BIOORGANIC CHEMISTRY 4, 166–180 (1975)

Stereochemistry of the Reaction Catalyzed by Mevaldate Reductase[1]

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Received November 6, 1974

3*RS*-[5-D_1]Mevalonate was prepared by the reduction of *RS*-mevaldate with [4*R*-4-D_1]NADH and mevaldate reductase and was resolved enzymically into the 3*R*- and 3*S*-isomers. Spectropolarimetric measurements gave nearly mirror-image optical rotatory dispersion curves with a minimum and maximum at 240 nm and a negative and positive Cotton effect, λ_0 being at 227 nm, for the 3*R*- and 3*S*-lactone, respectively. Since the H-atoms at C-5 of mevalonolactone form a virtual ABX_2 system in nmr, the chemical shifts of the equatorial and axial H-atoms being at δ 4.33 and 4.58, respectively, it was possible to show by nmr that the two [5-D_1]-lactones were diastereoisomers, both having the 5*R* absolute configuration. This conclusion was confirmed by the finding that specimens of 3-methyl[5-D_1]pent-2-eno-5-lactone made by the dehydration of 3*S*-[5-D_1]mevalonolactone and of 3*RS*-[5-D_1]mevalonolactone had identical optical activities and of the same sign. The implications of the observations and a correlation between the stereochemistry of the reactions catalyzed by mevaldate reductase and 3-hydroxy-3-methylglutaryl-CoA reductase are discussed.

INTRODUCTION

Mevaldate reductase catalyzes the reduction of mevaldic (3-hydroxy-3-methyl-5-oxovaleric acid) to mevalonic acid with NADH (EC 1.1.1.32) or NADPH (EC 1.1.1.33) as the coenzyme depending on whether the enzyme is prepared from pig liver or rat liver (*1–3*). Although it was claimed at first (*1*) that the enzyme exhibited stereospecificity in respect of one undefined enantiomer of *RS*-mevaldate, it was subsequently shown that the product of the reduction of *RS*-mevaldate with the enzyme prepared from pig liver was the racemic *RS*-mevalonate (*4*). In spite of this disregard by the enzyme for a chiral center of the substrate, the enzyme had shown stereospecificity in respect of the coenzyme, transferring only the pro-*R* hydrogen atom from C-4 of the dihydronicotinamide ring of NADH or NADPH to the substrate, the enzyme thus falling into the category of "A"-side specific oxido-reductases (*4*). The insertion of the hydride ion from NADH into mevaldate was also stereospecific, at least into the 3*R*-enantiomer, as it was shown that the 3*R*-component of 3*RS*-[5-D_1]- or of 3*RS*[5-$^3H_1$]mevalonate

[1] Research supported by United States Public Health Service Research Grant No. HL-12745.

[2] Work carried out in partial fulfillment of the requirements for the degree of Doctor of Philosophy in biochemistry at the Department of Biological Chemistry, UCLA School of Medicine.

obtained by the reduction of *RS*-mevaldate with [4*R*-4-D_1]- or [4*R*-4-$^3H_1$]NADH and mevaldate reductase contained a chiral center also at C-5, the absolute configuration of which was assigned to be *R* (*4*). This conclusion was reached from the following experiments and assumptions: A specimen of 3*RS*-[5-$^3H_1$]mevalonate was made by the reduction of *RS*-mevaldate with [4*R*-4-^{3}H]NADH and pig liver mevaldate reductase, *RS*-[4-^{14}C]mevalonate being added afterwards to the specimen. The 3*RS*-[5-^{3}H,4-^{14}C]mevalonate was then converted with a soluble multienzyme system of pig liver into farnesyl pyrophosphate which was then hydrolyzed with intestinal alkaline phosphatase. The [1,5,9-$^3H_3$-2,6,10-^{14}C]farnesol was then used as substrate for horse liver alcohol dehydrogenase with NAD^+ as electron acceptor. In the resulting [^{3}H, ^{14}C]-farnesal the atomic ratio of ^{3}H:^{14}C was reduced to 2:3 as compared to a ratio of 3:3 in the farnesol. Since liver alcohol dehydrogenase abstracts the pro-*R* hydrogen atom from C-1 of ethanol (for review, see (*5*)), it was inferred that the absolute configuration of the [$^3H_3$, $^{14}C_3$]farnesol at C-1 was *R* (*4*). The [$^3H_3$, ^{14}C]farnesol must have been derived only from the 3*R*-component of the mevalonates, because mevalonate kinase, the first enzyme acting on mevalonate, has an absolute specificity for the 3*R*-enantiomer (*6, 7*). It was argued that the absolute configuration at C-1 of the [$^3H_3$, $^{14}C_3$]farnesol had to be the same as at C-5 of the 3*R*-[5-^{3}H]mevalonate from which it was synthesized, because in the phosphorylation of mevalonate at C-5 the C–O bond should not have been affected, in analogy with other enzymic phosphorylations with ATP (*4*). Another assumption in the arguments (*4*) was that hydrolysis of farnesyl pyrophosphate by alkaline phosphatase would probably not cause a change of configuration at C-1 of farnesol. These assumptions were sound, although to this day they have not been proved rigorously, but they have not been disproved either.

There have been many experiments made, since the report of Donninger and Popják (*4*), with such specimens of [5-D_1]- and [5-$^3H_1$]mevalonates in studies of polyisoprenoid biosynthesis (cf., e.g., *8*); the validity of the conclusions drawn from such experiments depended on the correctness of the assignment of the absolute configuration of the [5-D_1]- or [5-$^3H_1$]mevalonates at C-5.

