Determination of mevalonate in blood plasma in man and rat. Mevalonate "tolerance" tests in man

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Abstract A method is described for the determination of mevalonate in ultrafiltrates of blood plasma. The method depends on the phosphorylation of mevalonate with [γ-^{32}P]ATP and mevalonate kinase to 5-[^{32}P]phosphomevalonate, and the subsequent isolation of the 5-[^{32}P]-phosphomevalonate together with known amounts of added 5-phospho[^{14}C]mevalonate by ion-exchange chromatography. The ^{32}P/^{14}C ratio in the isolated 5-phosphomevalonate is a linear function of the mevalonate content of the samples. The smallest amount that can be determined is 1–2 pmol. The fasting level in human plasma varied between 20 and 75 pmol/ml. Human red blood cells absorb mevalonate from plasma relatively slowly; their maximum storage capacity is about 1.3 pmol/10^6 red cells. An oral and intravenous "mevalonate tolerance test" in man is described that can be carried out with 200 and 30 μmol, respectively, of the unlabeled (*RS*)-mevalonate in a 70-kg man. Beer and wine contain mevalonate at a concentration of 3–8 μM, too low to provide a significant amount of mevalonate even for heavy drinkers. The mevalonate content of the plasma from the blood of the vena cava inferior of male rats varied between 81 and 502 pmol/ml and is positively related to the levels of liver 3-hydroxy-3-methylglutaryl-CoA reductase, suggesting that the liver is probably the main source of mevalonate circulating in blood. The plasma of renal venous blood contained only 33–85% as much mevalonate as the arterial plasma.—**Popják, G., G. Boehm, T. S. Parker, J. Edmond, P. A. Edwards, and A. M. Fogelman.** Determination of mevalonate in blood plasma of man and rat. Mevalonate "tolerance" tests in man. *J. Lipid Res.* 1979. **20:** 716–728.

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It was shown first in our laboratory (1–3) that mevalonate was not used in vivo exclusively for polyisoprenoid or sterol synthesis, but that either mevalonate or some intermediate of polyisoprenoid biosynthesis derived from it was diverted to a metabolic pathway that contributed to the C_2-pool of the body. It was found further that the kidneys played a paramount role in diverting mevalonate from the sterol biosynthetic pathway (4, 5), as nephrectomy reduced the extent of the "shunt" by over 50%, an observation confirmed by Wiley, Howton, and Siperstein (6). It was also shown that the shunt could be demonstrated in vitro with tissue slices of various organs (7–9). An interesting extension of these observations, made by Wiley et al. (10), was the finding that the extent of the shunt in female rats was twice that seen in males and that this sex difference was abolished by nephrectomy.

All the in vivo experiments cited were carried out with [^{14}C]mevalonate given to the experimental subjects either parenterally or by mouth. The question arose, therefore, whether mevalonate was normally circulating in the blood, and if so, what was its origin and whether some organs (e.g., the kidneys) used the blood mevalonate specifically. Hagenfeldt and Hellström (11) identified mevalonate in extracts of rat blood at a concentration of 0.02 and 0.04 μg/ml by gas–liquid chromatography coupled with mass spectrometry. They relied for their determinations on the appearance in the mass spectrometer of the fragment ion *m/e* 71 (one of the two most intense ions in the mass spectrum of mevalonolactone) at a retention time characteristic for mevalonolactone. Hagenfeldt and Hellström (11) reported that the identification was difficult on account of many interfering substances in the extracts with retention times similar to that of mevalonolactone. Their method is not suita-

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ble for large numbers of determinations; each of the two measurements they made required the pooled blood of three rats. We now offer further proof for the existence of mevalonate in the blood of man and rat and an enzymic method for its determination by the use of mevalonate kinase and [γ-^{32}P]ATP. The method can measure as little as 1–2 pmol of mevalonate, or much larger amounts, in 50–100 μl of an ultrafiltrate of blood plasma. We used the method to measure mevalonate in the blood plasma of man and rats and for the definition of an oral and intravenous "mevalonate tolerance test" in man. We also demonstrate the presence of mevalonate in beer and wine, but at concentrations far too low to have much effect on the body's pool of mevalonate, even in heavy drinkers.

MATERIALS

Mevalonate kinase (EC 2.7.1.36)

A partially purified preparation (758 mg protein, sp act 0.27 units/mg; 1 unit equals 1 μmol of mevalonate phosphorylated per min), prepared from pig liver by the method of Levy and Popják (12), was fractionated further by the reverse ammonium sulfate gradient elution method of King (13). The reverse gradient was made up from 700 ml of 70% and 800 ml of 20% saturated ammonium sulfate, both containing 10 mM 2-mercaptoethanol and 1 mM EDTA, and 0.1 M potassium phosphate buffer. The enzyme was eluted between 30 and 25% saturation of the gradient (131 mg protein and 109 units) and was precipitated again with ammonium sulfate at 65% saturation. The precipitate, collected by centrifuging, was redissolved in 10 ml of 1 mM potassium phosphate buffer, pH 7.3, 5 mM 2-mercaptoethanol, and 1 mM sodium EDTA. It was then dialyzed against several changes of the same buffer. The preparation was then applied to a 1 × 33 cm column of Bio-Gel hydroxyapatite equilibrated with the above buffer. The column was developed with a linear gradient of buffer made from 200 ml each of 1 mM and 200 mM potassium phosphate buffers, pH 7.2, containing 5 mM 2-mercaptoethanol and 1 mM sodium EDTA. The enzyme was eluted after 100 ml of the gradient had passed through the column. The two fractions (about 5 ml each) containing the enzyme with the highest specific activity (5.35 units/mg, total 54.5 units) were pooled and the enzyme was precipitated at 60% saturation with ammonium sulfate and stored as a suspension at 4°C without much deterioration over 2 years. Mevalonate kinase was assayed by the spectrophotometric method of Levy and Popják (12); the preparation was free of hexokinase.

5-Phospho[^{14}C]mevalonate

This substance was made from (*RS*)-[2-^{14}C]mevalonate (13 Ci/mol; Amersham-Searle Corp., Arlington Heights, IL) with purified mevalonate kinase as described before (12, 14) except that the ethanolic supernatant of the reaction mixture (after concentration to a small volume) was chromatographed on sheets of Whatman 3 MM paper with propan-1-ol–conc. NH_4OH (sp. gr. 0.85)–water 6:3:1 (by vol) for the separation of (*R*)-5-phospho-[2-^{14}C]mevalonate (R_f 0.2–0.25) and the residual (*S*)-[^{14}C]mevalonate (R_f 0.68). The 5-phospho[^{14}C]mevalonate was eluted from the paper with 10 mM ammonium hydroxide by a centrifugal elution method described previously (15). The 5-phospho[^{14}C]mevalonate can be made by these procedures on scales varying from 10 to 100 μmol in yields better than 80%, but the preparations may be contaminated with small amounts of ADP. This contamination was of no consequence to us as the 5-phospho[^{14}C]mevalonate was used as an internal standard for measuring recoveries of 5-[^{32}P]-phosphomevalonate.

