution (60 g of naphthalene, 10 ml of ethylene glycol, 4 g of 2,5-diphenyloxazole, and 0.2 g of 2,5-bis[5-(*tert*-butylbenzoxazole)]-thiophene per liter of dioxane) in a Packard Tri-Carb model 3365 liquid scintillation spectrometer.

RESULTS

Conversion of acetyl CoA to HMG

[2-^{14}C]Acetyl CoA was incubated with protein of the 16,000 *g* supernatant fraction. After incubation, coenzyme A esters in the incubation mixture were hydrolyzed, and the incorporation of radioactivity into compounds migrating with HMG on paper chromatography was measured. Radioactivity corresponding to authentic HMG was eluted, and the material was examined, after methylation, by gas–liquid chromatography. Approximately 60% of the radioactivity eluted from paper coincided with authentic nonradioactive dimethyl HMG (Fig. 1). The remainder appeared in a peak which preceded dimethyl HMG and which seemed to be composed of at least two poorly resolved components.

[2-^{14}C]Acetyl CoA, 1.3 nmoles, was incorporated into HMG during a 1-hr incubation with the protein from 0.1 g of tissue. Increasing the amount of protein caused greater incorporation of ^{14}C into compounds other than HMG and decreased the incorporation into HMG. When the 105,000 *g* supernatant fraction was used as the enzyme source, conversion of acetyl CoA to HMG occurred to approximately the same extent as in the presence of the 16,000 *g* supernatant fraction. No significant conversion occurred with microsomes alone.

An attempt was made to isolate HMG CoA from the reaction mixture. The reaction was stopped by careful adjustment of the pH to 4.5. The incubation mixture was extracted, and the extract was chromatographed on paper as reported under Materials and Methods. No radioactivity was found to coincide with carrier nonradioactive HMG CoA. It does not appear likely that a significant loss of CoA esters occurred during the isolation procedure because the unreacted substrate could be recovered in good yield from zero-time incubations. Instead, it appears probable that certain degradative enzymes such as HMG CoA deacylase and HMG CoA lyase are present in the 16,000 *g* supernatant fraction. When 150 nmoles of [^{14}C]HMG CoA were incubated with 4 mg of 16,000 *g* supernatant protein under the conditions described for the incubation of acetyl CoA with this fraction, approximately 75% of the HMG CoA was degraded within 1 hr.

HMG CoA reductase

Evidence for the presence of HMG CoA reductase activity in hog aortal microsomes was obtained by following the incorporation of radioactivity from [^{14}C]HMG CoA into mevalonate. The radioactive mevalonate was converted to the lactone. This compound has an R_F value identical with that of authentic mevalonolactone on thin-layer chromatography. Confirmation of the identity of the enzymatically formed mevalonate was provided by subjecting the radioactivity from the mevalonolactone area on thin-layer chromatography to gas–liquid chromatography. Radioactivity was found to coincide with the mass of carrier mevalonolactone (Fig. 2). The enzymic activity of aortal microsomes was found to be linear with respect to protein concentration and time of incubation over the ranges given in Table 1 and Fig. 3.

HMG CoA reductase activity was also observed in crude microsomes from homogenates of monkey aorta.

Conversion of mevalonate to phosphorylated intermediates

A significant conversion of [^{14}C]mevalonate to various phosphorylated intermediates of sterol biosynthesis was observed when an ammonium sulfate-precipitated fraction of the crude 105,000 *g* supernatant solution was used as the enzyme source. The phosphorylated products of this conversion were separated by paper chromatography and located by either dipping the chromatograms in Rosenberg's reagent (20) or assaying 1-cm sections for radioactivity (Fig. 4). In early experiments in which the crude 105,000 *g* supernatant fraction was used as enzyme source, very little conversion could be demonstrated. Maximal synthesis of isopentenyl pyrophosphate required the addition of both iodoacetamide, an inhibitor of isopentenyl pyrophosphate isomerase, and

