oxidation of C_{20} alcohols as compared with that of 11β-hydroxyl groups.

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The Chemical Synthesis of Mevalonic Acid 5-Phosphate, Isopentenyl Pyrophosphate, and Related Compounds*

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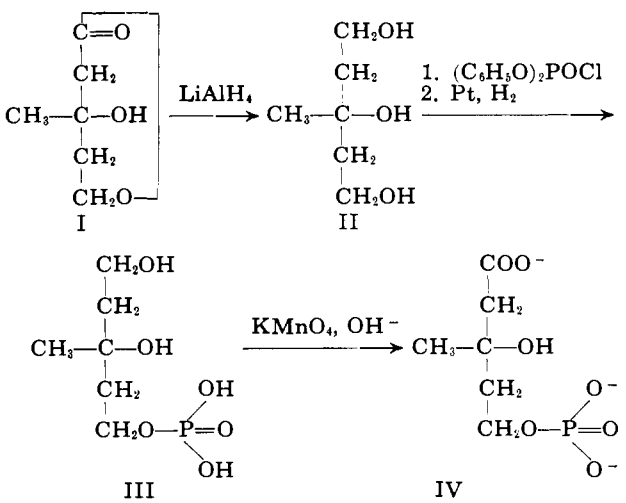
DL-Mevalonic acid 5-phosphate has been prepared by reduction of mevalonic lactone to 3-methlyl-1,3,5-pentanetriol and phosphorylation of this compound to 3-methyl-1,3,5-pentanetriol 5-phosphate, followed by oxidation to the desired product. 3-Methyl-1,5-pentanediol-5-phosphate and 5-hydroxy-3-methylpentanoic acid 5-phosphate have been prepared in a similar manner. The anhydride-anion exchange procedure for the synthesis of phosphate anhydrides has been successfully applied to the preparation of isopentenyl pyrophosphate.

Mevalonic acid 5-phosphate is well established as an intermediate in the enzymatic conversion of mevalonic acid to isopentenyl pyrophosphate (e.g., see the review by Popjak and Cornforth, 1960), but no satisfactory chemical synthesis of the compound is available (Lynen, 1959; Hellig and Popjak, 1961). In this report we describe a series of reactions which leads to mevalonic acid 5-phosphate in good yield.



Mevalonic lactone (I) was reduced to the corresponding triol (II) with lithium aluminum hydride. The phosphorylation of (II) to give the monophosphate ester (III) in reasonable yield without blocking the other alcohol functions was accomplished by reacting 1 mole of triol with 0.5–0.7 mole of diphenyl phosphorochloridate at room temperature. Under these conditions tertiary alcohols do not react (Wold and Ballou, 1959) and the ratio of monoester to diester produced was quite favorable. The monoester was separated from the diester by ion-exchange chromatography, and in the same step the unreacted triol was readily recovered. The over-all yield based on consumption of triol was 60–70%. Attempts to prepare the monoester after first blocking two of the three alcohol functions by preparing either the isopropylidene or ethylidene derivatives of the triol did not result in any improvement of this yield.

Oxidation of 3-methyl-1,3,5-pentanetriol 5-phosphate (III) with an excess of alkaline permanganate (Ballou and Hesse, 1956) yielded a compound which analyzed correctly for mevalonic acid 5-phosphate (IV). Potentiometric titration of this compound demonstrated that it contained one carboxyl group (pK about 4) per mole of phosphorus. The only structure for this final product which is consistent with these results is that of mevalonic acid 5-phosphate (IV). The over-all yield from mevalonic lactone was 43%.

A crude enzyme extract prepared from rabbit liver was used to prepare mevalonic acid 5-phosphate from 2-C^{14}-mevalonic lactone and ATP. 2-C^{14}-mevalonic acid 5-phosphate was separated from 2-C^{14}-mevalonic acid 5-pyrophosphate and isopentenyl pyrophosphate by paper chromatography (Tchen, 1958; Witting and Porter, 1959), and after it was eluted from the paper the enzymatically prepared 2-C^{14} compound was mixed with the synthetic product and chromatographed on an ion-exchange column. Figure 1 shows the exact match-