Because of the circumstantial nature of the evidence cited (*4*), it seemed worthwhile to deduce the absolute configuration at C-5 of the 3*R*-component of the 3*RS*-[5-D_1]-mevalonate resulting from the reduction of *RS*-mevaldate with [D]-NADH by an independent and unambiguous method. Besides, the absolute configuration at the same position in the 3*S*-[5-D_1]mevalonate was also of interest because a knowledge of this should give information about the orientation of the binding of the substrate to mevaldate reductase (*5*).

The apparent disregard by mevaldate reductase of the chiral center in its substrate raised two possibilities. One was that mevaldate interacted with the enzyme only through its carboxyl and aldehydo groups, the substituents at C-3 not being involved in the binding. If this were the case, the absolute configuration at C-3 of 3*S*-[5-D_1]-mevalonate should be the same as in the 3*R*-isomer. On the other hand, in the somewhat remote event that the enzyme had a "right-" and "left-handed" binding site, or if two enzymes were involved, one specific for the *R*- and the other for the *S*-mevaldate, the labeling at C-5 of 3*S*-[5-D_1]mevalonate might be enantiotopic to that seen in the 3*R*-isomer. The general problem has been defined previously (*5*); the main points of our observations, presented here in detail, have been summarized briefly before (*9*).

EXPERIMENTAL APPROACH

An unambiguous solution to the problems outlined presented itself by von Mühll's analysis of the nuclear magnetic resonance (nmr) spectrum of mevalonolactone (*10*) in which the three pairs of methylenic protons are magnetically nonequivalent and, furthermore, the chemical shifts of the axial (H_a) and equatorial (H_e) hydrogen atoms at C-5 differ by as much as 0.25 ppm, the chemical shift of H_a being at δ 4.58, on account of the deshielding effect of the axial 3-OH group, as compared to δ 4.33 for H_e at C-5 (see Fig. 3). Examination of the atomic models of *R* and *S* mevalonolactones shows that in the former the pro-*R* hydrogen atom at C-5 is equatorial and in the latter it is axial (cf. formulae in Fig. 3). Thus the stereospecific replacement of either the pro-*R* or pro-*S* hydrogen atom with deuterium at C-5 in either 3*R*- or 3*S*-mevalonolactone would cause distinct changes in the NMR spectra.

We have prepared, therefore, a large specimen of 3*RS*-[5-D_1]mevalonate by the reduction of *RS*-mevaldate with [4*R*-4-D_1]NADH. The mixture was resolved enzymically by the conversion of the 3*R*-mevalonate into 5-phosphomevalonate with mevalonate kinase, and by the isolation of the phosphate ester as the Ba-salt and of the unused 3*S*-isomer as the lactone. The 3*R*[5-D_1]mevalonolactone was obtained after hydrolysis of the 5-phospho-mevalonate with intestinal alkaline phosphatase. Each specimen was analyzed by mass spectrometry for deuterium-content, by nmr spectrometry and by spectropolarimetry. Additional evidence was obtained by the analysis of two specimens of 3-methyl[5-D_1]pent-2-eno-5-lactone, one derived by the dehydration of 3*S*-[5-D_1]-mevalonolactone and the other by the dehydration of 3*RS*-[5-D_1]mevalonolactone.

EXPERIMENTAL

Enzymes. Mevaldate reductase (EC 1.1.1.32) was partially purified from pig liver as described by Schlesinger and Coon (*1*); the preparation used reduced mevaldate to mevalonate with NADH at an initial rate of 0.2 μmoles min^{-1} mg^{-1} (specific activity, 0.2 units per mg of protein).

Mevalonate kinase (EC 2.7.1.36) was made from pig liver according to Levy and Popják (*6*); the preparation phosphorylated mevalonate at an initial rate of 0.4 μmoles min^{-1} mg^{-1}.

Pyruvate kinase, crystalline preparation from rabbit muscle (EC 2.7.1.40), calf intestinal alkaline phosphatase (EC 3.1.3.1), lactate dehydrogenase, crystalline preparation from rabbit muscle (EC 1.1.1.27), and yeast alcohol dehydrogenase (EC 1.1.1.1), as a dry preparation, were supplied by the Sigma Chemical Co.

Preparation of 3RS-[5-D_1]mevalonolactone. This was made on a large scale by the reduction of *RS*-mevaldate (prepared freshly from mevaldic acid dimethyl acetal) with mevaldate reductase and [4*R*-4-D]NADH as described in detail before (*4*). Five hundred milligrams (3.8 mmoles) of the lactone, purified by liquid–liquid partition chromatography (*4*), were obtained in a yield of 78%. *RS*-[2-^{14}C]mevalonolactone (specific activity, 5.85 Ci per mole; supplied by the Amersham-Searle Corporation) was added to the preparation to a final specific activity of 21×10^3 dpm/μmole.

Preparation of 4R-[4-D_1]NADH. The stereospecifically labeled [D_1]NADH was

made on a 10 mmolar scale by the reduction of NAD^+ with yeast alcohol dehydrogenase and CD_3CD_2OH essentially by the method of Rafter and Colowick (*12*) and as described in detail before (*4*).