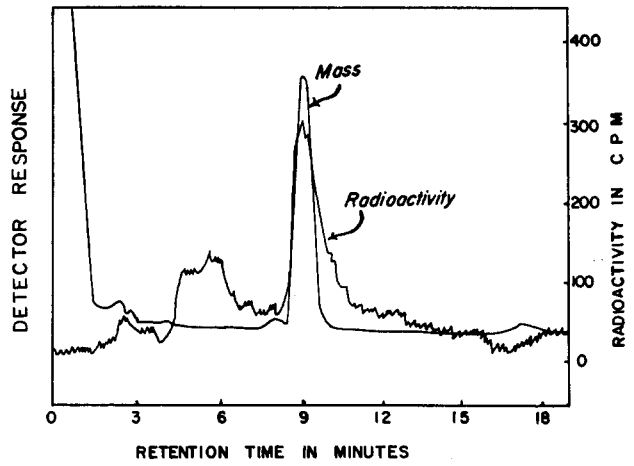

FIG. 1. Gas–liquid chromatography of dimethyl HMG. Protein, 2.2 mg of the 16,000 *g* supernatant fraction of hog aorta, was incubated with [2-^{14}C]acetyl CoA as described under Materials and Methods. HMG was isolated by paper chromatography and converted to the dimethyl ester. The sample was chromatographed in a Barber-Colman model 5000 on a column (6 ft × 6 mm) of butanediol succinate, 20%, on acid-washed Chromosorb W. The column temperature was 175°C and the helium flow rate was 100 ml/min. The radioactivity monitor was set at 1000 cpm full scale.

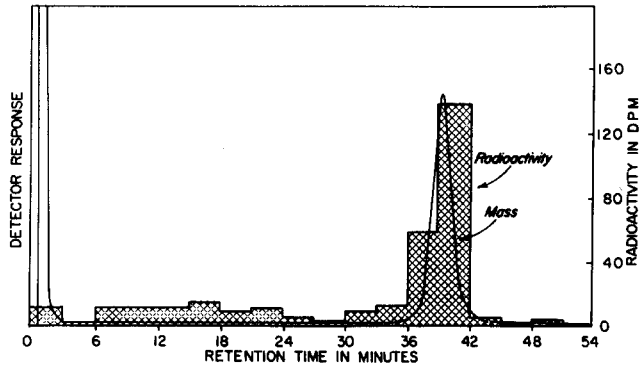

FIG. 2. Gas–liquid chromatography of mevalonolactone formed from [^{14}C]HMG CoA by hog aortal microsomes. Microsomal protein, 8 mg, was incubated with substrate and cofactors for 4 hr as described under Materials and Methods. The biosynthesized mevalonate was isolated as the lactone by thin-layer chromatography and then eluted from the silica gel with acetone. Carrier mevalonolactone was added, and an aliquot was chromatographed in a Barber-Colman model 10 instrument on a column (6 ft × 6 mm) of butanediol succinate, 20%, on acid-washed Chromosorb W. The column temperature was 190°C and the effluent gas flow was 100 ml/min.

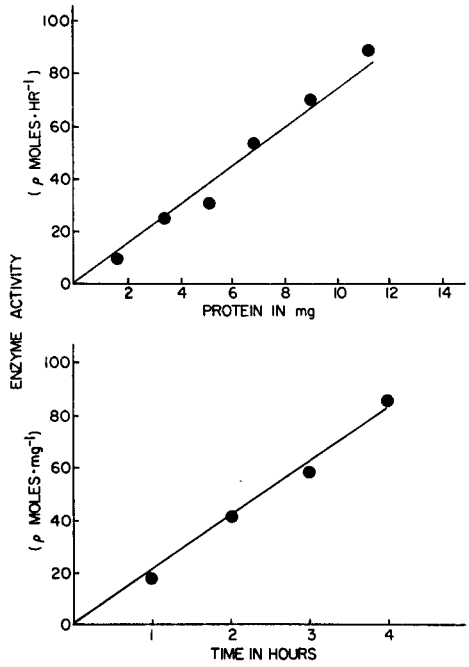

FIG. 3. Linearity of hog aortal HMG CoA reductase activity with respect to microsomal protein (*top*) and time of incubation (*bottom*). Enzyme activity was measured as described under Materials and Methods.

potassium fluoride, an inhibitor of phosphatases (Fig. 4). Isopentenyl pyrophosphate, 3.8 nmoles, was synthesized during 3 hr of incubation with protein from 1 g of arterial tissue.

The identities of phosphomevalonate, pyrophosphomevalonate, and isopentenyl pyrophosphate were established by chromatography on paper. Confirmation of the identities of the pyrophosphate compounds was obtained after elution from paper and cleavage of the pyrophosphate moieties with alkaline phosphatase. The resultant products were then subjected to gas–liquid chromatography. Coincidence of carrier isopentenol and radioactivity derived from isopentenyl pyrophosphate was observed (Fig. 5*A*). Similarly, radioactivity derived from phosphomevalonate and pyrophosphomevalonate coincided with carrier mevalonolactone (Fig. 5*B*).