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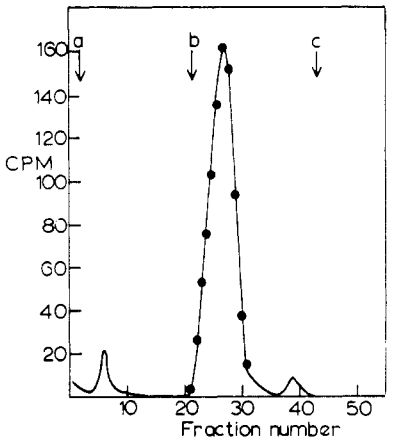


FIG. 1.—Ion-exchange chromatography of synthetic mevalonic acid 5-phosphate (10 mg) together with enzymatically produced 2-C^{14}-mevalonic acid 5-phosphate. The solid line gives the distribution of C^{14} in the eluate and the circles represent the calculated distribution of C^{14} based on the phosphorus analysis and using the specific activity of the peak tube (No. 27) as reference. A column of Dowex 1 × 10 (100-200 mesh) (Cl^- form) (0.9 × 7.0 cm) was used and was eluted with the following series of gradients: (a) 30 ml of 0.001 N HCl in the mixing flask and 62 ml of 0.05 M LiCl in 0.001 N HCl in the reservoir. When empty the reservoir was successively charged with (b) 62 ml of 0.08 M LiCl in 0.001 N HCl and (c) 62 ml of 0.4 M LiCl in 0.001 N HCl. Fractions (2.8 ml) were collected at the flow rate of 1 ml/min. The total amount of phosphorus contributed by C^{14}-mevalonic acid phosphate was below the level of detection in the phosphorus assay.

ing of the distribution of phosphorus and C^{14}-label in the eluted peak. Furthermore, when the synthetic mevalonic acid 5-phosphate was incubated with the crude rabbit liver enzyme preparation, the rate of ATP-dependent production of inorganic phosphate was enhanced (Figure 2). This is as should be expected if the synthetic compound were converted to isopentenyl pyrophosphate according to the accepted reaction sequence:

mevalonic acid 5-phosphate + 2 ATP ⟶
isopentenyl pyrophosphate + 2 ADP + P_i + CO_2

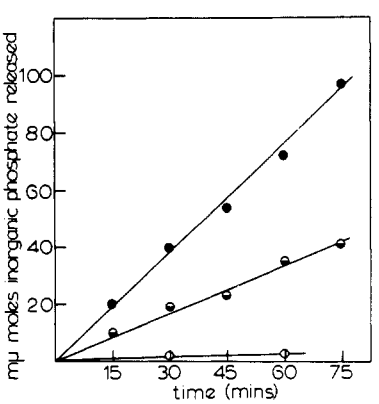
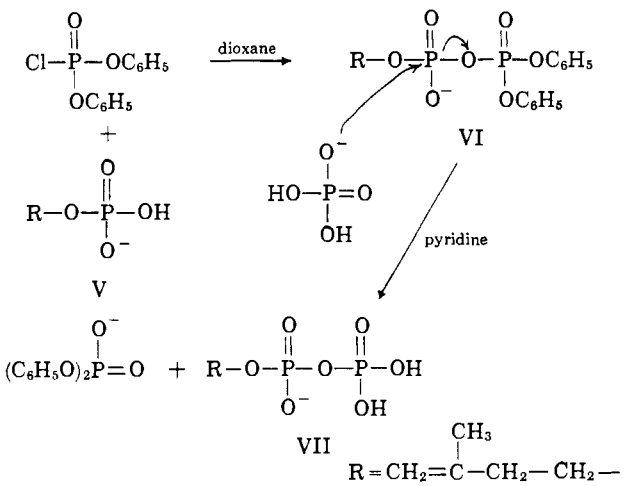


FIG. 2.—The release of inorganic phosphate from synthetic mevalonic acid 5-phosphate and ATP catalyzed by rabbit liver enzymes. The complete system contained, per 1-ml reaction volume, 100 μmoles of imidazole-HCl buffer (*p*H 7.4), 7 μmoles of ATP, 5 μmoles of $MgCl_2$, 10 μmoles of KF, 10 μmoles of lithium mevalonic acid 5-phosphate, and 2.2 mg of protein (from the rabbit liver ammonium sulfate fraction). Samples (0.1 ml) were removed at appropriate time and placed in 4.9 ml of 0.1 N HCl to stop the reaction. Complete system, ●——●; mevalonic acid 5-phosphate omitted, ▾——▾; ATP omitted, ○——○.

