McIlwain & Greengard (1957) to inhibit the metabolic response of cerebral tissues to electrical pulses. Lipids or lipoproteins are suspected of being involved in some of these actions, and the material is being further characterized.

SUMMARY

1. Slices of guinea-pig cerebral cortex have been exposed to a variety of adverse conditions with the object of diminishing their respiratory response to electrical stimulation, while maintaining relatively normal respiratory rates in the absence of stimulation.

2. The greatest differential effect occurred on exposure in tris-buffered glucose-containing media at 0° for 5 hr. These conditions lowered also the tissue's glycolytic response to electrical pulses and its respiratory response to high concentrations of potassium salts.

3. Restoration of the exposed tissue's respiratory response to electrical pulses was sought by adding a variety of substances to media in which the tissue was incubated. Additions included known metabolites, blood sera and extracts of brain, muscle and liver.

4. Blood sera from several animal species were the most active of these materials in restoring respiratory response. Dialysis and solvent precipitation suggested that the activity was associated with the serum proteins. Examination of plasma fractions obtained by the method of ethanol precipitation of Cohn *et al.* (1946) showed the most active fraction to be IV-4, which contains α - and β -globulin.

5. The inorganic phosphate and phosphocreatine of cerebral tissues have been determined after the chosen conditions of exposure and after incubation with blood-protein preparations, and relationships between the exposed tissue and restorative agents have been discussed.

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Studies on the Biosynthesis of Cholesterol

9. FORMATION OF PHOSPHORYLATED DERIVATIVES OF MEVALONIC ACID IN LIVER-ENZYME PREPARATIONS*

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After the discovery by Tavormina, Gibbs & Huff (1956) that mevalonic acid (3:5-dihydroxy-3-methylpentanoic acid) is efficiently converted into cholesterol by rat-liver homogenates, and after the

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demonstration that this acid furnishes the fundamental building unit in the synthesis of squalene (Cornforth, Cornforth, Popják & Gore, 1957, 1958; Dituri, Gurin & Rabinowitz, 1957) and hence of cholesterol (Isler, Rüegg, Wüsch, Gey & Pletscher, 1957), we studied the enzymic transformations of mevalonic acid in liver preparations. It has been

found that a combination of soluble enzymes and microsomes from rat liver catalysed the synthesis of squalene and of cholesterol from mevalonic acid in the presence of adenosine triphosphate, reduced diphosphopyridine nucleotide, triphosphopyridine nucleotide, Mg^{2+} ions, nicotinamide, oxygen and a reducing substance such as cysteine, glutathione or ascorbic acid (Popják, Gosselin, Gore & Gould, 1958). In this paper the isolation from liver-enzyme preparations of two phosphorylated derivatives of mevalonic acid are reported; both substances are intermediates in sterol biosynthesis. The results have been reported briefly at a Ciba Foundation Symposium in May 1958 (Popják, 1959).

METHODS AND MATERIALS

Preparation of liver enzymes. Soluble enzymes and microsomes from rat liver were prepared as previously described (Popják *et al.* 1958). The complete enzyme system, which synthesizes both squalene and cholesterol from mevalonic acid, contained (in a volume of 4 ml.): dialysed soluble enzymes (30–50 mg. of protein), suspension of microsomes (0.2 ml.), adenosine triphosphate (ATP; 10–15 μ moles), reduced diphosphopyridine nucleotide (DPNH; 1 μ mole), triphosphopyridine nucleotide (TPN; 0.5 μ mole), ascorbic acid (30 μ moles), DL-[2- ^{14}C]mevalonic acid (0.5–2.0 μ moles), potassium phosphate buffer (0.1 M, pH 7.5), $MgCl_2$ (4 mM) and nicotinamide (30 mM).

Coenzymes. All the coenzymes used were commercial preparations; their sources were given in an earlier paper (Popják *et al.* 1958).

[^{14}C]Mevalonic acid. DL-[2- ^{14}C]Mevalonic acid lactone and DL-[1- ^{14}C]mevalonic acid lactone were synthesized by Dr Rita Cornforth according to the method of Cornforth *et al.* (1958). Solutions of the lactone (0.1 M) were made in 0.1 M-potassium hydrogen carbonate and were incubated for 30 min. at 37° before use; this treatment caused the opening of the lactone ring and gave the potassium salt of mevalonic acid. Mevalonic acid preparations with specific activities of 0.04 and 0.2 μ C/ μ mole have been used. The efficiency of our instrument for assaying ^{14}C in infinitely thin samples is about 6%.