Conversion of isopentenyl pyrophosphate to allylic pyrophosphates

Incubation of [^{14}C]isopentenyl pyrophosphate with the 105,000 *g* supernatant solution of aortal homogenates resulted in the rapid formation of acid-labile, petroleum ether-extractable compounds. The quantities of nerolidol and farnesol in this extract were determined. The sum of these compounds was taken as the quantity of farnesyl pyrophosphate synthesized by the enzyme system. Under these conditions, formation of farnesyl pyrophosphate was linear with respect to time and protein concentration over the ranges given in Table 1. When the products formed during the incubation were not cleaved with acid but instead chromatographed on paper, the results shown in Fig. 6 were obtained. After 20 min of incubation (chromatogram *A* of Fig. 6), radioactivity from iso-

TABLE 1. Relative rates of intermediate reactions of sterol formation

Reaction	Range of Linearity		Rate[a]	
	Protein[b]	Time[c]	Hog Aorta	Rat Liver[d]
	mg	*min*	*pmoles · min⁻¹ · g tissue⁻¹*	
Acetyl CoA → HMG		*e*	201[e]	
HMG CoA → mevalonate	1.7–11.2	60–240	1.0	570
Mevalonate → isopentenyl pyrophosphate		*e*	21[e]	138,000[f]
Isopentenyl pyrophosphate → farnesyl pyrophosphate	0.4–4.4	5–30	417	12,400[f]
Farnesyl pyrophosphate → squalene	1.2–4.8	20–90	26	780
Mevalonate → squalene		*e*	3[e]	

[a] The rates are reported as picomoles of substrate converted to product.

[b] The lower limits represent either the smallest amount tested experimentally or the smallest amount that gave a readily measurable rate. The upper limits are amounts beyond which the rate of incorporation was no longer proportional to the amount of protein, with the exception of HMG CoA → mevalonate.

[c] The lower limits are the shortest times that gave a readily measurable conversion of substrate to product. HMG CoA reductase was not measured for times longer than 4 hr. The other reactions were measured for longer times, and the conversion was found to be proportional to time only for the intervals stated.

[d] These data are calculated from the results reported by Slakey et al. (17).

[e] The dependence of the extent of these reactions on the amount of protein or time of incubation was not measured. Values are given for the amount of product formed under the conditions described in the text and may not represent the maximal conversion rate for these reactions.

[f] These values are for the slowest step in each reaction sequence.

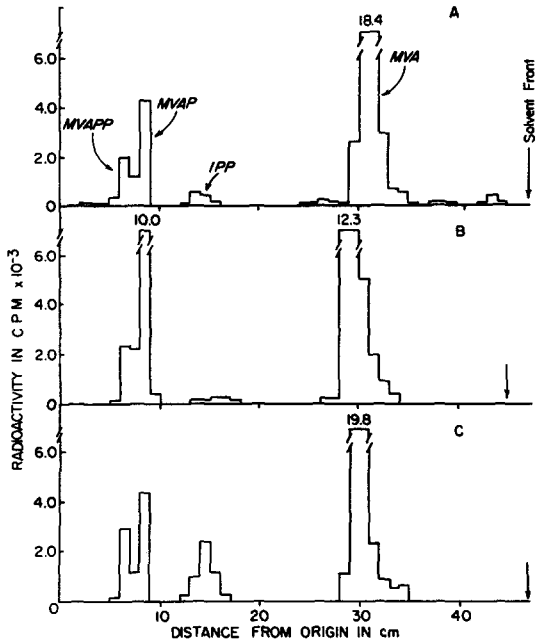

FIG. 4. Paper chromatography of phosphorylated compounds formed from [^{14}C]mevalonate. Incubations were carried out (*A*) without potassium fluoride or iodoacetamide, (*B*) without potassium fluoride, and (*C*) in the complete incubation system described under Materials and Methods for 3 hr in the presence of 4 mg of ammonium sulfate-precipitated proteins. The products of the incubation were separated by paper chromatography. Portions of the paper chromatograms were cut into 1-cm sections and then assayed for radioactivity in dioxane–phosphor solution. The location of the phosphorylated compounds formed from mevalonic acid on the paper chromatograms was also determined by dipping in Rosenberg's reagent (20). Abbreviations used in the figure are: MVA, mevalonate; MVAP, phosphomevalonate; MVAPP, pyrophosphomevalonate; and IPP, isopentenyl pyrophosphate.