(*R*)-[5-^{14}C]Mevalonate and mevalonolactone

These preparations were used to study transfer of mevalonate or of lactone from plasma to cells. The preparation of pure (*R*)-[5-^{14}C]mevalonate from the racemic mixture through the enzymically generated (*R*)-5-phospho[^{14}C]mevalonate has been previously described (14). An amount of (*R*)-[5-^{14}C]mevalonolactone needed for the experiment was made in the incubation flask from 20 μl of a solution of potassium (*R*)-[5-^{14}C]mevalonate by acidifying it with 20 μl of 0.1 N HCl and warming the solution to 50°C, followed by drying in vacuo at 50°C for several hours. The residual lactone was dissolved in 10 ml of heparinized blood and was incubated as described below.

[γ-^{32}P]Adenosine triphosphate ([γ-^{32}P]ATP)

This labeled coenzyme, with a sp act >1,000 Ci/mol, was obtained from New England Nuclear, Boston, MA, and its specific activity was adjusted to about 300 Ci/mol by the addition of unlabeled ATP. Although the [γ-^{32}P]ATP preparations contained varying amounts of inorganic ^{32}P and occasionally [^{32}P]ADP, we found it unnecessary to purify the ATP from these contaminants, as they were removed from 5-[^{32}P]phosphomevalonate by ion-exchange chromatography.

Unlabeled mevalonolactone

(*RS*)-Mevalonolactone, obtained from Sigma Chemical Co., St. Louis, MO, was purified by crystallization from diethyl ether and was kept in a vacuum desiccator over Drierite. Solutions of lactone in water were made

alkaline (pH 10) with a small excess of 1 N KOH and were heated at 50°C for 30 min for the hydrolysis of the lactone; the pH of the solutions was then adjusted to about 7.5 with dilute HCl. The solutions were standardized with mevalonate kinase (12, 14). For reference standard a 1 μM solution was prepared. For human use, and intravenous injection, a 30 mM solution of potassium (*RS*)-mevalonate was made in sterile pyrogen-free 0.9% NaCl and was further sterilized by two passages through Amicon bacterial filters and collected in sterile vials. The solutions were not pyrogenic, as tested in rabbits, and were free of bacterial or fungal contamination as tested in the UCLA clinical laboratory.

METHODS

Uptake of mevalonate and mevalonolactone by blood cells

For these experiments 1.38 μCi of the pure potassium (*R*)-[5-^{14}C]mevalonate in 10 μl was added to 5 ml of freshly drawn heparinized[4] human blood (2.76 μCi in 20 μl to 10 ml of blood) in a 25-ml stoppered flask. For the experiment with the lactone, 10 ml of heparinized blood was added to a flask containing the lactone made from 20 μl of the (*R*)-[5-^{14}C]mevalonate. The samples, vigorously shaken, were incubated at 33.5°C in an atmosphere of 95% O_2 and 5% CO_2. At various intervals 0.7-ml samples were withdrawn and centrifuged in hematocrit tubes at 1000 *g* for 10 min. Then 0.1 ml of plasma was added to 0.9 ml of ethanol, the precipitate was centrifuged off, and 0.5 ml of the supernatant was counted. Standards were made by diluting 10 μl of the mevalonate solution with 5 ml of water and by adding 0.1 ml of this diluted solution to 0.9 ml of ethanol; a 0.5-ml volume of the ethanolic solution was then counted. From the counts of the standards and the hematocrit values of the blood we could calculate the concentration of mevalonate in the plasma at the moment of mixing with the blood (zero time values) before the cells had taken up any mevalonate.

Blood samples

Blood (most conveniently, 2 ml) drawn from vein or artery was added to tubes containing dry heparin and packed in ice. The chilled heparinized blood was then transferred to disposable hematocrit tubes (1 ml each) and centrifuged at 500 *g* and 4°C for 15 min. The plasma was removed with a Pasteur pipette to an Amicon CF-50 cone and was centrifuged in the Amicon cone-assembly at 1000 *g* and 4°C for 30 to 60 min. One ml of plasma usually gives 0.75 ml of nearly protein-free ultrafiltrate[5] which is sufficient for several replicate analyses. [^{14}C]- or [^{3}H]mevalonate or mevalonolactone added to plasma is recovered quantitatively in such ultrafiltrates, which can be kept frozen at −20°C for several weeks without change in mevalonate content. The pH of such ultrafiltrates of plasma is usually between 8.2 and 8.5 and hence mevalonolactone, if it existed in blood, would be changed to the anion during the preparation.[6]

A 400-ml batch of human plasma was obtained from the heparinized blood of four of us and its ultrafiltrate was made by passing it through an Amicon PM-30 membrane. The ultrafiltrate (300 ml) was acidified to pH 1.7 with HCl, then 70 g of Na_2SO_4 were dissolved in it at 50°C; the addition of the salt raised the pH to 3.0. The whole solution was transferred to a continuous extractor and was extracted with diethyl ether for 3 days. The ether extract was evaporated, leaving a small oily residue which was taken up in 3 ml of MeOH and 1 ml of water. The pH of this solution was brought to 10 with 0.5 ml of 1 N KOH whereupon a small precipitate formed, which was removed by a single extraction with 5 ml of diethyl ether. The remaining solution was then concentrated to about 0.2 ml to remove organic solvents and was made up to 700 μl with water and a few μl of 6 N HCl to bring its pH to 7.6. Samples of this concentrate of plasma ultrafiltrate were then used to verify the presence of mevalonate in human plasma (cf. Results).

Animals

Male Sprague-Dawley rats (260–300 g) were kept under normal or reverse illumination cycles (normal cycle, lights on 7 AM to 11 PM; reverse cycle, lights off 10:30 AM to 10:30 PM). Some of the animals were fed 5% cholestyramine in their diet for 3 days, and others were given mevalonolactone by stomach tube. The latter animals were subjects of a previous study (16). A few animals were fasted for 24 hr.

3-Hydroxy-3-methylglutaryl-CoA reductase

This enzyme was assayed in liver microsomes of some of the animals as described in detail previously (16, 17).

Mevalonate tolerance test

A scalp-vein needle, fitted with a two-way stopcock and heparin lock, was inserted into the right cubital

[4] EDTA should not be used as an anticoagulant because it chelates Mg^{2+} ions essential for the action of mevalonate kinase.

[5] Examination of the filtrates by sodium dodecylsulfate–polyacrylamide gel electrophoresis revealed only traces of serum albumin.

[6] Mevalonate kinase does not react with the lactone.

vein before the administration of a test dose of mevalonate to one of us (G. P.), a 63-year-old normocholesterolemic man. For the oral test 200 μmol of (*RS*)-mevalonate was swallowed in 50 ml of water and washed down with 100 ml of water. For the intravenous test 30 μmol of (*RS*)-mevalonate in 1 ml of sterile pyrogen-free 0.9% NaCl was injected into the left cubital vein. The two tests were made 2 months apart. Two-ml samples of blood were drawn at the time intervals indicated in Figs. 7 and 8.

Determination of mevalonate with mevalonate kinase

For the determination of mevalonate in the range of 0–50 pmol, 50 μl of a reaction mixture of the following composition is added to 100 μl of plasma filtrate, and to 100 μl of mevalonate standards containing 0, 5, 25, or 50 pmol of (*R*)-mevalonate [0, 10, 50, or 100 pmol of (*RS*)-mevalonate, respectively]: 1 M potassium phosphate buffer, pH 7.5, 15 μl; 0.3 M $MgCl_2$, 5 μl; 1 mM [γ-^{32}P]ATP, 7.5 μl, 2 to 3 μCi; mevalonate kinase (10 units/ml), 3 μl; and water, 19.5 μl. We usually carry out 16 simultaneous determinations (4 standards and 12 samples); for such a set we prepare 17 × 50 = 850 μl of the reaction mixture which allows the delivery of 50 μl of identical composition to each sample tube. The assays are carried out in 10 × 75 mm disposable glass tubes.