Resolution of 3RS-[5-D_1-2-^{14}C]mevalonate. The two isomers of the labeled mevalonate were obtained by the conversion of the 3*R*-isomer into 5-phosphomevalonate with mevalonate kinase and ATP, followed by the isolation of the phosphate ester as the Ba salt and of the unreacted 3*S*-mevalonate as the lactone. For this purpose a 340-ml incubation, at 37°C and for 1 hr, was set up containing 0.1 *M* Tris–HCl buffer, pH 7.4, 10 m*M* mercaptoethanol, 5 m*M* $MgCl_2$, 7.5 m*M* ATP, 5 m*M* 3*RS*-[5-D_1-2-^{14}C]mevalonate, 340 units of mevalonate kinase, 2.5 m*M* phosphoenol pyruvate and 9600 units of pyruvate kinase. The inclusion of pyruvate kinase and phosphoenolpyruvate ensured the continuous regeneration of ATP and the phosphorylation of all utilizable 3*R*-mevalonate. The 5-phosphomevalonate was isolated as the ethanol-insoluble barium salt as described by Levy and Popják (*6*). The yield of the barium salt after purification and conversion to the potassium salt (*4*) was 0.45 mmole (53%); it contained no free mevalonate nor nucleotide phosphates as judged by thin layer chromatography and by spectrophotometric measurements.

The residue of the reaction mixture after removal of the Ba 3*R*-5-phospho-[5-D_1-2-^{14}C]mevalonate by centrifugation was evaporated to dryness and was dissolved in dilute HCl. The solution was then lyophilized and the residue triturated several times with $CHCl_3$. The combined chloroform extracts were clarified by centrifugation and concentrated to about 5 ml. The mevalonolactone was purified by liquid–liquid partition chromatography (*4*); 100 mg (0.76 mmole) of the pure lactone were obtained. Assay with mevalonate kinase (*6*) showed the preparation to contain no more than 1.3% of the 3*R*-isomer. The preparation contained no impurities as judged by thin layer chromatography, mass spectrometry and nmr spectrometry.

3R-[5-D_1-2-^{14}C]Mevalonolactone from barium 3R-5-phospho-[5-D_1-2-^{14}C-mevalonate. The barium salt of the labeled 5-phosphomevalonate was converted into the potassium salt as described (*4*). For the hydrolysis of the phosphate ester a 40-ml incubation was set up containing 50 m*M* Tris–HCl buffer pH 9.7, 5 m*M* $MgCl_2$, 11.2 m*M* potassium 3*R*-5-phospho-[5-D_1-2-^{14}C]mevalonate and 1720 units of calf intestinal alkaline phosphatase. After 2 hr at 37°C the reaction mixture was acidified to pH 1 with 5 *N* HCl and was lyophilized. The mevalonolactone was extracted from the dry residue and purified as described for the preparation of the 3*S*-[5-D_1-2-^{14}C]mevalonolactone. The yield was 40 mg (306 μmoles; 68%).

3-Methyl[5-D_1-2-^{14}C]pent-2-eno lactone. This was obtained by the dehydration of mevalonolactone at 120°C *in vacuo* with fused $KHSO_4$ as described (*11*), except that the dehydration product and some unchanged mevalonolactone were collected on the tip of a cold-finger inserted into the dehydration vessel. The crude product was then purified by liquid–liquid partition chromatography on the same column (stationary phase: 0.5 *N* H_2SO_4 on Celite; moving phase: $CHCl_3$ saturated with the stationary phase) as was used for the purification of mevalonolactones (*4*). The methylpentenolactone was eluted immediately after the solvent front in a narrow band. Two specimens were dehydrated: (a) 3*RS*-[5-D_1-2-^{14}C]mevalonolactone, 33.2 mg; and (b) 3*S*-[5-D_1-2-^{14}C]mevalonolactone, 52.1 mg. The former gave 12.0 mg, and the latter 23.3 mg of the 3-methyl[5-D_1-2-^{14}C]pentenolactone. Nmr spectrometry (cf. Figs. 6 and 7) showed

that the preparations contained 88% of the methylpent-2-eno-5-lactone and 12% of the methylpent-3(4)-eno-5-lactone. These isomers were not resolved. A reference specimen of 3-methylpent-2-eno-5-lactone was also made by the dehydration of 100 mg of unlabeled *RS*-mevalonolactone.

Physical measurements. Mass spectra were taken with an MS-902 high resolution instrument (A.E.I. Ltd., Manchester, England) at 70-eV ionization potential. Nmr spectra were obtained in a Varian HA-100 spectrometer (Varian Associates, Palo Alto, CA) in $CDCl_3$ with tetramethylsilane as internal standard. Proton and deuteron spin–spin decoupling facilities were available. Optical rotary dispersions (ORD) were measured in ethanol with a Carey 140 spectropolarimeter. The values of rotations were checked in the visible and near-ultraviolet regions at selected wavelengths with an optical spectropolarimeter (Perkin–Elmer, Model 141).

Measurements of ^{14}C were made with a Packard Tri-Carb scintillation spectrometer, Model 3320. Thin layer chromatographic plates containing ^{14}C-labeled substances were examined with a Packard Radiochromatogram scanner, Model 7201.

Chromatography. In addition to the liquid–liquid partition chromatography of mevalonolactone described before (*4*), thin layer chromatography on plates (5 × 20 cm) coated with silica gel G (Merck, Darmstadt, Germany) were used with *n*-propanol–concd ammonia–1% sodium ethylenediamine tetraacetate in water (6:3:1, by volume) for the analysis of the radiopurity of 3*R*-5-phospho-[5-D_1-2-^{14}C]mevalonate and 3*S*-[5-D_1-2-^{14}C]mevalonolactone.