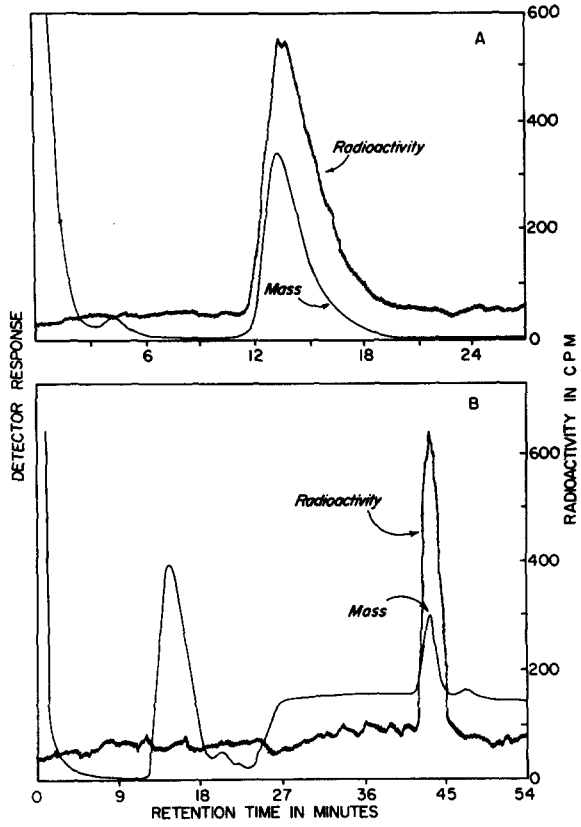

FIG. 5. Gas–liquid chromatography of isopentenol and mevalonolactone. *A*, radioactive isopentenyl pyrophosphate, biosynthesized from [^{14}C]mevalonate and isolated by paper chromatography, was eluted from the paper and cleaved with alkaline phosphatase as described under Materials and Methods. Chromatography of the resultant isopentenol was effected on a Carbowax column in a Barber-Colman model 5000 instrument equipped with a system monitoring for radioactivity. The column (6 ft × 6 mm) was packed with 20% Carbowax 20M and 3% terephthalic acid on Gas-Chrom Q support. The column temperature was 45°C and the effluent gas flow was 60 ml/min. *B*, radioactive phosphomevalonate and pyrophosphomevalonate, biosynthesized from [^{14}C]mevalonate and isolated by paper chromatography, were eluted from the paper and cleaved with alkaline phosphatase. The liberated mevalonate was converted to the lactone. Chromatography of mevalonolactone was effected on a Carbowax column. The temperature setting was changed from 45°C to 190°C at 24 min, i.e., after the elution of isopentenol. A temperature of 190°C was reached after 4 min. The effluent gas flow was 60 ml/min.

pentenyl pyrophosphate was found in farnesyl pyrophosphate and in a second, less polar compound (R_F 0.9). The radioactivity in the second compound increased markedly during another 20 min of incubation (chromatogram *B* of Fig. 6).

No significant amount of radioactivity was found on paper chromatograms in the areas corresponding to dimethylallyl pyrophosphate or geranyl pyrophosphate (Fig. 6). The identity of the enzymatically formed farnesyl pyrophosphate was confirmed by gas–liquid chromatography of the product obtained on cleavage with alkaline phosphatase. Radioactivity was found to be coincident with carrier *trans*-farnesol (Fig. 7).

The other major radioactive compound (R_F 0.9) was eluted from paper and incubated with an enzyme preparation from tomato plastids capable of converting geranylgeranyl pyrophosphate to phytoene, a C_{40} intermediate of carotene biosynthesis (21). Chromatography of the petroleum ether extract of this reaction on an alumina column and on a thin layer of silica gel G (22) showed that the radioactivity coincided with carrier phytoene. These results lead us to suggest that this compound is geranylgeranyl pyrophosphate.

Squalene synthetase

Conversion of [^{14}C]farnesyl pyrophosphate to squalene was obtained with twice-washed microsomes in the presence of bovine serum albumin. This reaction was linear with respect to time and protein concentration (Table 1). In earlier experiments with unwashed microsomes without bovine serum albumin, considerable radioactivity was found in farnesol, thereby suggesting the presence of phosphatases in this preparation.