Chromatography. For chromatography on Whatman no. 1 paper a variety of solvent systems was used; isobutyric acid-aq. NH_3 soln.-water (66:3:30, by vol.) was found the most convenient and results obtained with this system alone will be shown. Photographic prints with ultraviolet light (Markham & Smith, 1949) were made of the paper strips, which were also scanned for radioactivity with an automatic and recording instrument (G. Popják, unpublished work).

Chromatography on ion-exchange resins Dowex-2 (formate form) (Dow Chemical Co., Midland, Mich., U.S.A.) and Amberlite IRA-400 (Cl^- form) (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) were made according to the gradient-elution technique of Hurlbert, Schmitz, Brumm & Potter (1954). Details of the elution schedules are shown on Figs. 2 and 3.

Determinations of phosphorus. These were made according to the method of Allen (1940), except that the digestion of the samples was made by combustion with $Mg(NO_3)_2$, and the method was adapted as a microprocedure. To a dried

sample of the compound to be analysed in a hard-glass tube 2 drops of a $Mg(NO_3)_2$ solution (10%, w/v, in 95% ethanol) were added and the solvent was evaporated off. The mixture was then ignited by heating in a microflame until all the brown fumes were driven off and a white ash remained; the pyrophosphate which may have been formed was cleft by heating the ash in a stoppered tube with 0.2 ml. of $N-HCl$ in a boiling-water bath for 15 min. After the contents of the tube had cooled down, there were added 0.6 ml. of water, 0.08 ml. of 60% (w/v) perchloric acid, 0.08 ml. of Allen's (1940) amidol (2:4-diaminophenol hydrochloride) reagent and 0.04 ml. of 8.3% (w/v) ammonium molybdate solution. The colour was measured in a 1 ml. microcuvette of 1 cm. light path at 660 m μ in a spectrophotometer 15 min. after addition of the reagents. By this method amounts of orthophosphate as small as 0.02–0.08 μ mole could be accurately determined.

RESULTS

Isolation of phosphorylated derivatives of mevalonic acid

The enzymic formation of two new derivatives of mevalonic acid was first observed in short-term (5–10 min.) incubations of the complete enzyme system synthesizing cholesterol from mevalonic acid (cf. Methods and Materials). In such experiments the incubation mixtures were deproteinized with 5% (w/v, final concentration) trichloroacetic acid and the pH of the protein-free filtrate was adjusted to about 8.3 by the addition of $Ba(OH)_2$; the water-insoluble precipitate of barium salts contained, among other things, the new derivatives of mevalonate, which were recognized on paper chromatograms. Further experiments showed, however, that the same derivatives of mevalonic acid were also formed, and in better yield, with the dialysed soluble enzymes alone (without microsomes) in the presence of ATP and Mg^{2+} ions. Subsequently the formation of these substances was studied in incubations containing, in a volume of 3 ml., dialysed soluble enzymes (30–50 mg. of protein), ATP (10 μ moles), potassium phosphate buffer (0.1 M, pH 7.5), $MgCl_2$ (4 mM), nicotinamide (30 mM) and either [1- ^{14}C]- or [2- ^{14}C]-mevalonate (0.5–2.0 μ moles) as the potassium salt. After incubation of such mixtures for 5–10 min. at 37° the proteins were precipitated by the addition of 3 ml. of ice-cold 10% (w/v) perchloric acid and were removed by centrifuging. The protein-free extracts were neutralized with KOH and the insoluble $KClO_4$ was centrifuged off at 0°. The perchlorate-free extracts were dried from the frozen state *in vacuo* and the dry residues dissolved in 0.5 ml. of water. Chromatography of samples of this solution on Whatman no. 1 paper with the isobutyric acid-aq. NH_3 soln.-water system revealed the presence of four radioactive substances whether [1- ^{14}C]mevalonate or [2- ^{14}C]mevalonate was used as substrate. A typical chromatogram is shown in Fig. 1. The two least

Fig. 1. Paper-chromatographic identification of two derivatives (MV_1 and MV_2) of $[^{14}C]$ mevalonic acid. Photographic prints were made with ultraviolet light and the records of scanning for radioactivity are shown. The lower chromatogram is the control from an incubation mixture containing no enzyme, the upper one shows the appearance of two new radioactive substances after incubation of $[2-^{14}C]$ mevalonate with soluble enzymes, ATP and Mg^{2+} ions for 5 min. at 37° . O, Origin; s.f., solvent front.