Although the final concentration of [^{32}P]ATP in the assay mixture at 50 μM is not optimal for the mevalonate kinase reaction (12), we have found it adequate with a relatively large excess of the kinase and a long incubation time. It is unnecessary to determine the specific activity of the ATP, as this value is not needed in the calculations; the 2–3 μCi/7.5 nmol per assay is a convenient value and can be varied within fairly wide limits.[7] If the amounts of mevalonate to be determined are larger than 100 pmol, the samples are diluted. It is essential to include mevalonate standards with each set of determinations; the standards should cover the range of mevalonate values expected in the samples.

The 150-μl (final volume) reaction mixtures are left at room temperature (about 22°C) for 2.5 hr. After this, 25 μl of conc. HCl, *precisely* 20 μl of 5-phospho[^{14}C]-mevalonate (about 2 × 10^5 dpm) and 25 μl of a solution containing 0.5 M ATP, 0.1 M ADP, and 0.5 M KH_2PO_4, in that order, are stirred into each reaction mixture. Absolute ethanol (0.9 ml) is then added[8] and the mixtures are chilled in ice for at least 30 min; the precipitate that is formed is then centrifuged down at 500 *g* for 10 min at 0°C. The supernatant is taken off with a Pasteur pipette and is added to 5 ml of 0.1 M triethylammonium carbonate buffer, pH 9.7, at room temperature. It is immaterial whether precisely the same volume of supernatant is drawn off from each sample tube, as the method is essentially one of isotope dilution. The diluted samples are applied to 0.7 × 7 cm AG 1-X 8 (carbonate) ion-exchange columns (Bio-Rad Laboratories, Richmond, CA), equilibrated with 0.1 M triethylammonium carbonate buffer, pH 9.7, and are allowed to drain in by gravity. The samples are washed onto the columns twice with 2 ml of the 0.1 M carbonate buffer. After the second wash has drained in, the outflow stopcocks are turned off and the columns are topped up with the starting buffer and are connected to the linear gradient.

Ion-exchange chromatography of 5-phosphomevalonate

Bio-Rad AG 1-X 8 resin, 200–400 mesh, washed repeatedly with methanol and water and cycled with 1 N HCl and 1 N NaOH in the usual way, was treated with 2 N Na_2CO_3 over several days and then washed with water. It was then equilibrated in a large column for several days with 20 mM triethylammonium carbonate buffer, pH 9.7. After equilibration the resin was removed from the column and kept as an approximately 50% (w/v) slurry in 20 mM triethylammonium carbonate buffer.

For each set of determinations 1 ml of 0.1 M buffer is added to each of 16 0.7 cm × 10 cm "Bio-Rad" glass "Econo-Columns" fitted with Luer-type three-way nylon stopcocks (Bio-Rad) at their outflow. Then 4 ml of the slurry of the AG 1-X 8 (carbonate) are added to each. After the resin has settled, the height of the packings should be about 7 cm and they should not differ from column to column by more than 0.5 cm. The columns can be used immediately without further equilibration.

Usually 16 columns are developed from one gradient connected to the columns through a manifold made up of ⅛ inch o.d. "Nalgene-type" Y-shaped connectors (Beckman Instruments, Inc., Fullerton, CA). The length of the connections from the gradient to each column is identical, as is the length of tubing from the tip of the columns to the needles delivering the eluates to the fraction collector.

The columns are fed by gravity (initial hydrostatic pressure 90 cm) from the linear gradient made up

[7] As a precaution against exposure to the 1.5 meV β-rays of ^{32}P (harzard mostly to the eyes), we handle ^{32}P samples behind a 1-cm thick Lucite (Perspex) shield.

[8] It is essential to inactivate the enzyme with HCl before the addition of the 5-phospho[2-^{14}C]mevalonate, as aged preparations of the latter may contain traces of mevalonate, the phosphorylation of which can produce erratic results.

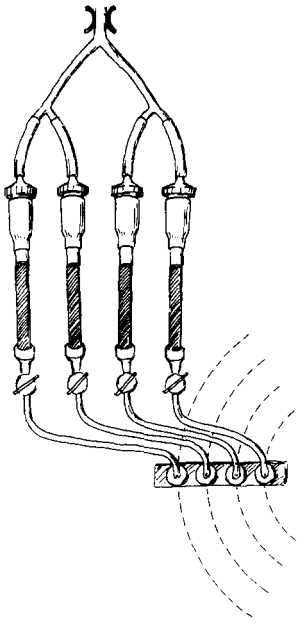

Fig. 1. Schematic representation of one group of four columns fed through a manifold from a common linear gradient and placed over a circular fraction collector. Fifteen fractions can be taken from each column in one quadrant of the collector. Three other groups of four columns are placed over three other quadrants of the collector.

from 800 ml each of 0.1 M and 0.7 M triethylammonium carbonate buffers, pH 9.7, contained in 1-liter graduated measuring cylinders. The initial flow rate through each column was 25–30 ml/hr. The mixing "chamber" (cylinder) is connected to a four-way manifold, the branches of which lead to four other four-way manifolds. The branches of each feed a bank of four ion-exchange columns (total of 16). The tube from the gradient to the first four-way manifold, and each of the four tubes leading to each "secondary" four-way manifold, needs to have a clamp on it. The eluate from each of the four banks of columns is collected in 15 tubes placed in the quadrant of a circular fraction collector which has 60 places in each of four concentric circles (American Optical Corp., Richmond, CA, Model 12063). The effluents from each bank of four columns are connected to 22-gauge injection needles put through holes in four horizontal metal bars fixed at 90° to one another over the radii of the fraction collector's turntable. This arrangement is illustrated in **Fig. 1.**

The filling of the manifold and the tubing leading to the columns with buffer needs special care to avoid air locks which may stop the flow to some of the columns. The following steps avoid this problem. First, the buffer solutions used in the gradient must be at room temperature. Second, after the columns have been loaded with the samples and topped up with the 0.1 M buffer, the stopcocks at the outflow are turned off and the plastic tops of the columns, smeared with vacuum grease, are firmly inserted into their sockets so that there shall be no air-leaks in the system. The branches of the manifold are not yet connected to the inlet of the columns. Every outlet of the manifold but one is plugged and, while the outlets are held upright, the clamp on the main lead from the reservoir of the 0.1 M buffer to the first four-way manifold, and also the clamp on the lead to the "secondary" four-way manifold on which one outlet was left unplugged, are opened so that buffer can flow through the tubings. When the air bubbles have been cleared, the clamp on this secondary lead is tightened and the other three outlets of this secondary four-way manifold are unplugged and all four outlets are connected to the inlets of one bank of four columns. The steps are then repeated, one by one, with each of the other three secondary four-way manifolds. **Fig. 2** illustrates the filling of one of the four-way manifolds free of air locks. When all the 16 columns have been connected, the cylinder holding the 0.1 M buffer is topped up to 800 ml, the syphon between the cylinders holding the 0.1 M and 0.7 M buffer is established, and the clamps on all four secondary four-way manifolds are released and then the stopcocks at the outlet of the columns are opened.