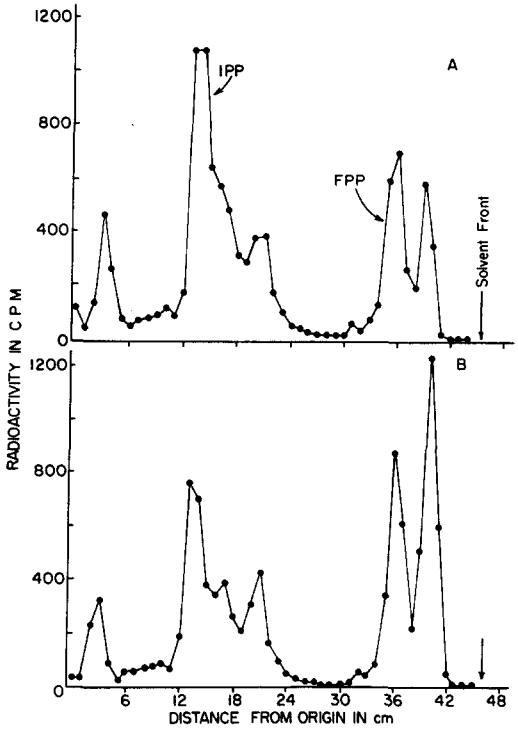

FIG. 6. Paper chromatography of phosphorylated compounds synthesized from [^{14}C]isopentenyl pyrophosphate on incubation with hog aortal 105,000 *g* supernatant solution. Chromatograms *A* and *B* represent 20 and 40 min of incubation, respectively. The paper chromatograms were assayed for radioactivity in 1-cm sections. Samples were assayed in dioxane–phosphor solution. The location of the phosphorylated compounds was determined with Rosenberg's reagent (20). Abbreviations used in the figure are: IPP, isopentenyl pyrophosphate; FPP, farnesyl pyrophosphate.

Radioactive squalene formed during the incubation was identified by thin-layer chromatography and gas–liquid chromatography (Fig. 8).

Conversion of mevalonate to squalene

The overall conversion of mevalonate to squalene was demonstrated by incubation of [^{14}C]mevalonate with an ammonium sulfate-precipitated fraction (15–60%) of the 105,000 *g* supernatant fraction and twice-washed microsomes in the presence of bovine serum albumin. The biosynthesized squalene cochromatographed with authentic squalene on thin-layer and gas–liquid chromatography. 1.6 nmoles of squalene were synthesized during 4 hr of incubation with the protein obtained from 2 g of tissue (Table 1).

DISCUSSION

The data presented in this paper provide proof that subcellular fractions of homogenates of hog aorta are able to carry out each of the reactions that occur in the conversion of HMG CoA to squalene. In addition, the 105,000 *g* supernatant fraction converts acetyl CoA to HMG, and the recombined subcellular fractions carry out the overall conversion of mevalonate to squalene.

Evidence has been presented by a number of laboratories that the reduction of HMG CoA to mevalonate is the rate-limiting step in cholesterol synthesis in liver (17, 23–25). The observed rate of reduction of HMG CoA catalyzed by hog aorta microsomes in comparison with the rates of each of the other reactions or reaction sequences measured in this study (Table 1) suggests that HMG CoA reductase is also rate limiting in aorta. If HMG CoA reductase is in fact rate limiting in the intact tissue, then the rate observed would support the production of approximately 0.1 μg of squalene or sterol/g of tissue/day.

It appears that hog aorta supernatant fraction also catalyzes the synthesis of geranylgeranyl pyrophosphate, as evidenced by the conversion of this compound to phytoene by a tomato enzyme system (21, 22). It is not clear whether the formation of geranylgeranyl pyrophosphate is of physiological significance to aortal tissue or simply the result of the activity of the prenyl transferase enzyme on farnesyl pyrophosphate in the absence of microsomal squalene synthetase. Possibly, geranylgeranyl pyrophosphate is an intermediate in some as yet unidentified process in aortal tissue.

During these studies it became apparent that phosphatases present in crude homogenates interfered with attempts to demonstrate the presence of some of the enzymes studied. Possibly, these phosphatases were released from intracellular organelles by the mechanical stress required to prepare homogenates of aortal tissue. It was found that maximal conversion of [^{14}C]mevalonate to phosphomevalonate, pyrophosphomevalonate, and isopentenyl pyrophosphate required an enzyme system prepared by ammonium sulfate fractionation and the presence of added potassium fluoride, an inhibitor of phosphatases, and iodoacetamide, an inhibitor of isopentenyl pyrophosphate isomerase. In this regard, it is of interest to note that Salokangas, Rilling, and Samuels (26) noted very little conversion of mevalonate to squalene by homogenates of rat testicular tissue but observed marked incorporation of [^{14}C]isopentenyl pyrophosphate into squalene. In a later paper (27) they showed that this result was due to the presence of a microsomal adenosine triphosphatase in the 700 *g* supernatant fraction of their homogenates.