Fig. 2. Results of chromatography on Dowex-2 formate of perchloric acid extract of liver enzymes incubated with $[2-^{14}C]$ mevalonate, according to the gradient-elution technique of Hurlbert, Schmitz, Brumm & Potter (1954). The mixing flask for the eluent contained at the start 100 ml. of water and the reservoir 100 ml. of 4N-formic acid. At each arrow 50 ml. of ammonium formate solution of the strength indicated, and contained in 4N-formic acid, was added to the reservoir. Peaks I and II are mevalonic lactone and mevalonic acid respectively, peak III is MV_1 and peak IV is MV_2 .

polar substances (R_f 0.75 and 0.66) were identified as mevalonic lactone and mevalonic acid respectively; these two alone were found when mevalonic acid was incubated in the buffer without enzymes. In the *isobutyric acid*-aq. NH_3 soln.-water system there was always found an equilibrium mixture of the lactone and the acid irrespective of whether mevalonic lactone or acid has been applied to the papers. The two more polar substances, MV_1 and MV_2 (R_f 0.37-0.40 and 0.20-0.25), formed from either $[1\text{-}^{14}\text{C}]$ - or $[2\text{-}^{14}\text{C}]$ -mevalonic acid, could be detected in significant amounts only when the incubation time was 10 min. or less; at 5-7 min. as much as 30 % of the added DL-mevalonic acid could be accounted for in these two new products. The amount of MV_2 was always less than that of MV_1 (cf. Figs. 1 and 2). In incubations lasting longer than 10 min. the amounts of MV_1 and MV_2 gradually diminished, so that after 20 or 30 min. only traces of them could be found, suggesting their utilization for the synthesis of products other than squalene and sterol, which are not formed with the soluble enzymes alone.

The prints of the chromatograms made with ultraviolet light showed that nucleotides were associated with both MV_1 and MV_2 (cf. Fig. 1), but, as subsequent work showed, this was a fortuitous circumstance, adenosine diphosphate (ADP) having an R_f identical with that of MV_1 and ATP an R_f identical with that of MV_2 in the *isobutyric acid*-aq. NH_3 soln.-water system. Furthermore, ADP and ATP were found to migrate in at least three other chromatographic systems to the same places as the two derivatives of mevalonic acid. For this reason we believed at first that nucleotide complexes of mevalonic acid were formed.

The four compounds mevalonic acid and lactone, MV_1 and MV_2 , were also separated on Dowex-2 formate ion-exchange resin by gradient-elution technique (Hurlbert *et al.* 1954). Mevalonic lactone was eluted from the Dowex column with the solvent front (water) (Fig. 2, peak I) and mevalonic acid with low concentrations of formic acid (Fig. 2, peak II). Elution with increasing concentrations of ammonium formate (in 4N-formic acid) yielded peaks III and IV, which were identified as MV_1 and MV_2 by paper chromatography. Even after chromatography on this resin ADP and ATP were still associated with MV_1 and MV_2 .

Purification of compounds MV_1 and MV_2

For purposes of identification and for testing in enzymic reactions, compounds MV_1 and MV_2 , formed from $[2\text{-}^{14}\text{C}]$ mevalonic acid, were isolated from a large-scale incubation (equivalent to 40 incubations of 3 ml.) by chromatography of the deproteinized mixture on Dowex-2 resin. The compounds MV_1 and MV_2 , as obtained from the

Dowex column, were finally freed from ADP and ATP as follows. The eluates from the resin were dried from the frozen state; most of the formic acid and ammonium formate was sublimed off in high vacuum, the samples being warmed with an infra-red lamp during sublimation. The dried specimen of peak III, containing MV_1 , was dissolved in a few millilitres of water and applied to a small charcoal (Nuchar C190, N; West Virginia Pulp and Paper Co., Tyrone, Penn., U.S.A.) column (5 cm. height, 1 cm. in diameter); the charcoal was then washed with water to remove formic acid and ammonium formate. MV_1 was eluted with 7 ml. of 55 % pyridine in water; 67 % of the applied radioactive substance was thus recovered. After removal of pyridine from the eluate by CHCl_3 and ether extractions, MV_1 was chromatographed again on an Amberlite IRA-400 ion-exchange resin (Cl^- form; column height 7 cm., diameter 1.5 cm.). Gradient elution of this column was carried out with 0.015N-HCl containing increasing concentrations of KCl (0.02-0.5M). MV_1 was eluted when the concentrations of KCl in the eluate was between 0.1 and 0.2M (Fig. 3). The dilute solution of the compound, still containing traces of ADP, was dried from the frozen state *in vacuo* and the dry powder extracted with 90 % ethanol, in which MV_1 dissolved completely whereas the ADP did not. The solutions of MV_1 now did not absorb light at 260 $\text{m}\mu$ and were free from inorganic phosphate.