The first 45 ml of eluate from each column are collected in three tubes; thereafter, when the level of the buffer in the two cylinders has dropped to about 440 ml, fractions of 2.5 ml are collected. The 5-phosphomevalonate is usually eluted between fractions 5 and 9. By then the level of the buffers in the two cylinders has dropped to about 300 ml and the elution

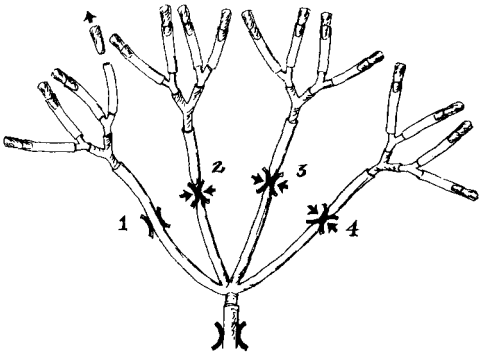

Fig. 2. Schematic representation of the method for filling a 16-way manifold with buffer to eliminate air locks. All but one of the outlets (to be connected to the column inlets) are plugged and the clamp to the main lead to the first four-way manifold and the clamp to the one secondary four-way manifold on which one of the outlets is unplugged are released to allow free flow of buffer through the tubings and to clear air locks. See also text.

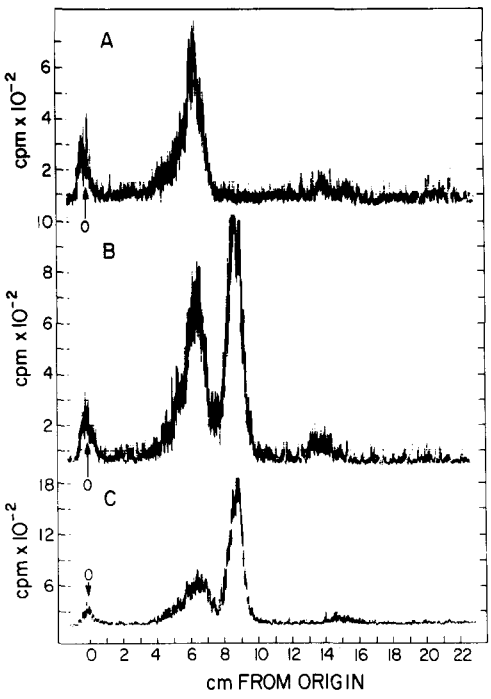

Fig. 3. Photograph of radiochromatograms of extracts of incubations of mevalonate kinase with [γ-^{32}P]ATP without and with a source of mevalonate. *A*, one of three identical records of control incubations in the absence of added mevalonate. *B*, record of incubation identical with that from which chromatogram *A* was obtained except that it contained (in 100 μl of final volume) 40 μl of the concentrate of an extract of human plasma. *C*, the same as *B* but after 5-phospho[2-^{14}C]mevalonate (~30,000 dpm of ^{14}C) was added to the sample. The papers were developed with propan-1-ol–conc. ammonia–water 6:3:1 (by volume); the solvent fronts were at 35 cm. For further details see text. Note that the scale of the ordinate for *C* is different from that for *A* and *B*.

of the columns may be stopped.[9] Two-ml samples are taken from the fractions with a "Selectopipette" (Clay Adams, Division of Becton, Dickinson and Co., Parsippany, NJ), fitted with tip extension and disposable tips, and are added to 10 ml of scintillator and are counted briefly (say, for 10 sec) to identify the two or three fractions giving the highest ^{14}C counts. These are then counted for ^{32}P and ^{14}C for longer intervals.

Radioactivity measurements

Radioactivity of samples, dissolved in 10 ml of RPI-3A70B scintillator (Research Products International Corp., Elk Grove Village, IL), was determined in a Packard Tri-Carb Scintillation Spectrometer. The gain and window of two channels for ^{32}P counting (efficiency about 72%) was so adjusted as to exclude ^{14}C; the third channel, set for ^{14}C counting (efficiency for ^{14}C about 52%), contained 12–14% of the counts of the ^{32}P channels. Corrections were made for such cross-contamination of the ^{14}C counts. Blanks, standards of [^{32}P]ATP, and of 5-phospho-[^{14}C]mevalonate and their mixtures were made by the addition also of 2 ml of 0.4 M triethylammonium carbonate buffer to the RPI scintillator. All specimens were counted repeatedly immediately after their preparation for 2 min (usually three times without interruption), and the mean of the repeat counts was calculated. There was no need to correct for decay of ^{32}P. Paper chromatograms were scanned for radioactivity with a Packard Radiochromatogram Scanner, Model 7201.

RESULTS AND DISCUSSION

Enzymic determination of mevalonate

The principle of the method is the phosphorylation of (*R*)-mevalonate with ATP to 5-phosphomevalonate by mevalonate kinase and the use of [γ-^{32}P]ATP in the reaction:

$$\text{mevalonate} + [\gamma\text{-}^{32}\text{P}]\text{ATP} \xrightarrow[Mg^{2+}]{\text{kinase}} 5\text{-}[^{32}\text{P}]\text{phosphomevalonate} + \text{ADP}.$$

In theory, if one knew the specific activity of the ATP and there were a good method for the isolation of the 5-[^{32}P]phosphomevalonate from residual [^{32}P]ATP and from possible ^{32}P-labeled contaminants, pmol or even smaller amounts of mevalonate could be determined.

Determination of the specific activity of [^{32}P]ATP is not easy, particularly as the various batches of [γ-^{32}P]-ATP may contain various amounts of inorganic $^{32}P_i$ and, furthermore, radiolysis of the substance, initially of very high specific activity, can produce compounds of unknown nature during the few weeks of the use of a batch. In one instance we found a batch of [γ-^{32}P]ATP in which only 39% of the ^{32}P was in ATP, the remainder probably being in inorganic phosphate. Also, we could not at first find any chromatographic system either on paper or on several thin-layer media that would adequately separate 5-[^{32}P]phosphomevalonate from ^{32}P-labeled contaminants.

After the many trials and failures, it occurred to us that since 5-phosphomevalonate was soluble in 60–70% ethanol (12), but ATP, ADP, and inorganic phosphate were mostly precipitated, adequate separation might be achieved by adding relatively large amounts of un-

[9] Since residual [^{32}P]ATP is not eluted from the columns with this particular gradient, the column packings are removed after each use and stored for several half-lives of the ^{32}P and then regenerated in the usual way and used again. The glass "Econo-Columns" and their fittings, after being soaked in detergent and rinsed with water, can be reused immediately.