5-Hydroxy-3-methylpentanoic acid 5-phosphate was prepared from 3-methyl-1,5-pentanediol by application of the same phosphorylation and oxidation reactions. Thus mevalonic acid 5-phosphate and three analogs, 3-methyl-1,3,5-pentanetriol 5-phosphate, 3-methyl-1,5-pentanediol 5-phosphate, and 5-hydroxy-3-methylpentanoic acid 5-phosphate, have been obtained by use of this reaction scheme.

The preparation of isopentenyl pyrophosphate has been described by Lynen *et al.* (1958) and by Yuan and Bloch (1959). It was felt of interest to attempt to prepare this compound by the relatively simple anhydride anion-exchange method of Michelson's (1960) with the hope of subsequently applying the same method to the synthesis of mevalonic acid 5-pyrophosphate.

Isopentenyl phosphate (V) was prepared by phosphorylation of isopentenol with excess phosphorus oxychloride in ether (R. C. Blume, personal communication, 1961). After hydrolysis to the free phosphate ester, the inorganic phosphate formed from the excess phosphorus oxychloride was removed by precipitation with lithium hydroxide at *p*H 12. The lithium salt of isopentenyl phosphate was then recovered in an essentially pure state.



The intermediate P^1-isopentenyl-P^2-diphenyl pyrophosphate (VI) was prepared by reaction of isopentenyl phosphate (V) and diphenyl phosphorochloridate in dry dioxane and, without isolation, was reacted with inorganic phosphate in pyridine. Isopentenyl pyrophosphate (VII) was isolated from the reaction mixture by ion-exchange chromatography in an over-all yield from isopentenol of 25–30%.

Subsequent attempts to prepare mevalonic acid 5-pyrophosphate by this method had little success. Approximately 15% yield of a pyrophosphate derivative which was identical with enzymatically produced 2-C^{14}-mevalonic acid 5-pyrophosphate could be detected by ion-exchange chromatography, but isolation and purification of this component from the column eluate proved unsuccessful. Michelson and Wold (1962) showed that the anion-exchange reaction will give a good yield of pyrophosphate even if the attacking anion contains both a phosphate and a carboxylate function. In the case of mevalonic acid 5-phosphate, however, the carboxyl group is present in the starting material and it is possible that the formation of the desired trisubstituted pyrophosphate (VI above) may be complicated by the presence of the unblocked carboxylate.

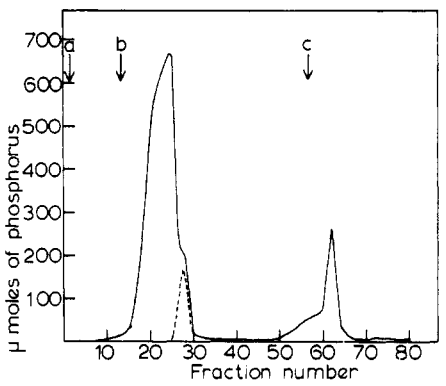


FIG. 3.—Ion-exchange chromatography of phosphate esters of 3-methyl-1,3,5-pentanetriol. The 1.5 × 40-cm column of Dowex 1 × 10 (Cl^- form) was eluted with (a) 200 ml of 0.001 N HCl, (b) 900 ml of 0.06 M LiCl in 0.001 N HCl, and (c) 720 ml of 0.4 M LiCl in 0.001 N HCl. Fractions (20 ml) were collected at a flow rate of 1 ml/min. The solid line represents total phosphorus and the dotted line inorganic phosphorus. The peak eluting at the high LiCl concentration was identified as the 1,5-diphosphate ester of the triol.

EXPERIMENTAL

3-Methyl-1,3,5-pentanetriol.—Mevalonic lactone was reduced to 3-methyl-1,3,5-pentanetriol by the careful addition of a solution of 3.92 g (30.1 mmole) of the lactone in 250 ml of dry ether to a stirred suspension of 2.00 g (52 mmole) of lithium aluminum hydride in 75 ml of dry ether. The excess hydride was decomposed by the careful addition of 200 ml of water. The ether was evaporated in a stream of air. The precipitate of aluminum oxide was removed by centrifugation and washed with water. The lithium was removed from the combined supernatant and wash by the addition of an excess of Dowex 50 (H^+ form), followed by filtering and washing the resin with water. Evaporation of the clear water solution on a rotary evaporator, followed by two evaporations from absolute alcohol, gave a colorless syrup weighing 3.72 g (91% based on the microanalysis given below).