The purified MV_1 was chromatographed again on paper and its R_f values were found to be identical with that of the substance identified as MV_1 in the original perchloric acid extracts of enzyme incubations. On chromatography of the pure MV_1 together with added ADP in the *isobutyric acid*-aq. NH_3 soln.-water system the two substances ran together as originally observed. Since radioactive MV_1 was formed from either $[1\text{-}^{14}\text{C}]$ - or $[2\text{-}^{14}\text{C}]$ -mevalonate, the substance contains in all probability all six carbon atoms of mevalonate. The polar behaviour of the substance during chromatography was accounted for by finding that it contained phosphorus. Assuming that MV_1 was a C_6 compound and that no dilution of the $[^{14}\text{C}]$ mevalonate with unlabelled material in the incubations occurred, the analyses showed that MV_1 contained one atom of phosphorus per mevalonic acid unit (the actual figure gave 0.8 atom of phosphorus/molecule of mevalonate). The phosphorus is probably present in the compound as a phosphate ester as it proved to be stable to hydrolysis for 7 min. in N-HCl or N-KOH at 100°. As it failed to give a lactone, the esterification probably involves the 5-hydroxy group of mevalonic acid. Judging from these characteristics, MV_1 appears to be identical with the substance isolated by Tehen (1957) and independently by Lynen (1959) from incubations of

yeast enzymes with [^{14}C]mevalonate and claimed to be 5-phosphomevalonate. Lynen (1959) phosphorylated mevalonic acid with POCl_3 in pyridine and showed that the product of the reaction was identical with the substance formed in enzyme incubations.

The compound MV_2 , obtained as Dowex peak IV (cf. Fig. 2), was separated from contaminating ATP in a manner similar to that used for MV_1 . After adsorption to and elution from charcoal with 55% pyridine, the radioactive MV_2 was further chromatographed on Amberlite IRA-400 resin (Cl^- form) as described for MV_1 (Fig. 3). MV_2 was eluted from the resin when the concentration of KCl in the eluate was about 0.2 M. The eluate was dried from the frozen state *in vacuo* and the dry residue extracted with 90% methanol, yielding MV_2 uncontaminated by nucleotides or by inorganic phosphate and which, as judged by its behaviour on paper chromatograms, was radiochemically homogeneous. It had the same properties as the substance identified in the original perchloric acid extracts of enzyme incubations. Phosphorus analysis showed that MV_2 contained two atoms of

phosphorus (the actual figure gave 1.82 atoms) per mevalonic acid unit, the calculations being based on the same assumptions as those for MV_1 . We cannot offer any further evidence at present on the chemical properties of MV_2 ; it might be 3:5-diphosphomevalonate, but it is equally possible that the second phosphate group is present in some form other than the tertiary phosphate. The chromatographic behaviour of this substance suggests that it is identical with compound II of Chaykin, Law, Phillips, Tchen & Bloch (1958), who have found this in incubations of yeast extracts with mevalonic acid, ATP and Mg^{2+} ions and suggest that it is mevalonic acid 5-pyrophosphate. For convenience this substance might be referred to as diphosphomevalonate.

5-Phosphomevalonate and diphosphomevalonate as precursors of cholesterol

The two purified compounds were tested as precursors in the biosynthesis of sterol with the complete liver-enzyme system under our standard conditions (Popják *et al.* 1958). As the results in Table 1 show, both 5-phospho-[2- ^{14}C]mevalonate and diphospho-[2- ^{14}C]mevalonate were more effective precursors of cholesterol than DL-mevalonic acid itself, but both still required the addition of ATP to the incubation mixtures: in the absence of ATP only negligibly small labelling of sterol occurred. These experiments were repeated with three preparations of each compound with identical results.