Reagents. ATP, β-NAD^+, phosphoenolpyruvate, and Tris base were the products of Sigma Chemical Company. Deuterio-ethanol, CD_3CD_2OD, was supplied by the International Chemical and Nuclear Corporation, City of Industry, CA. N_1N'-dibenzylethylenediammonium - *bis* - 5,5′ - dimethoxy - (3*RS* -)3 - hydroxy - 3 - methyl - pentanoate was obtained from Mann Research Laboratories, Inc., New York, and was converted to the potassium salt of mevaldate as described by Knauss et al. (*3*). All solvents were of analytical or nanograde quality.

RESULTS

3RS-[5-D_1-2-^{14}C]Mevalonolactone

The authenticity and purity of each preparation examined was of prime importance in this study. Analyses by mass spectrometry and by nmr revealed no impurities in the 3*RS*-[5-D_1-2-^{14}C]mevalonolactone. After conversion to potassium mevalonate, precisely one-half of the specimen was convertible into 5-phosphomevalonate with mevalonate kinase and ATP.

Mass spectrometry of the lactone indicated that the specimen contained 80% monodeuterated molecules as judged by the ratios of the molecular ions at *m/e* 131 and 130 in the experimental sample and as compared to the ratios of the same ions in an unlabeled preparation. The mass spectra of this specimen of 3*RS*-[5-D_1]mevalonolactone as well as of those of the resolved 3*R*- and 3*S*-[5-D_1]lactones (see below) were very similar to those previously published (*4*) and were in complete accord with Gray's analysis (*13*) of the fragmentation of mevalonolactone in the mass spectrometer on electron-impact ionization.

Nuclear magnetic resonance spectrometry gave a similar value, 80.3% for the

deuterated molecules, by a comparison of the integrals of proton resonances at C-5 with those at C-4 and C-2 of the lactone. The nmr spectra located the deuterium at C-5 (see below).

Optical rotatory dispersion measurements made on the 3*RS*-[5-D_1-2-^{14}C]mevalonolactone showed it to be optically active giving a negative Cotton effect (λ_0 at 227 nm) with a minimum at 240 nm, $[\alpha]_{240}^{25°} = -84°$ (cf. insert in Fig. 2).

The optical activity of this deuterated mevalonolactone contrasted with the complete lack of optical activity in an unlabeled specimen of mevalonolactone prepared by the reduction of *RS*-mevaldate with mevaldate reductase and unlabeled NADH (*4*). The optical activity of our specimen of deuterated mevalonolactone might have been due to a slight preponderance of the 3*R*-isomer in the specimen, or to the presence in the 3*R*- and 3*S*-isomers of another asymmetric center of the same chirality. It will be shown further on that the latter is the correct explanation for the observed phenomenon.

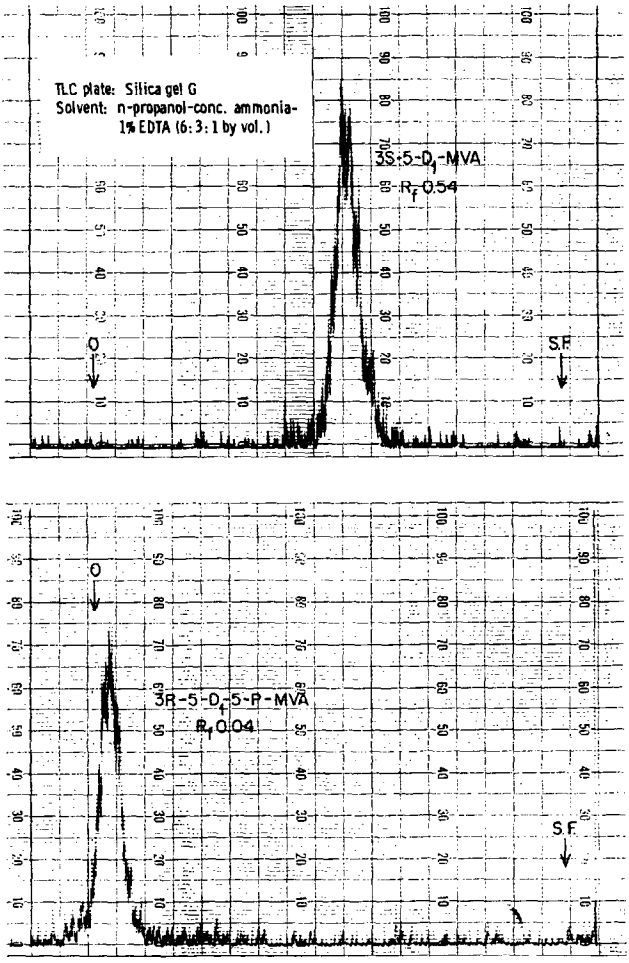

FIG. 1. Radiochromatograms on thin layer plates of 3*S*-[5-D_1-2-^{14}C]mevalonolactone (top) and of the 3*R*-5-phospho[5-D_1-2-^{14}C]mevalonate (bottom) obtained from the resolution of 3*RS*-[5-D_1-2-^{14}C]mevalonate.

3R- and 3S-[5-D_1-2-^{14}C]Mevalonolactones

Figure 1 is the record of the scans for radioactivity of the thin layer chromatograms of the potassium 3*R*-5-phospho-[5-D_1-2-^{14}C]mevalonate prepared, through the Ba salt, with mevalonate kinase from the 3*RS*-[5-D_1-2-^{14}C]mevalonolactone, and of the 3*S*-[5-D_1-2-^{14}C]mevalonolactone purified as described in the Experimental section. Neither substance was contaminated by the other and each specimen contained only one radioactive species.