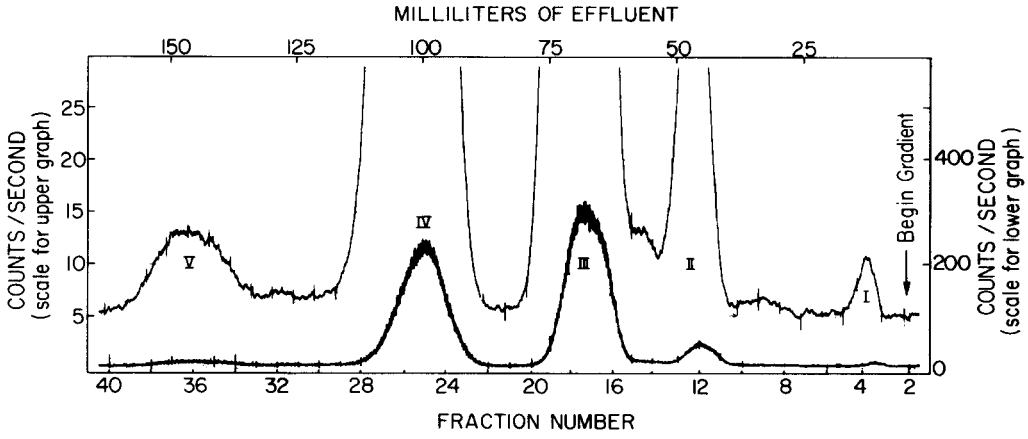

Fig. 4. Chromatography of the extract of the same incubation as recorded in Fig. 3*B* with added 5-phospho[2-^{14}C]mevalonate (120,000 dpm) on a 0.7 × 8 cm column of Dowex AG 1-X 8 (carbonate) with a linear gradient of 0.1–1.0 M triethylammonium carbonate buffer, pH 9.5; the total volume of the gradient was 200 ml. The effluent was monitored continuously for radioactivity with a homemade instrument similar to that described previously (18). The recording was made simultaneously at two levels of sensitivity. Except for 600 cpm of ^{14}C (and 5600 cpm of ^{32}P) in fraction I, all the ^{14}C was found in fraction IV, 87,372 cpm, together with 100,990 cpm of ^{32}P as counted on a portion of the pooled fraction IV. Fractions II, III, and V contained only ^{32}P; the chemical identity of these fractions was not established. Residual [^{32}P]ATP was not eluted by this gradient.

labeled ATP, ADP, and phosphate to the reaction mixtures at the end of the incubations, and then adding ethanol. One of many such experiments is presented in **Figs. 3** and **4.** Five 0.1-ml incubations were set up with the batch of [^{32}P]ATP that contained only 39% of the ^{32}P in the ATP as mentioned above. One incubation (number I) contained 20 μl and another, number II, contained 40 μl (out of the total of 700 μl) of the concentrate of human plasma extract (made from 300 ml of plasma ultrafiltrate; cf. Methods); three controls, numbers III–V, contained no source of mevalonate. These incubations contained 10 μmol of potassium phosphate buffer, pH 7.5; 1 μmol of $MgCl_2$; 10.9 nmol of [γ-^{32}P]ATP (3.9 μCi ^{32}P in ATP and 6.1 μCi in P_i) and 50 mU of mevalonate kinase in a final volume of 100 μl. After a 2-hr incubation at 37°C, the mixtures were acidified with 25 μl of conc. HCl, and then 0.1 ml of a solution containing 0.5 M ATP, 0.1 M ADP, and 0.5 M KH_2PO_4, and 0.9 ml of absolute ethanol were added. After 1 hr on ice, the precipitate was centrifuged down at 500 *g* for 10 min, and 0.9 ml of the supernatant was taken off, to which we again added 0.1 ml of the ATP, ADP, and KH_2PO_4 solution and 0.5 ml of absolute ethanol. After another hour in ice the precipitate was centrifuged off and 1.2 ml of the supernatant were removed. Twenty-five μl from each sample of the second supernatants were counted and also put on strips of Whatman 3 MM paper which were developed with propan-1-ol–ammonia–water 6:3:1 (by volume) for a 35-cm length. The control blanks gave a mean of 8,108 ± 144 cpm/25 μl; the two experimental samples gave 12,523 and 15,379 cpm, respectively; or, after correction for the control blanks, 4,415 and 7,271 cpm/25 μl. The latter values were nearly proportional to the volumes of plasma extracts used in the two incubations, but could not be regarded accurate enough, nor taken as proof for the existence of mevalonate in plasma. The cause of the uncertainty in this type of analysis became apparent from the chromatographic examination of the ethanolic supernatants of the incubations (Fig. 3). Fig. 3*A* shows at least four ^{32}P-containing fractions in one of the three identical control samples. Fig. 3*B* is the chromatogram of the ethanolic supernatant of incubation number II and shows the presence of a ^{32}P-labeled compound not found in the control incubation at an R_f value of 0.25 which is that expected for 5-phosphomevalonate. Indeed, 5-phospho[2-^{14}C]-mevalonate co-chromatographed precisely with the suspected 5-[^{32}P]phosphomevalonate (Fig. 3*C*).

To prove further the synthesis of 5-[^{32}P]phosphomevalonate in the extract of human plasma, we took 400 μl of the ethanolic supernatant of incubation number II and added to it 120,000 dpm (88,000 cpm) of 5-phospho[2-^{14}C]mevalonate in 20 μl. Then we diluted the mixture with water to 10 ml and applied the whole to a 0.7 × 8 cm Bio-Rad Ag 1-X 8 (carbonate) column which was developed with a linear gradient of 0.1 to 1.0 M triethylammonium carbonate buffer, pH 9.5, the gradient being made up of 100 ml each of the two buffers. The effluent was monitored continuously for radioactivity by a homemade instrument similar to that previously described (18). The photograph of the original record is shown in Fig. 4. Five distinct radioactive fractions—three major ones—were seen. The fractions under each peak were pooled

and portions of the pools were counted for ^{32}P and ^{14}C. Peak I contained 600 cpm of ^{14}C and 5,600 cpm of ^{32}P; peaks II, III, and V contained only ^{32}P; in peak IV we found 87,372 cpm of ^{14}C and 100,990 cpm of ^{32}P. Within the errors of simultaneous counting of two isotopes and allowances for sampling factors (400 μl were counted out of 27.1 ml of the pooled peak IV), peak IV contained all the 5-phospho[2-^{14}C]-mevalonate added to the ethanolic extract of the incubation and the 100,990 cpm of ^{32}P. The symmetry of peak IV suggests that it was radiochemically homogeneous and that it represented 5-phosphomevalonate. Making an allowance for the decay of ^{32}P between the times of the experiment and counting and for the various sampling factors, and taking into account the specific activity of the [^{32}P]ATP used, we calculated that the mevalonate content of the pooled human plasma was 39 pmol/ml. This was a much lower value than that reported by Hagenfeldt and Hellström (11) for rat blood (150 and 300 pmol/ml) and we thought at first that the extraction of mevalonolactone and mevalonic acid from the acidified ultrafiltrate of the pooled human plasma might have been inefficient. Further experiments established, however, that the plasma of men and women contained much less mevalonate than the lowest values found for the rat (see below).

To check further on the components eluted from the ion-exchange column, the pools of peaks II, III, and IV (cf. Fig. 4) were lyophilized. The residues were dissolved in 1 ml of water and 100 μl of each sample were put on strips of Whatman 3 MM paper which were developed with the propan-1-ol–conc. ammonia–water solvent to a 40-cm length. Peak IV containing ^{32}P and ^{14}C gave a single radioactive spot with an R_f value of 0.248, characteristic for 5-phosphomevalonate. Peak II also gave a single radioactive spot at an R_f value of 0.41; but peak III was resolved into two components, one at R_f 0.0 (20%) and another at R_f 0.18 (80%). These ^{32}P-containing fractions correspond to the contaminants of the 5-[^{32}P]phospho-[^{14}C]mevalonate seen on the chromatograms in Figs. 3*B* and 3*C*, but their identities were not established. We present these experiments because they convinced us of the genuine presence of mevalonate in human plasma and because they led us to the final method and the use of ion-exchange chromatography for the separation of the 5-[^{32}P]phosphomevalonate from ^{32}P-containing contaminants.