Infrared analysis of the product demonstrated the disappearance of the carbonyl absorption (at 5.78 μ) of mevalonic lactone (Wolf *et al.*, 1957). Gas chromatographic analysis demonstrated complete disappearance of mevalonic lactone. Analysis for carbon was low.

Anal. Calcd. for $C_6H_{14}O_3$ (134.12): C, 53.78; H, 10.45. Found: C, 52.70; H, 10.25.

The analysis indicated that the product contained about 1.7% water (by weight). The product contained two hydroxyl groups which could be phosphorylated under conditions where tertiary alcohols are unreactive (Wold and Ballou, 1959) and where mevalonic lactone itself was found to be unreactive.

3-Methyl-1,3,5-pentanetriol 5-Phosphate.—Diphenyl phosphorochloridate (2.00 ml, 9.56 mmole) in 8 ml of dry pyridine was added dropwise to a cooled (ice bath), stirred solution of 2.57 g (18.9 mmole) of 3-methyl-1,3,5-pentanetriol in 8 ml of dry pyridine. The cold solution was left at room temperature for 20 hours in a tightly stoppered flask. After removing most of the excess pyridine by evaporation, the resulting syrup was dissolved in water-ethanol (5:7, v/v) and the solution deionized by passage through a 2.5 × 30-cm column of Amberlite MB3 (H^+ and OH^- form). The column was washed with 10 bed volumes of the water-ethanol solvent, and the effluent was evaporated to dryness. The evaporation was repeated once from absolute alcohol, and, after several hours' evacuation with an oil pump, the colorless syrup weighed 4.21 g.

The syrup, dissolved in 100 ml of absolute ethanol, was hydrogenated by shaking in a hydrogen atmosphere in the presence of 0.45 g of platinum oxide and 1.4 g of carbon, and 1540 ml of hydrogen was consumed in 190 minutes. The catalyst was removed by filtration and about 40 ml of water was added to the filtrate. The *p*H was brought to above 8 with cyclohexylamine and the solution was concentrated to about 10 ml. The concentrated solution was titrated to *p*H 4 with Dowex 50 (H^+ form), the resin was removed and washed by filtration, and the filtrate was finally fractionated by ion-exchange chromatography (Figure 3). Fractions 17-26 were pooled and concentrated to about one-half volume on a rotary evaporator (bath temperature at 30°). The *p*H of the resulting solution was adjusted to 8 with lithium hydroxide and the solution was lyophilized. Lithium chloride was removed from the dry white residue by extraction with absolute ethanol–ethyl ether (1:1.2, v/v). The precipitate of the lithium salt of the phosphate ester (which is insoluble in ethanol-ether) was collected by centrifugation, washed with ether, and air dried. The product weighed 1.132 g (69% yield based on the amount of triol consumed, see below). It gave a single spot (R_F 0.43) on paper chromatography in isobutyric acid–ammonia–water (66:3:30, v/v). One hundred ten mg of the amorphous material was converted to the cyclohexylammonium salt which was crystallized from water-acetone at −15°. The crystals after drying *in vacuo* over potassium hydroxide pellets weighed 185 mg (92%).

Anal. Calcd. for $C_{18}H_{41}O_6N_2P \cdot 1/2H_2O$ (421): C, 51.31; H, 9.98; N, 6.65; P, 7.36. Found: C, 51.44; H, 9.83; N, 6.66; P, 7.10.

The fractions 1-16 in Figure 3, containing the unreacted 3-methyl-1,3,5-pentanetriol, were pooled and concentrated to one-third volume. The resulting solution was deionized by passage through a 2.5 × 10-cm column of Amberlite MB3 resin. The column was thoroughly washed with water and the triol was recovered from the effluent by removal of solvent and drying as described for its preparation. Triol, 1.57 g (61% of the starting material), was recovered. As it was found advantageous in terms of yield and ease of work-up to use a minimum of resin in the fractionation shown in Figure 3, this column was frequently overloaded with respect to the monophosphate ester. When the fractions containing the unreacted triol also contained ester from such an overload, the ester could easily be recovered from the Amberlite MB3 column used in the subsequent deionization step by eluting with four bed volumes of 0.8 M LiCl in 10^{-3} N HCl. The lithium salt of the phosphate ester could then be obtained from this eluate as described above.