The mevalonolactone derived from the 5-phospho-[5-D_1-2-^{14}C]mevalonate by hydrolysis with intestinal alkaline phosphatase, the 3*R*-isomer, was indistinguishable chromatographically and by its mass spectrum from the presumed 3*S*-isomer. Enzymic analysis, measurements of optical activity and nmr spectra revealed, however, great

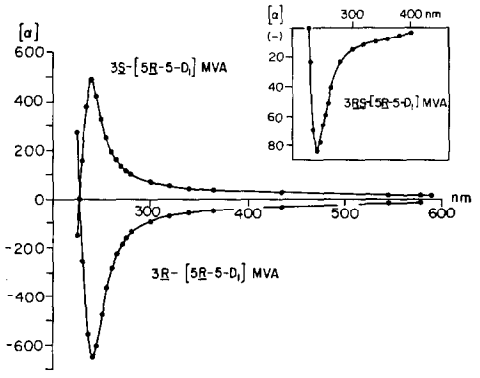

FIG. 2. Optical rotatory dispersions (in ethanol) of 3*R*- and 3*S*-[5-D_1-2-^{14}C]mevalonolactones. The insert is the ORD curve of the unresolved 3*RS*-[5-D_1]mavalonolactone. The measurements were made at the following concentrations: 3*R*-isomer, 9.74 mg/ml and 0.72 mg/ml; 3*S*-isomer, 9.69 mg/ml and 0.22 mg/ml; mixture of 3*R*- and 3*S*-isomers, 4.5 mg/ml and 1.29 mg/ml. The higher concentrations were used for measurements with the Perkin–Elmer polarimeter and the lower ones for measurements with the Carey spectropolarimeter.

differences between the two preparations. While all of the mevalonate obtained from the hydrolysis of 5-phosphomevalonate reacted with mevalonate kinase, only 1.30% of the presumed 3*S*-isomer reacted similarly. ORD measurements gave curves for the two preparations that were mirror images, with maxima and minima for the 3*S*- and 3*R*-isomers at 240 nm, and positive and negative Cotton effects, respectively, and λ_0 at 227 nm, but the specific rotation of the 3*S*-specimen at its maximum was +500° and that of the 3*R*-specimen at its minimum was −650° (Fig. 2). The difference between the optical activities of the two specimens is far greater than could be accounted for by the small (1.3%) contamination of the 3*S*-isomer by the 3*R*-isomer. If it were assumed that the difference in the maximum and minimum of the optical activity of the two specimens was attributable to a chiral center, other than position 3, giving a negative rotation in each, we calculate that this center accounts for an $[\alpha]_{240}$ of −75° ([−650° + 500°]/2 = −75°) rotation in the two specimens at 240 nm. This value is reasonably close to the $[\alpha]_{240} = -84°$ determined for the 3*RS*-[5-D_1-2-^{14}C]mevalonolactone.

Nuclear magnetic resonance spectra of 3RS-, 3R- and of 3S-[5-D_1-2-^{14}C]mevalonolactones. The nmr spectra of the 3*RS*-, 3*R*- and 3*S*-[5-D_1-2-^{14}C]mevalonolactones confirmed the assumption that the 3*R*- and 3*S*-isomers contained an additional asymmetric center created by the stereospecific replacement of one hydrogen atom by deuterium at C-5.

Figure 3 is the nmr spectrum of unlabeled 3*RS*-mevalonolactone and is identical with that reported by von Mühll (*10*). The multiplet (four triplets) pertaining to the

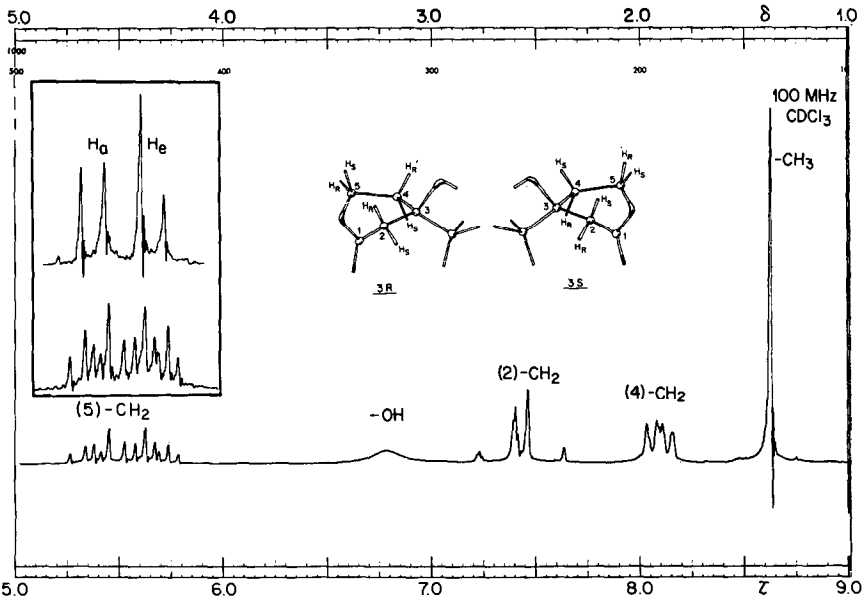

FIG. 3. Nmr spectrum of unlabeled 3*RS*-mevalonolactone. The insert shows the multiplet of the C-5 methylenic protons before and after decoupling with the frequencies of the C-4 methylenic protons; H_a = axial, H_e = equatorial proton. The formulae have been drawn from Dreydig models of the 3*R*- and 3*S*-lactone.

C-5 methylenic protons (a virtual ABX_2 system) was reduced to two doublets (H_a, δ 4.58; H_e, δ 4.33; $J_{H_aH_e}$ 11.3 Hz) by spin–spin decoupling with the frequencies (nearly identical) of the C-4 methylenic protons.