The problem of phosphatases was also encountered in attempts to demonstrate squalene synthetase activity in aortal microsomes. In initial experiments, approximately 10 times as much farnesol as squalene was formed from [^{14}C]farnesyl pyrophosphate. In later experiments in which the microsomes were isolated in potassium phosphate buffer containing $MgCl_2$ and nicotinamide,

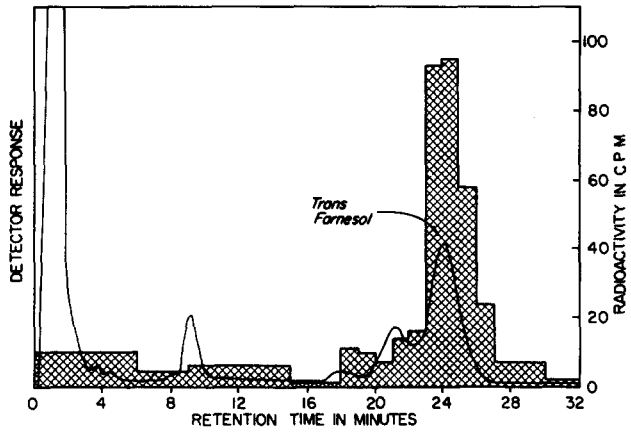

FIG. 7. Gas–liquid chromatography of farnesol. Radioactive farnesyl pyrophosphate, biosynthesized from [^{14}C]isopentenyl pyrophosphate and isolated by paper chromatography, was eluted from the paper and cleaved with alkaline phosphatase to yield farnesol as described under Materials and Methods. Chromatography of the farnesol was carried out on a Barber-Colman model 10 instrument. The column (6 ft × 6 mm) was packed with butanediol succinate, 20%, on acid-washed Chromosorb W. The column temperature was 175°C and the effluent gas flow was 100 ml/min.

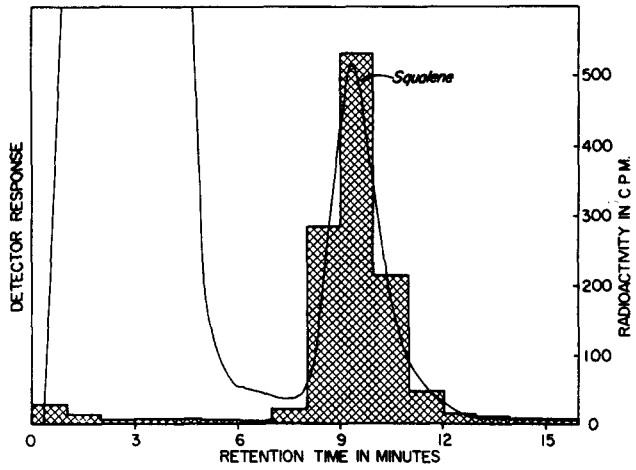

FIG. 8. Gas–liquid chromatography of radioactive squalene synthesized from [^{14}C]farnesyl pyrophosphate on incubation with hog aortal microsomes. Biosynthesized squalene was isolated by thin-layer chromatography. Carrier squalene was then added, and the sample was chromatographed in a Barber-Colman model 10 instrument. The column (8 ft × 6 mm) was packed with SE 30, 2%, on Gas-Chrom Q. The column temperature was 232°C and the effluent gas flow was 100 ml/min.

washed twice, and then incubated with added bovine serum albumin, the amount of farnesol formed was markedly reduced with an increase in the conversion to squalene. Christophe and Popják (28) have demonstrated the presence of a microsomal phosphatase in rat liver capable of dephosphorylating farnesyl pyrophosphate to farnesol as the initial step in the formation of farnesoic acid. Interestingly, they noted that bovine serum albumin was a potent inhibitor of this microsomal phosphatase.