If peak IV shown on Fig. 4 was truly a homogeneous mixture of 5-[^{32}P]phosphomevalonate and of 5-phospho[^{14}C]mevalonate, then the $^{32}P/^{14}C$ ratio in every fraction of the peak should be the same. To test this point two 1-ml reaction mixtures were set up: one contained 0.2 nmol of (*R*)-mevalonate and

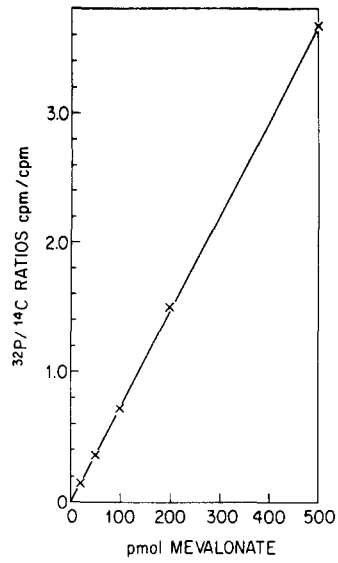

Fig. 5. Proportionality between $^{32}P/^{14}C$ ratios and amount of mevalonate in assays.

the other 846 μl of plasma filtrate from one of us (G. B.). The final concentrations of the reagents were: 0.1 M potassium phosphate buffer, pH 7.5; 10 mM $MgCl_2$; 0.1 mM [γ-^{32}P]ATP (3 μCi), and 50 mU mevalonate kinase. After a 1-hr incubation 60,000 cpm of 5-phospho[^{14}C]mevalonate were added to each of the samples which were then diluted to 10 ml with water. These diluted samples were applied to two 0.7 × 9 cm AG 1-X8 (carbonate) ion-exchange columns, which were then developed with the same linear gradient of triethylammonium carbonate buffer as used in the experiment of Fig. 4. Fractions corresponding to peak IV of Fig. 4 were counted for ^{32}P and ^{14}C, and were found to have the same $^{32}P/^{14}C$ ratios throughout the peak within the experimental error of counting radioactivity. Thus in the sample containing the 0.2 nmol of (*R*)-mevalonate the mean ratio in the three fractions containing the highest ^{14}C counts was 0.174 ± 0.004 and in the sample of human plasma filtrate the mean ratio was 0.031 ± 0.001. On the assumption that the $^{32}P/^{14}C$ ratios in the isolated 5-phosphomevalonate fractions were linearly proportional to the mevalonate content of the original samples (proved subsequently correct; cf. next section), we calculated that the 846 μl of human plasma contained (200 × 0.031)/0.174 = 35.63 pmol of mevalonate, or 42.12 pmol/ml, a value very close to the 39 pmol/ml measured on the extract of the large pooled sample of human plasma.

Proportionality of $^{32}P/^{14}C$ ratios in the isolated 5-phosphomevalonate to amount of mevalonate content of samples

To test whether the $^{32}P/^{14}C$ ratios in the 5-phosphomevalonate samples isolated by ion-exchange chroma-

TABLE 1. Proportionality of $^{32}P/^{14}C$ ratios in the standard assays of mevalonate by mevalonate kinase and [γ-^{32}P]ATP

Sample	Corrected $^{32}P/^{14}C$ Ratio ± SD[a]	Mevalonate Measured
		pmol
Mevalonate:		
20 pmol	0.1455 ± 0.0020	19.84
20 pmol	0.1448 ± 0.0020	19.74
50 pmol	0.3664 ± 0.0029	49.95
50 pmol	0.3700 ± 0.0031	50.44
100 pmol	0.7315 ± 0.0035	99.73
100 pmol	0.7221 ± 0.0015	98.45
200 pmol	1.5048 ± 0.0026	205.15
200 pmol	1.5171 ± 0.0131	206.83
500 pmol[b]	3.6676 ± 0.0077	500.01
Plasma filtrate:		
90 μl	0.0398 ± 0.0027	5.42
90 μl	0.0401 ± 0.0018	5.46
90 μl + 50 pmol mevalonate	0.4081 ± 0.0050	55.59
90 μl + 50 pmol mevalonate	0.4051 ± 0.0016	55.19

Sixteen incubations containing 0–500 pmol of (*R*)-mevalonate, 90 μl of human plasma filtrate, or 90 μl of plasma filtrate plus 50 pmol of (*R*)-mevalonate, were set up in duplicates in the standard 150-μl assays. The samples were worked up, with the addition to each acidified mixture of 120,000 dpm of 5-phospho[2-^{14}C]mevalonate, as described under Methods, and were chromatographed on the 0.7 × 7.0 cm AG 1-X 8 (carbonate) ion-exchange columns; the peak of the ^{14}C activity was located in the effluents from each column, and three samples were counted for ^{32}P and ^{14}C, the peak and one on each side of the peak. The mean $^{32}P/^{14}C$ ratios were calculated from the three determinations.

[a] Calculated from the observed $^{32}P/^{14}C$ ratios minus the $^{32}P/^{14}C$ ratio of blank controls, the mean of which from the duplicates (six determinations) was 0.0071 ± 0.0010, corresponding to 0.97 pmol of mevalonate.

[b] The duplicate sample was lost during the processing.

tography were linearly proportional to the mevalonate content of the samples, sixteen duplicate incubations were set up containing 0, 20, 50, 100, 200, and 500 pmol of (*R*)-mevalonate, 90 μl of human plasma filtrate, and 90 μl of plasma filtrate plus 50 pmol of (*R*)-mevalonate in the standard 150-μl incubations described under Methods. The results are shown in **Fig. 5.**

The true accuracy of our method cannot be fully appreciated from Fig. 5 in which the $^{32}P/^{14}C$ ratios are plotted against the mevalonate content of the samples on a relatively low scale. In **Table 1** we present the mean $^{32}P/^{14}C$ ratios obtained on each sample with the SD of measurements taken on three fractions eluted from the ion-exchange columns. From the many determinations we have done, we feel confident that it is justifiable to calculate the ratios to four decimal places.

There is always a small blank value to be taken into account. We have always found a small ^{32}P-containing "peak" eluted with the 5-phospho[^{14}C]-mevalonate fractions of control samples set up with distilled water alone. We cannot attribute these blank values to the "trailing" of some ^{32}P-labeled contaminant into the 5-phosphomevalonate fraction as, invariably, we found the highest ^{32}P values in the blank controls associated also with the highest ^{14}C counts. Thus in the experiment recorded in Fig. 5 and Table 1 the ^{32}P counts in the three "peak" fractions counted from the two blank controls were 138, 168, and 98 cpm, and 105, 185, and 101 cpm, respectively, the highest ^{32}P counts coinciding with the highest ^{14}C counts. For this reason we corrected the counts on the experimental samples by subtracting the mean $^{32}P/^{14}C$ ratio of the controls rather than by subtracting just the blank ^{32}P counts from the similar counts of the samples. The data of Fig. 5 and Table 1 were presented after such corrections. The blank values usually correspond to about 1 pmol of mevalonate; occasionally we had values as high as 2–3 pmol.[10]

The slope of the line in Fig. 5 gives the numerical values of the $^{32}P/^{14}C$ ratio per pmol of mevalonate; in practice, we do not construct such reference graphs, but instead calculate the slope of the regression line by the method of least squares with the aid of a simple computer program.