The complex multiplet in the nmr spectrum of the mixture of 3*R*- and 3*S*-[5-D_1]-mevalonolactones, representing the resonances of the hydrogen atoms at C-5 (Fig. 4), is the result of the overlap of the resonances of the hydrogen atoms at this position in the nondeuterated molecules (about 20%) with the split resonances of one equatorial and one axial proton coupled to a deuterium atom and to the methylenic protons at C-4. The integral of the spectrum and spin–spin decoupling experiments indicated that about 40% of the molecules in the specimen contained one axial proton and another 40%, one equatorial proton (cf. middle column of Fig. 5). The presence of a deuterium atom at C-5 was also indicated by the reduction of the multiplet of the resonances of the C-4 methylenic protons, seen in the spectrum of the unlabeled specimen, to a broad doublet (cf. Figs. 3 and 4).

The mass spectra of the separated 3*R*- and 3*S*-[5-D_1]mevalonolactones were in-

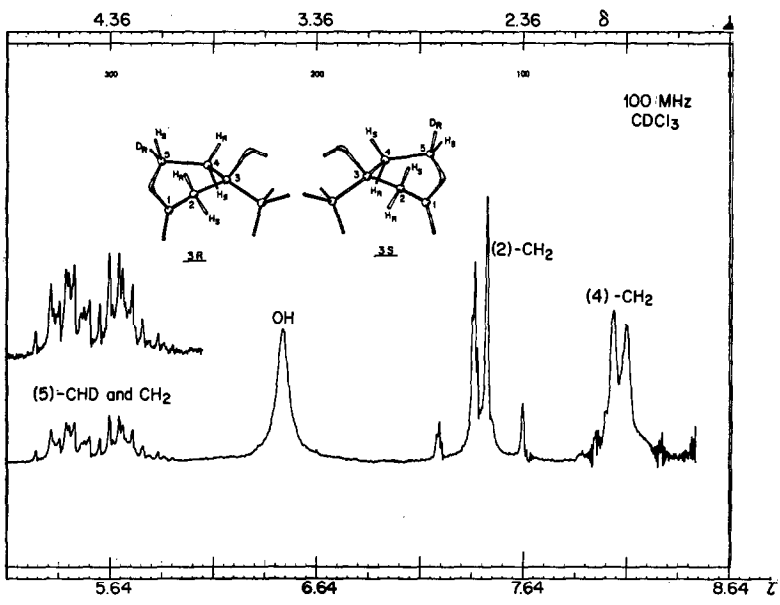

FIG. 4. Nmr spectrum of the mixture of 3*R*- and 3*S*-[5-D_1]mevalonolactones formed by the reduction of mevaldate with mevaldate reductase and [4*R*-4-D_1]NADH. The part of the spectrum below the field of the resonance of the 3-methyl group is shown.

distinguishable and indicated that each contained the same proportion (80%) of deuterated molecules. Their nmr spectra were, however, different and simpler than the spectrum of the 3*RS*-[5-D_1]mevalonolactone, but only in respect of the resonances of the protons at C-5. Figure 5 shows the resonances of the protons at C-5 in the mixture of the 3*R*- and 3*S*-[5-D_1]]lactones (middle column) and of the separated isomers, in the 3*R*-lactone on the left, and in the 3*S*-lactone on the right, before spin–spin decoupling (spectra at the bottom), after deuterium decoupling (spectra in the middle) and after deuterium plus proton spin–spin decoupling with the frequencies of the protons at C-4. The broad doublet of the C-4 methylenic protons in the deuterated specimens was reduced to a singlet by irradiation with the frequencies of the C-5 protons.

These spectra show that, excepting some nondeuterated molecules, the 3*R*-specimen contained at C-5 an axial, pro-*S*, proton (δ 4.58) and the 3*S*-lactone an equatorial, pro-*S*, proton (δ 4.33). Thus the nmr spectra showed clearly that the 3*R*-[5-D_1]mevalonolactone and the 3*S*-[5-D_1]mevalonolactone, resulting from the reduction of *RS*-mevaldate with 4*R*-[4-D_1]NADH and mevaldate reductase, were diastereoisomers, and they also specified that the absolute configuration at C-5 in each isomer was *R*.

3-Methyl[5-D_1]pent-2-eno-5-lactones

We have obtained further evidence that the absolute configuration at C-5 was the same in the 3*S*-[5-D_1-2-^{14}C]mevalonolactone as in the 3*R*-[5-D_1-2-^{14}C]mevalonolactone. We have converted a specimen of the pure 3*S*-[5-D_1]mevalonolactone and one of the 3*RS*-[5-D_1]mevalonolactone into 3-methyl[5-D_1]pent-2-eno-5-lactone (cf. Experimental) and compared their nmr and mass spectra and their ORD properties. The two specimens were identical in every respect.