Repeating the entire procedure with the recovered alcohol gave an additional 0.752 g of phosphate ester (Li salt), while 0.818 g of alcohol was recovered. The yield of monophosphate ester in this second step was 60% based on the disappearance of 3-methyl-1,3,5-pentanetriol.

Mevalonic Acid 5-Phosphate.—Potassium permanganate (746 mg, 14.3 meq) was added to a stirred solution of 400 mg (1.77 mmole) of lithium 3-methyl-1,3,5-pentanetriol 5-phosphate in 18 ml of 1 N lithium hydroxide. The flask was stoppered and stirred for 3 hours at room temperature. (As the oxidation proceeded, the original purple color of MnO_4^- was replaced by a deep green color, and a deposit of MnO_2 was visible after about 5 minutes. The green color persisted until the oxidation was complete, after which the purple

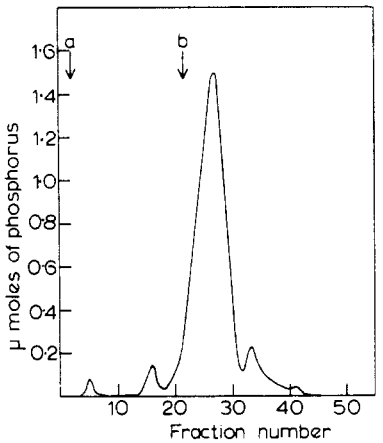


FIG. 4.—Ion-exchange chromatography of 10 mg of the impure crystalline product obtained after permanganate oxidation of 3-methyl-1,3,5-pentanetriol. The column and elution procedure was identical to that given in Fig. 1. The peak at tube 16 is inorganic phosphate. The slow peak (eluting after tube 32) has not been characterized. If the oxidation reaction were allowed to go for longer periods of time, the amount of this slow component would increase.

color of the excess MnO_4^- was again clearly visible. In the reaction being described, the oxidation was essentially complete after 1 hour.) Excess permanganate was reduced with hydrogen peroxide, and the resulting manganese dioxide removed by centrifugation and washed with water. Lithium and potassium were removed with an excess of Dowex 50 (H^+ form). The *p*H of the resulting filtrate was brought above 8 with cyclohexylamine and the solution was evaporated to dryness. The residue was fractionally crystallized from water-acetone at −15°. The first two crops of crystals were pooled and weighed 810 mg. Analysis by ion-exchange chromatography (Figure 4) demonstrated that this product contained at least three components of which the major one could be isolated in a 68% yield as follows: A water solution (384 mg of the cyclohexylammonium salt), adjusted to *p*H 4, was placed on a 1.5 × 30-cm column of Dowex 1 × 10 (chloride form) and the column was eluted with the following solutions: 750 ml of 0.001 N HCl; 100 ml of 0.03 M LiCl in 0.001 N HCl, 2000 ml of 0.06 M LiCl in 0.001 N HCl. The major peak was eluted in the 0.06 M LiCl solution, free of contaminants, and the lithium salt of the phosphate ester was freed of LiCl and isolated as described above. Crystallization of the cyclohexylammonium salt from water-acetone at −15° and drying *in vacuo* over potassium hydroxide pellets gave 259 mg of pure product.

Anal. Calcd. for $C_{24}H_{52}O_7N_3P \cdot H_2O$ (543); C, 53.04; H, 9.95; N, 7.73; P, 5.71. Found: C, 52.49; H, 9.77; N, 7.59; P, 5.81.

The compound gave a single spot (R_F 0.42) on paper chromatography in isobutyric acid–ammonia–water (66:3:30, v/v). The presence of a carboxyl group was confirmed by titration of an ionizable group with a *pK* in the range of 4. On the basis of the base (or acid) consumption, the ratio of carboxyl ionization to secondary phosphorus ionization was 1. The compound was completely stable to 1 N acid or base at 100° for 10 minutes.