Mevalonate in human plasma

The last entries in Table 1 show that the ultrafiltrate of human plasma does not contain a substance inhibitory to mevalonate kinase as mevalonate added to the plasma could be measured quantitatively. The mevalonate content of this plasma (60.2 ± 1.85 pmol/ml) was the highest measured for this individual (G.P.) in the course of 1 year; other values for this man varied between 30 and 40 pmol/ml. Up to now we have measured the plasma mevalonate content of 26 fasted individuals (not counting the large pooled plasma sample referred to earlier). The values found fell into three groups: *i*) eight had values between 20 and 30 pmol/ml with a mean of 23.4 ± 2.7; *ii*) 14 had values between 30 and 50 pmol/ml with a mean of 38.3 ± 5.9; and *iii*) four had values over 60 pmol/ml with a mean of 68.1 ± 7 pmol/ml. We do not know at present the physiological, or possible pathological, significance of these data.

Uptake of mevalonate and mevalonolactone by blood cells

As a preliminary to the study of administration of mevalonate on plasma levels of this substance, we examined whether there might be a rapid equilibra-

[10] It is imperative that all solutions are made with freshly distilled water. Stale distilled water, taken from containers contaminated with algae or mold, may contain some mevalonate.

tion of mevalonate between plasma and the blood cells. To this end we carried out the in vitro experiments with heparinized human blood described under Methods. The results obtained are shown in **Fig. 6.** Initially there was a moderately rapid uptake of mevalonate by the cells, but by 2 hr an equilibrium was reached with 70% of the mevalonate remaining in the plasma. In contrast to the behavior of isolated hepatocytes, which show a much greater avidity for the lactone than for the anion (16, 19), there was no difference between the uptake of the mevalonate anion and lactone by the cells. On the assumption that the red cells were mostly responsible for the disappearance of mevalonate from the plasma, we calculated that the maximum capacity of the red cells for mevalonate was about 1.3 pmol per 10^6 cells. The amount of mevalonate that disappeared in 3 hr from the plasma (about 1.84×10^5 dpm/ml whole blood) cannot be attributed to the utilization of this substance by the leukocytes for sterol synthesis (15), since we have found in one experiment only 332 dpm in the total unsaponifiable lipids from 1 ml of whole blood. It seems that equilibration of mevalonate between plasma and blood cells is determined by a relatively slow diffusion process, rather than by an active transport.

Mevalonate "tolerance test" in man

Figs. 7 and 8 show changes in plasma mevalonate levels in one of us (G.P.) after an oral dose of 200 μmol and after an intravenous dose of 30 μmol of (*RS*)-mevalonate (cf. Methods). It needs to be borne in mind that our method measures only the natural (*R*)-enantiomer of mevalonate. Two sets of data are shown in **Fig. 7:** one, marked with crosses, was obtained the day after the test, and the second, marked with circles, was obtained about one month later with a different batch of [^{32}P]ATP on samples stored at −20°C. Although the second set of determinations gave somewhat lower values, no value differed by more than 5% from the earlier one. We have also had similar experiences

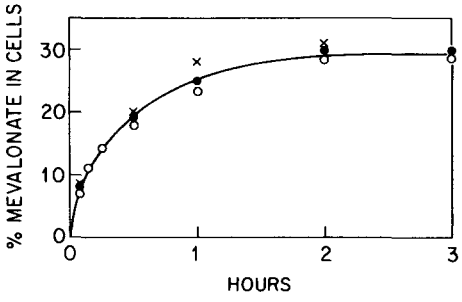

Fig. 6. Uptake of (*R*)-[^{14}C]mevalonate by cells in blood incubated in vitro. Symbols: × and ○, experiments with potassium (*R*)-[2-^{14}C]mevalonate; ●, experiment with (*R*)-[2-^{14}C]mevalonolactone.

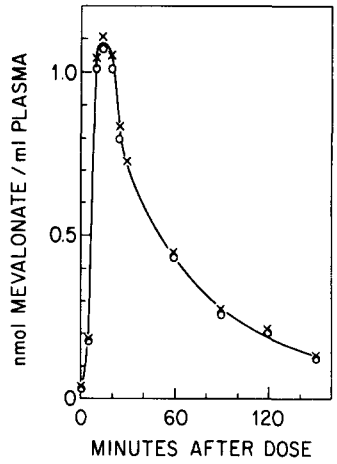

Fig. 7. Blood plasma mevalonate levels in man after oral dose of 100 μmol of (*R*)-mevalonate. Symbols: ×, values determined one day after experiment; ○, values determined on samples stored at −20°C for about one month.

with other samples of plasma filtrate: they can be kept at −20°C safely for several weeks without significant change of their mevalonate content as long as they remain free of fungal or bacterial infection.

The oral test, which was made 5 hr after the subject had a light breakfast, provided two surprises. The first one was that the peak level of mevalonate, 15 min after the administration, was only about one-fortieth of that which might have been expected from a rapid absorption of mevalonate. The much lower peak value could be the result of incomplete absorption or, more likely, of a rapid utilization of mevalonate during the early absorptive period, particularly if the main route of absorption led to the portal vein. The second surprise was the relatively slow rate of disappearance of mevalonate from plasma, after the peak, with an apparent initial half-life of about 27 min. After an intravenous dose of 30 μmol of (*RS*)-mevalonate (15 μmol of

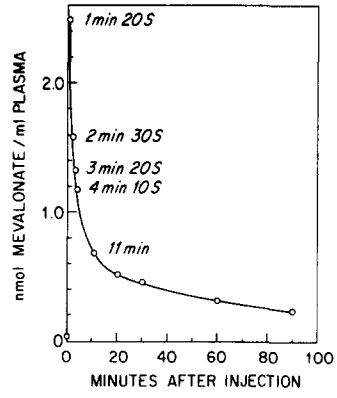

Fig. 8. Blood plasma mevalonate levels in man after an intravenous dose of 15 μmol of (*R*)-mevalonate. The taking of the samples was timed with a stopwatch.

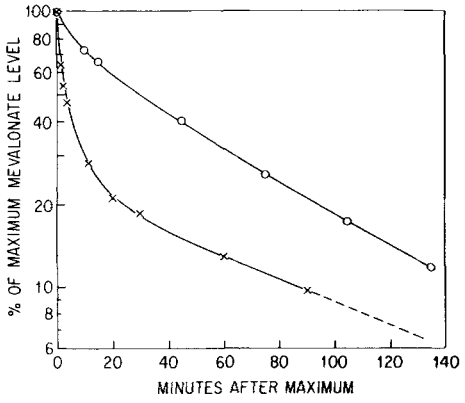

Fig. 9. Semilogarithmic plots of the data of Figs. 7 and 8. Upper curve from data of Fig. 7; lower curve from data of Fig. 8.