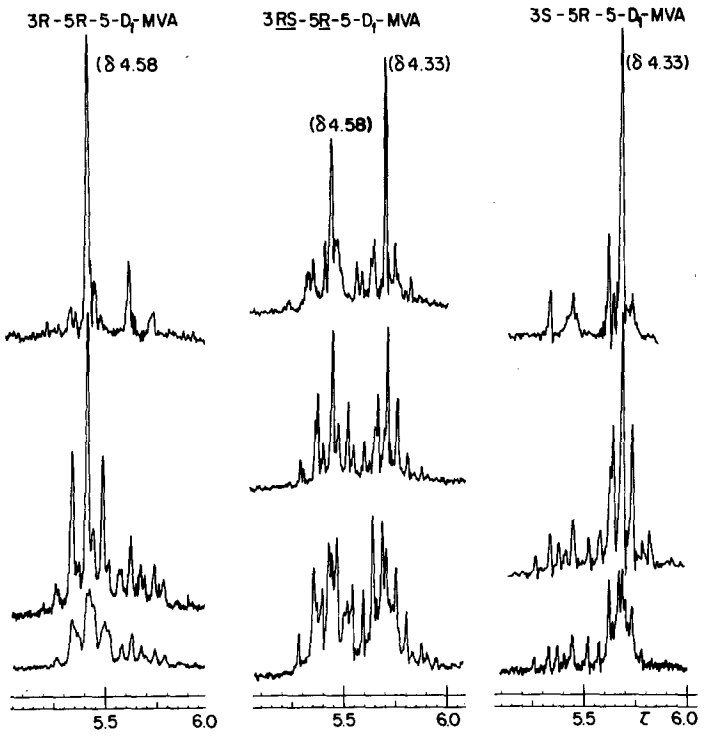

FIG. 5. The nmr resonances, from left to right, of the C-5 protons in the spectra of 3*R*-[5-D_1-2-^{14}C]-, 3*RS*-[5-D_1-2-^{14}C] and 3*S* [5-D_1-2-^{14}C]mevalonolactone: At the bottom, before decoupling; in the middle, after deuterium decoupling; and at the top, after deuterium and proton decoupling.

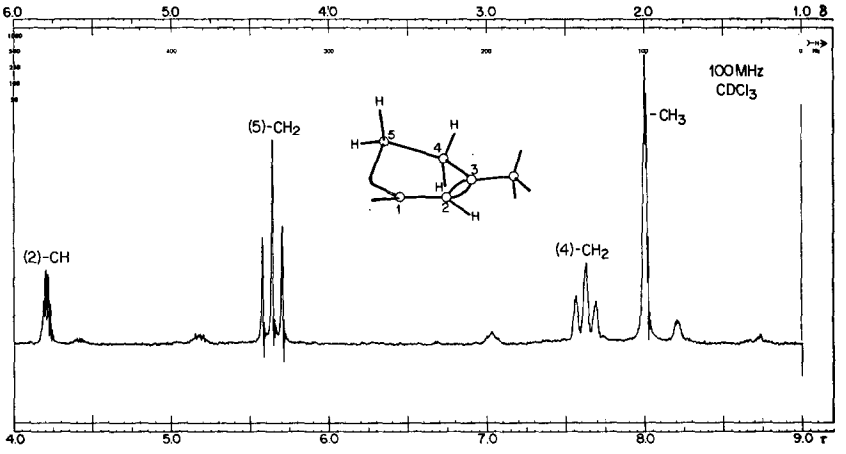

FIG. 6. Nmr spectrum of 3-methylpent-2-eno-5-lactone made by the dehydration of 3*RS*-mevalonolactone. The weak resonances seen at δ 1.80, 2.98, 4.84 and 5.60 are those attributed to 3-methylpent-3-eno-5-lactone also formed during the dehydration and not separated from the Δ^2-isomer.

The mass spectra indicated that 80% of the molecules in both specimens contained one atom of deuterium as judged by the ratios of the molecular ions at *m/e* 113 and 112, i.e., there was no loss of deuterium during the dehydration of mevalonolactones.

The nmr spectra of the two specimens were also identical but differed in two important aspects from that of the 3-methylpent-2-eno-5-lactone prepared from unlabeled mevalonolactone (Figs. 6–8). The triplets of the C-5 and of the C-4 methylenic protons, δ 4.36 and δ 2.37, respectively (Fig. 6), were replaced in the two deuterium-labeled specimens by a multiplet for the proton resonances at C-5 and by a broad doublet of

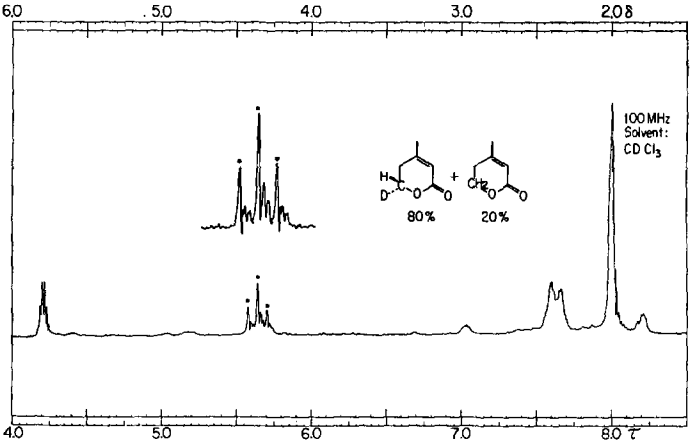

FIG. 7. Nmr spectrum of 3-methyl[5-D_1]pent-2-eno-5-lactone contaminated by 20% of the unlabeled substance. The specimen was obtained from the dehydration of 3*S*-[5-D_1]mevalonolactone. The specimen made by the dehydration of 3*RS*-[5-D_1]mevalonolactone gave an identical spectrum.

the resonances of the protons at C-4 (Fig. 7). The multiplet of the resonances of protons at C-5 in the spectrum of the deuterium-labeled specimen consisted of a triplet clearly identifiable as representing unlabeled molecules in the specimen. The remainder of the multiplet was attributable to the spin–spin coupling of one proton at C-5 with deuterium on the same carbon atom and with the methylenic protons at C-4. This interpretation was supported by the change of the multiplet of the resonances of the protons at C-5 to two triplets, the stronger of the two being upfield from the weaker one by 2 Hz, after deuterium decoupling (Fig. 8). The deuterium decoupling combined with the decoupling by the frequency of the C-4 methylenic protons reduced the two triplets, just referred to, to two unequal singlets, the stronger one at a field higher by 2 Hz than the weaker one (Fig. 8). The shielding effect of one deuterium atom at C-5 on the hydrogen atom at the same position was clearly evident.