5-Hydroxy-3-methylpentanoic Acid 5-Phosphate.—The lithium salt of 3-methyl-1,5-pentanediol 5-phosphate was prepared by procedures similar to those described above for preparation of 3-methyl-1,3,5-pentanetriol 5-phosphate. After oxidation of this material with

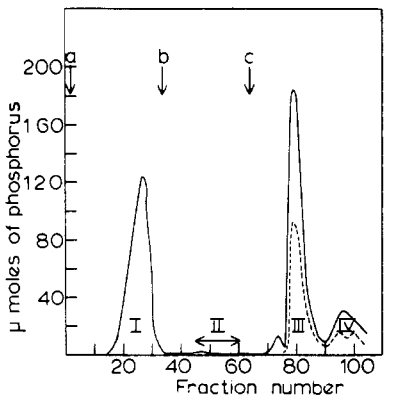


FIG. 5.—Ion-exchange chromatography of isopentenyl pyrophosphate. A column of Dowex 1 × 10 (100-200 mesh) (Cl^- form) (1.5 × 30 cm) was eluted with the following series of gradients: (a) 300 ml of 0.001 N HCl in the constant-volume mixing vessel and 620 ml of 0.03 M LiCl in 0.001 N HCl in the reservoir. When empty, the reservoir was successively charged with (b) 620 ml of 0.06 M LiCl in 0.001 N HCl and (c) 620 ml of 0.4 M LiCl in 0.001 N HCl. Fractions (20 ml) were collected at a flow rate of 1 ml/min. The solid line represents total phosphorus, the dashed line acid labile phosphorus. I is inorganic phosphate, III isopentenyl pyrophosphate, and IV an unidentified component. Isopentenyl phosphate if present would have been eluted in the region marked II.

excess permanganate (as above for preparation of mevalonic acid 5-phosphate), the cyclohexylammonium salt of 5-hydroxy-3-methylpentanoic acid 5-phosphate was isolated in 51% yield.

Anal. Calcd. for $C_{24}H_{52}O_6N_3P \cdot H_2O$ (527); C, 54.65; H, 10.25; N, 7.97. Found: C, 54.69; H, 10.26; N, 7.87.

Isopentenyl Phosphate.—A precooled solution of 2.58 g (30 mmole) of isopentenol and 3.63 ml (45 mmole) of dry pyridine in 6 ml of dry ether was added at a rapid dropwise rate to a cooled (ice-salt bath) stirred solution of 4.09 ml (45 mmole) of phosphorus oxychloride in 6 ml of dry ether. After 10 hours at −15° in a tightly stoppered flask, the crystals of pyridine hydrochloride were removed by filtration and washed with ether. The combined filtrate and wash were poured on 10 g of crushed ice, and 4.32 g of lithium hydroxide in 50 ml of water was added. The ether was evaporated in a stream of air. The milky aqueous suspension was titrated to *p*H 12 with lithium hydroxide to complete precipitation of the water-insoluble trilithium phosphate, which was removed by centrifugation. The clear supernatant was titrated to *p*H 8 with dilute hydrochloric acid and evaporated to dryness, and the residue was dried thoroughly *in vacuo* over potassium hydroxide pellets.

Lithium chloride was removed from the dry residue by extraction with 100 ml of ethanol-ether (1:1.2, v/v), leaving lithium isopentenyl phosphate as a precipitate which was collected by centrifugation and washed twice with ether. The white powder, after drying *in vacuo*, weighed 3.30 g (61%).

Anal. Calcd. for $C_5H_9O_4PLi_2$ (178): C, 33.71; H, 5.06; P, 17.4. Found: C, 33.31; H, 5.08; P, 17.2.

A single spot (R_F 0.88) was found on paper chromatography in isobutyric acid–ammonia–water (66:30:30, v/v). Treatment with 1 N HCl for 20 minutes at 100° resulted in quantitative conversion of isopentenyl phosphate to a chromatographically different phosphate ester, tentatively identified as the hydrated product 3-methyl-1,3-butanediol 1-phosphate (Lynen, *et al.*, 1958).