R-isomer), the maximum (2.4 nmol/ml) was found 1 min and 20 sec after injection, followed by a very rapid decline with a half-life about 2.5 min and a second, much slower, component with a half-life of about 60 min (**Fig. 8**). The plasma mevalonate level did not return to the initial concentration of 40 pmol/ml 2.5 hr after the oral dose, nor 1.5 hr after the intravenous dose. The semilogarithmic plots of the two tests are shown in **Fig. 9** and suggest that two (or more) organs may be competing for mevalonate. This competition appears to depend on the route of entry of mevalonate into the blood. The liver and kidneys (and probably the intestines) may be the main consumers of mevalonate.

Hellström et al. (20), during their studies on the metabolism of mevalonate in the rat with the aid of [1-^{14}C]- and [5-^{3}H]mevalonate, also found a biphasic "decay" of mevalonate in arterial blood after intravenous administration of the labeled substrates with half-lives of 5.1 to 5.2 min, and 24.6 and 28 min. These investigators were also the first to record the far greater utilization of intravenously administered mevalonate by the kidneys than by the liver, but that, after an oral dose, the liver took a greater share of the substrate than the kidneys.

Human consumption of mevalonate-containing drinks

Recalling that mevalonate was discovered in "distillers' solubles" (21–23)—the water-soluble residue left after the alcohol had been distilled from fermentation liquor—and that mevalonate added to incubations of hepatocytes, or given to animals, repressed the induction of HMG-CoA reductase in the cells and the postprandial rise of this enzyme in the liver of rats (16), it occurred to us that consumers of fermented drinks, beer and wine, might also be consumers of mevalonate.

We therefore applied our method to determine the mevalonate content of two types of beer and of a California chablis. The determinations were done on distillers' solubles made from 100 ml of the ultrafiltrate of each beverage. The values obtained were: for one batch of the American lager beer 7.81 ± 0.19 μM (four determinations), and for another brew of the same brand 8.31 ± 0.10 μM (five determinations), for a British light ale 3.23 ± 0.47 μM (three determinations), and for the California chablis 5.56 ± 0.36 μM (three determinations). Considering that an adult man synthesizes about 3 mmol of cholesterol per day requiring 18 mmol of mevalonate, the concentrations of mevalonate in beer and wine are too small to contribute significantly, even in a heavy drinker, to the body's pool of mevalonate.

Plasma mevalonate in rats

We have examined the mevalonate content of the blood plasma of rats kept under controlled conditions (**Table 2**). There appears to be a correlation between the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase levels in liver and the mevalonate content of the blood, in that animals in the middle or end of the

TABLE 2. Plasma levels of mevalonate in male rats under various conditions

State of Animal	Plasma Mevalonate	Liver Microsomal HMG-CoA Reductase
	pmol·ml^{-1}	*pmol·min*$^{-1}$*·mg*$^{-1}$
L-10	234	167
L-10	220	103
D-2	227	221
D-2	333	not determined
D-6	458	601
D-6	492	1,150
D-6; ChSt[a]	405	2,682
D-6; ChSt[a]	502	1,001
Starved; L-6	85	not determined
Starved; L-6	99	not determined
Starved; L-6	81	not determined
D-2; MVA[b]	379 × 10^{3}	23
D-2; MVA[b]	373 × 10^{3}	21
D-6; MVA[c]	6,500 × 10^{3}	322

Rats were housed either under normal light cycle (lights off at 7 PM to 7 AM) or under reverse cycle (lights off at 10:30 AM to 10:30 PM). In the column "State of Animal", L-10 means that the sample of blood was taken at the 10th hour of the light cycle; correspondingly, D-2 and D-6 indicate the 2nd and 6th hours of the dark cycle. All blood samples were drawn from the vena cava inferior under ether anesthesia.

[a] These rats were fed 5% cholestyramine in their diet for 3 days.

[b] These two animals (300 g) were given 12.3 μmol of (*RS*)-mevalonolactone per g body weight by stomach tube 2 hr before sampling. The samples of plasma filtrate had to be diluted 1:100 for the determination.

[c] Thirty minutes before sampling, this rat was given, by stomach tube, 12.3 μmol of (*RS*)-mevalonolactone per g body weight. The plasma filtrate from this animal had to be diluted 1:2000 for the determination.

light cycle have low plasma levels and those that are at the midpoint of the dark cycle, or have been treated with cholestyramine, have high levels. The lowest values were found in fasted animals. Although we did not determine the HMG-CoA reductase activity in the liver microsomes of the fasted animals, it is known that fasting profoundly suppresses the activity of this enzyme in the liver (24, 25). The data thus suggest that the liver is one of the main sources of blood mevalonate.

Administration of mevalonate, shown in our laboratory to result in rapid repression of HMG-CoA reductase (16), not unexpectedly caused very large rises in blood mevalonate. Even 2 hr after the administration of a dose of mevalonate its concentration in the blood plasma was about one thousand times greater than that observed under physiological conditions. The dose per g body wt given to the rats was, however, about 4000 times greater than that given to the man in the oral tolerance test (cf. Fig. 7). This may explain the persisting high level of plasma mevalonate in the rat.

Arterio-venous differences in mevalonate content

We have measured the mevalonate content of plasma from aortic and renal venous blood in six female rats starved for 24 hr. The rats were anesthetized with diethyl ether; 2 ml of blood were first drawn from the renal vein, which was clamped immediately afterwards, then 2 ml of arterial blood were taken from the aorta. The blood was drawn into syringes containing 50–100 μl of a heparin solution. The syringes were weighed dry, then with heparin and with blood. Thus, with the aid of the hematocrit values, we could calculate the degree of dilution of plasma by the heparin solution. We have found in every instance that the plasma from the renal venous blood contained less mevalonate than the plasma from aortic blood, which may be taken as representing the composition of the blood of the renal artery (**Table 3**). The mean ratio of the mevalonate content of venous to arterial plasma was 0.67 ± 0.19. Thus it appears that, even under physiological conditions, the kidneys are extracting mevalonate from the blood.

We have made similar comparisons between aortic blood and the blood from the portal vein, but in three experiments we found no difference between them in respect of their mevalonate content.

We presented our method for the determination of mevalonate and the data on blood mevalonate in the belief that the method should make it possible to examine some questions of cholesterol metabolism in animals and man, specifically whether there are conditions in which HMG-CoA reductase might be in a derepressed ("induced") state, or in which the "shunting" or mevalonate might be abnormal.

TABLE 3. Mevalonate content of plasma from aortic and renal venous blood in female rats starved for 24 hours

Experiment Number	State of Animal[a]	Mevalonate Content[b] of Plasma from: Aortic Blood A	Renal Venous Blood B	B/A
		pmol·ml^{-1}		
1	L-6	83	63	0.76
2	L-6	90	52	0.58
3	L-6	102	82	0.80
4	L-6	178	58	0.33
5	D-4	143	96	0.67
6	D-4	129	110	0.85

[a] L-6, animals at the midpoint of a 12-hr light cycle; D-4, animals at the 4th hr of the 12-hr dark cycle.

[b] The values are the means of two sets of determinations done 2 weeks apart and with two different batches of [γ-^{32}P]ATP. The values determined on the two occasions differed by less than 5%.

Addendum

Since this paper was submitted, we learned that blood plasma of man and other animals contains a very active lactone hydrolyase.[11] Hence, the lack of any difference between the uptake by the red cells of mevalonate anion and lactone may be attributed to the latter's rapid hydrolysis in the blood.

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