DISCUSSION

The finding of the same phosphorylated derivatives of mevalonic acid in a mammalian enzyme system

Fig. 3. Chromatography of MV_1 and MV_2 , as obtained from Dowex-2 column, on Amberlite IRA-400 (Cl^-) by gradient elution. The mixing flask for the eluent contained at the start 100 ml. of water; at each arrow 50–100 ml. of 0.015 N-HCl containing KCl in the concentrations indicated was added to the reservoir.

Table 1. Utilization of 5-phospho-[2- ^{14}C]mevalonate and of diphospho-[2- ^{14}C]mevalonate as compared with that of DL-[2- ^{14}C]mevalonate for cholesterol synthesis

Each flask contained: soluble dialysed enzymes (1 ml.; 50 mg. of protein); microsome suspension (0.2 ml.); ATP, 30 μmoles ; DPNH, 1 μmole ; TPN, 1 μmole ; ascorbic acid, 30 μmoles ; nicotinamide, 120 μmoles ; potassium phosphate buffer, pH 7.4, 400 μmoles ; Mg^{2+} ions, 16 μmoles ; final volume: 4 ml. Incubation was for 1 hr. at 37° with air as gas phase. The substrates were added in equal amounts (0.26 μmole , containing a total of 7600 counts of $^{14}\text{C}/\text{min.}$).

Specific activity of digitonides (counts/min./2 cm.² at infinite thickness)

Substrate added	
Mevalonate	1012
5-Phosphomevalonate	1705
5-Phosphomevalonate	30*
Diphosphomevalonate	1552
Diphosphomevalonate	1571
Diphosphomevalonate	40*

* No ATP was added to these incubations.

as were found in yeast extracts (Tchen, 1957; Bloch, 1959; Lynen, 1959) adds weight to the view that the biological synthesis of polyisoprenoid substances proceeds according to similar reactions both in plants and animals and that the key substance is mevalonic acid. The enzyme catalysing the formation of 5-phosphomevalonate from mevalonic acid and ATP, named mevalonic kinase, has been purified by Tchen (1958) from yeast extracts and by Levy & Popják (1959) from extracts of pig liver; apart from some minor differences the enzymes from these two sources have very similar characteristics.

The fact that ATP is still needed for the utilization of either 5-phosphomevalonate or diphosphomevalonate for cholesterol synthesis suggests a further phosphorylating step in the enzymic reactions of mevalonic acid. If the suggestion of Chaykin *et al.* (1958) that diphosphomevalonate is identical with mevalonic acid 5-pyrophosphate is proved correct, then this additional phosphorylation might involve the 3-hydroxy group of mevalonic acid. It seems that at least 3 moles of ATP are needed per mole of mevalonic acid converted into squalene or sterol. Considering that in the synthesis of mevalonic acid from acetate three high-energy bonds of acetyl-coenzyme A are needed (Rudney, 1959) and 6 moles of mevalonate are used for each mole of cholesterol, it follows that synthetic energy equivalent to at least 36 high-energy bonds is needed in the synthesis of one molecule of cholesterol from acetate.

The amounts of the two phosphorylated derivatives of mevalonate that were obtained with the rat liver enzymes were very variable and critically depended on the length of the incubations, 5–7 min. being the optimum period. After an incubation period of 10 min. or longer the yields became increasingly poor owing to the conversion of mevalonic phosphates into other products. The [^{14}C]mevalonic acid and the [^{14}C]mevalonic acid lactone, isolated by chromatography on Dowex-2 resin after incubating DL-[2- ^{14}C]mevalonate with the enzyme mixture for 20 min., were ineffective as precursors of cholesterol. Presumably they represent the unnatural enantiomorphs of the acid and lactone, which, in agreement with the suggestion of Tavormina *et al.* (1956), are not used in sterol biosynthesis.

Since the work described here has been completed, a method based on the adsorption to and

elution from charcoal followed by chromatography on Dowex-2 resin has been successfully employed for the large-scale preparation of 5-phosphomevalonate with purified liver mevalonic kinase (Levy & Popják, 1959).

SUMMARY

1. The formation of two phosphorylated derivatives of mevalonic acid by liver enzymes in the presence of adenosine triphosphate and Mg^{2+} ions is described.
2. The two substances were purified by various chromatographic procedures. One of the compounds, MV_1 , is thought to be 5-phosphomevalonate. The second, MV_2 , contains two atoms of phosphorus per molecule of mevalonate.
3. Both of these substances are converted into cholesterol more efficiently than is DL-mevalonate, but adenosine triphosphate is still needed in this conversion.

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