In the present experiments, several attempts were made to convert either farnesyl pyrophosphate or squalene to cholesterol because Schwenk and Werthessen (6) had previously shown the synthesis of cholesterol by intact hog aorta. Invariably, a low amount of radioactivity was found in the digitonin precipitate. However, this radioactivity did not coincide with cholesterol on gas–liquid chromatography. The lack of cholesterol synthesis could be due to an extremely low level of these enzymes in hog aorta, the denaturation of these enzymes during the homogenization procedure, or to an extensive dilution of [^{14}C]squalene with a relatively large pool of endogenous squalene. The possibility that one or more of these enzymes might be denatured during our homogenization procedure is suggested by the observation of Scallen, Dean, and Schuster (29) that homogenization of rat liver with a tight-fitting pestle results in a marked decrease in the conversion of squalene to cholesterol. St. Clair et al. (8) have previously commented upon the squalene content of arterial tissue and the possibility of a large isotope dilution (1000-fold).

This study has reinforced the previous observation (6) that no single means of identification is sufficient to prove the presence of biosynthesized cholesterol. For example, farnesol and squalene were found to contaminate cholesterol–digitonide precipitates. Farnesol also migrated with cholesterol on thin-layer chromatography. Further, it appears to us that the radioactivity which Teal and Gamble (30) attributed to cholesterol was more likely present in polyisoprene alcohols, since their enzyme preparations from bovine aorta probably contained no microsomes.

An increased concentration of cholesterol in aortal tissue has long been known as one of the earliest chemical changes in the process of atherosclerotic plaque formation. The relative contributions of transport and local synthesis to this accumulation of cholesterol have been extensively investigated; however, no final conclusion has been reached. Data from several laboratories have established beyond doubt that arterial wall is capable of cholesterol synthesis from acetate (2–8). This paper describes procedures for measuring the activity of the enzymes catalyzing the intermediate reactions of sterol synthesis in cell-free preparations of aorta. Measurements of the activities of these enzymes, particularly HMG CoA reductase, under atherogenic conditions should now permit more definitive studies of the enzymic response of arterial wall tissue during early stages of atherosclerosis.