Isopentenyl Pyrophosphate.—Mono(tributylammo-

nium)isopentenyl phosphate (1 mmole) in 6 ml of dry dioxane was reacted with 1 mmole of diphenyl phosphorochloridate in the presence of 1 mmole of tributylamine for 2.5 hours at room temperature. The solvent was removed under vacuum and the residue was shaken with 15 ml of pentane to remove unreacted diphenyl phosphorochloridate. After decanting the pentane, the residue was dried and dissolved in 2 ml of dry pyridine containing 2 mmole of mono(tributylammonium) phosphate. After 1 hour at room temperature the solvent was removed under vacuum and the residue was washed with 15 ml of pentane. After drying, the precipitate was dissolved in 4 ml of water by adding 95% ethanol, and the resulting solution was fractionated by ion-exchange chromatography (Figure 5).

The fractions containing the product (tubes 76–88) were pooled, adjusted to *p*H 8 with lithium hydroxide, and concentrated to about 35 ml. Two volumes of absolute ethanol were added and the mixture was set at 4° overnight. The precipitate of lithium isopentenyl pyrophosphate was collected by centrifugation, washed with 95% ethanol, and dried by dissolving in water and lyophilizing. One hundred five mg (40% of theory) was recovered.

Anal. Calcd. for $C_5H_9O_7P_2Li_3$ (264): P, 23.5. Found: P, 25.9.

The material was free of inorganic phosphate and contained 1.01 moles of acid-labile phosphorus for every 2.00 moles of phosphorus. Chromatography in the isobutyric acid solvent gave a single spot (R_F 0.79) different from isopentenyl phosphate (R_F 0.88).

Phosphorous Analyses.—Phosphorus was analyzed as the phosphomolybdate complex in butanol by the method of Marsh (1959). "Total phosphorus" was obtained after a 1-hour digestion of suitable aliquots with 0.5 ml 1 N sulfuric acid in open tubes on a sand bath (150–170°). Pyrophosphates formed during the digestion were hydrolyzed in a boiling water bath for 10 minutes. "Acid labile phosphorus" was obtained after hydrolysis with 1 N sulfuric acid for exactly 10 minutes in a boiling water bath.

Preparation of Rabbit Liver Enzyme Extracts.—The preparation of acetone powder of frozen rabbit livers (Pel-Freese Biologicals, Inc.), as well as the subsequent extraction, was carried out at 4° according to Markley and Smallman (1961). The resulting 20,000 × *g* supernatant (in 0.1 M potassium phosphate buffer, *p*H 7.4) was brought to 55% saturation with ammonium sulfate, and the precipitate was redissolved in enough phosphate buffer to give about 50 mg of protein per ml. After dialysis overnight against 0.01 M potassium phosphate buffer (*p*H 7.4) containing 0.01 M cysteine, and subsequent removal of small amounts of insoluble material by centrifugation, a clear solution was obtained which contained all the enzymes necessary to convert mevalonic acid to isopentenyl pyrophosphate. The half-life of this activity in the extract was only about 72 hours at 4°. In spite of the addition of fluoride, the ATPase activity of this preparation was always high (see Fig. 2).

Preparation of 2-C^{14}-Mevalonic Acid 5-Phosphate.—One-tenth ml incubation mixtures each containing 5 μmoles of imidazole-HCl buffer (*p*H 7.4), 0.35 μmole of ATP, 0.25 μmole of magnesium chloride, 0.285 μmole of potassium mevalonate 2-C^{14} (20,000 cpm), and 100–500 μg of protein from the above liver extract were incubated at 37°. The reaction was stopped by placing the tubes in a boiling water bath, and the entire content of each tube was streaked on 1.5-cm-wide strips of Whatman No. 1 paper. The strips were developed by descending chromatography in *n*-butanol–formic acid–water (77:10:13, v/v). The positions of radioactive peaks on the dried paper strips were determined with a Vanguard Model 800 Autoscanner. The areas corresponding to mevalonic acid 5-phosphate (Tchen, 1958; Witting and Porter, 1959) were cut out from a number of strips, and the radioactive material was eluted with dilute ammonia in the normal fashion. The pooled material was mixed with synthetic mevalonic acid 5-phosphate and chromatographed on Dowex-1 as given in Figure 1. The radioactivity of the eluted fractions was determined in the Packard Tri-Carb liquid scintillation spectrometer, Model 500B, after mixing appropriate aliquots of eluate with Kinard (1957) solution.

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