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REFERENCES

1. Ness, G. C., L. L. Slakey, N. Qureshi, and J. W. Porter. 1972. Demonstration of the presence of enzymes effecting the intermediate reactions of cholesterol synthesis in homogenates of aortal tissue. *Federation Proc.* **31:** 220. (Abstr.)
2. Siperstein, M. D., I. L. Chaikoff, and S. S. Chernick. 1951. Significance of endogenous cholesterol in arteriosclerosis: synthesis in arterial tissue. *Science*. **113:** 747–749.
3. Werthessen, N. T., L. J. Milch, R. F. Redmond, L. L. Smith, and E. C. Smith. 1954. Biosynthesis and concentration of cholesterol by the intact surviving bovine aorta *in vitro*. *Amer. J. Physiol.* **178:** 23–29.
4. Azarnoff, D. L. 1958. Species differences in cholesterol biosynthesis by arterial tissue. *Proc. Soc. Exp. Biol. Med.* **98:** 680–683.
5. Daly, M. M., Q. B. Deming, V. M. Raeff, and L. M. Brun. 1963. Cholesterol concentration and cholesterol synthesis in aortas of rats with renal hypertension. *J. Clin. Invest.* **42:** 1606–1612.
6. Schwenk, E., and N. T. Werthessen. 1952. Studies on the biosynthesis of cholesterol. III. Purification of C^{14}-cholesterol from perfusions of livers and other organs. *Arch. Biochem. Biophys.* **40:** 334–341.
7. Chobanian, A. V. 1968. Sterol synthesis in the human arterial intima. *J. Clin. Invest.* **47:** 595–603.
8. St. Clair, R. W., H. B. Lofland, Jr., R. W. Prichard, and T. B. Clarkson. 1968. Synthesis of squalene and sterols by isolated segments of human and pigeon arteries. *Exp. Mol. Pathol.* **8:** 201–215.
9. Walsh, M. R., S. W. Teal, and W. Gamble. 1969. Biosynthesis of cholesterol by the bovine aorta and the mechanism of action of *α-para*-chlorophenoxyisobutyric acid. *Arch. Biochem. Biophys.* **130:** 7–18.
10. Goldfarb, S., and H. C. Pitot. 1971. Improved assay of 3-hydroxy-3-methylglutaryl coenzyme A reductase. *J. Lipid Res.* **12:** 512–515.
11. Hilz, H., J. Knappe, E. Ringelmann, and F. Lynen. 1958. Methylglutaconase, eine neue Hydratase, die am Stoffwechsel verzweigter Carbonsäuren beteiligt ist. *Biochem. Z.* **329:** 476–489.
12. Brodie, J., and J. W. Porter. 1960. The synthesis of mevalonic acid by non-particulate avian and mammalian enzyme systems. *Biochem. Biophys. Res. Commun.* **3:** 173–177.
13. Lynen, F., and M. Grassl. 1958. Zur Biosynthese der Terpene. II. Darstellung von (−)-Mevalonsäure durch bakterielle Racematsspaltung. *Hoppe-Seyler's Z. Physiol. Chem.* **313:** 291–295.
14. Yuan, C., and K. Bloch. 1959. Synthesis of 3-methyl-3-butenyl-1-pyrophosphate. *J. Biol. Chem.* **234:** 2605–2608.
15. Popják, G., J. W. Cornforth, R. H. Cornforth, R. Ryhage, and DeW. S. Goodman. 1962. Studies on the biosynthesis of cholesterol. XVI. Chemical synthesis of 1-H_2^3-2-C^{14}- and 1-D_2^2-2-C^{14}-*trans*-*trans*-farnesyl pyrophosphate and their utilization in squalene biosynthesis. *J. Biol. Chem.* **237:** 56–61.
16. Dugan, R. E., E. Rasson, and J. W. Porter. 1968. Separation of water-soluble steroid and carotenoid precursors by DEAE-cellulose column chromatography. *Anal. Biochem.* **22:** 249–259.
17. Slakey, L. L., M. C. Craig, E. Beytia, A. Briedis, D. H. Feldbruegge, R. E. Dugan, A. A. Qureshi, C. Subbarayan, and J. W. Porter. 1972. The effects of fasting, refeeding, and time of day on the levels of enzymes effecting the conversion of β-hydroxy-β-methylglutaryl-coenzyme A to squalene. *J. Biol. Chem.* **247:** 3014–3022.
18. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177:** 751–766.
19. Goodman, DeW. S. 1961. Studies on the conversion of squalene to sterol with rat liver enzymes. *J. Biol. Chem.* **236:** 2429–2434.
20. Rosenberg, H. 1959. The detection of phosphates on chromatograms. *J. Chromatogr.* **2:** 487–489.
21. Shah, D. V., D. H. Feldbruegge, A. R. Houser, and J. W. Porter. 1968. Conversion of ^{14}C-labeled geranylgeranyl pyrophosphate to phytoene by a soluble tomato plastid enzyme system. *Arch. Biochem. Biophys.* **127:** 124–131.
22. Qureshi, A. A., F. J. Barnes, and J. W. Porter. 1972. Lycopersene and prelycopersene pyrophosphate. Intermediates in carotene biosynthesis. *J. Biol. Chem.* **247:** 6730–6732.
23. Bucher, N. L. R., P. Overath, and F. Lynen. 1960. β-Hydroxy-β-methylglutaryl coenzyme A reductase, cleavage and condensing enzymes in relation to cholesterol formation in rat liver. *Biochim. Biophys. Acta.* **40:** 491–501.
24. Linn, T. C. 1967. The effect of cholesterol feeding and fasting upon β-hydroxy-β-methylglutaryl coenzyme A reductase. *J. Biol. Chem.* **242:** 990–993.
25. White, L. W., and H. Rudney. 1970. Regulation of 3-hydroxy-3-methylglutarate and mevalonate biosynthesis by rat liver homogenates. Effects of fasting, cholesterol feeding, and Triton administration. *Biochemistry*. **9:** 2725–2731.
26. Salokangas, R. A., H. C. Rilling, and L. T. Samuels. 1964. Terpene metabolism in the rat testes. I. The conversion of isopentenyl pyrophosphate to squalene and sterols. *Biochemistry*. **3:** 833–837.
27. Salokangas, R. A., H. C. Rilling, and L. T. Samuels. 1965. Terpene metabolism in the rat testes. II. Metabolism of mevalonic acid by cell-free preparations. *Biochemistry*. **4:** 1606–1611.
28. Christophe, J., and G. Popják. 1961. Studies on the biosynthesis of cholesterol: XIV. the origin of prenoic acids from allyl pyrophosphates in liver enzyme systems. *J. Lipid Res.* **2:** 244–257.
29. Scallen, T. J., W. J. Dean, and M. W. Schuster. 1968. Enzymatic conversion of squalene to cholesterol by an acetone powder of rat liver microsomes. *J. Biol. Chem.* **243:** 5202–5206.
30. Teal, S. W., and W. Gamble. 1965. Effect of *p*-chlorophenoxyisobutyric acid, ethyl *p*-chlorophenoxyisobutyrate, and Atromid on the synthesis of nonsaponifiable material and cholesterol in the bovine aorta. *Biochem. Pharmacol.* **14:** 896–898.