The pro-*R* and pro-*S* hydrogen atoms at C-5 of 3-methylpent-2-eno-5-lactone have, unlike the H-atoms in mevalonolactone, identical chemical shifts, and hence nmr spectrometry cannot identify the steric position of a deuterium atom specifically at either the pro-*R* or pro-*S* position. However, since we have demonstrated that the deuterium atom at C-5 of both 3*R*- and 3*S*-[5-D_1]mevalonolactone occupied the pro-*R* position, the specimens of 3-methyl[5-D_1]pent-2-eno-5-lactone obtained from the 3*S*-[5-D_1]- and from the 3*RS*-[5-D_1]mevalonolactone should have the same absolute

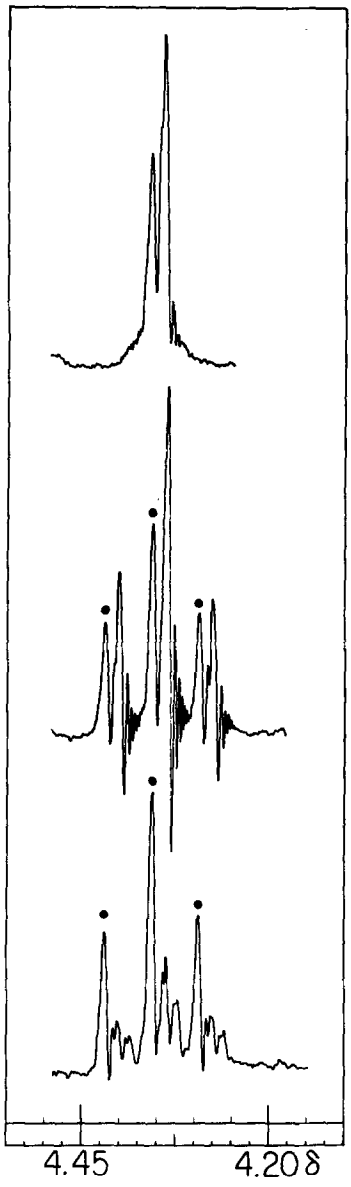

FIG. 8. The nmr resonances of the proton(s) at C-5 of 3-methyl[5-D_1]pent-2-eno-5-lactone (cf. Fig. 7) before decoupling (bottom), after deuterium decoupling (middle), and after deuterium and proton decoupling, with the frequency of the C-4 methylenic protons (top).

configuration at C-5 and identical optical activities. The data of Fig. 9 show this to be the case.

DISCUSSION

We have demonstrated by direct unambiguous methods that the absolute configuration at C-5 of the 3*R*-component of the 3*RS*-[5-D_1]mevalonate generated by the reduction of *RS*-mevaldate with mevaldate reductase and [D]NADH was *R*, confirming

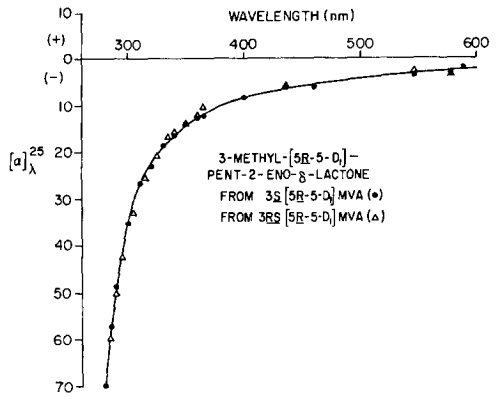

FIG. 9. Optical rotatory dispersion measurements on 3-methyl[5-D_1]pent-2-eno-5-lactone specimens made by the dehydration of 3*S*-[5-D_1]mevalonolactone (●), measured at 5.57 mg/ml; and of 3*RS*-[5-D_1]mevalonolactone (△), measured at 3.43 mg/ml.

the former conclusion arrived at by Donninger and Popják (*4*) by an indirect approach. We have also shown that the absolute configuration at C-5 of the 3*S*-component of the 3*RS*-[5-D_1]mevalonate was also *R*. It might be argued that our studies contain an element of uncertainty in that the 3*R*-[5-D_1]mevalonolactone was obtained by isolation of the enzymically synthesized 5-phospho-[5-D_1]mevalonate and the hydrolysis of the latter by alkaline phosphatase. Thus if either of these two enzymic reactions caused an inversion at C-5, our main conclusion, that the absolute configuration at C-5 of both 3*R*- and 3*S*-[5-D_1]mevalonate, as generated by mevaldate reductase, was *R*, might be in error. Two observations refute such an argument: First, the nmr spectrum of the original specimen of 3*RS*-[5-D_1]mevalonolactone, as obtained from the mevaldate reductase reaction, demonstrated that in 40% of the molecules an axial and in another 40% an equatorial protium atom at C-5 must have been replaced by deuterium. Thus it become apparent that the 3*R*- and 3*S*-isomers were not enantiomers but diastereoisomers, although the absolute configuration at C-5 could not be defined without the resolution of the 3*RS*-mixture (a diastereomeric mixture at 3*RS*-[5*S*-5-D_1]mevalonolactones would give an nmr spectrum similar to that of the mixture of 3*RS*-[5*R*-5-D_1]-mevalonolactones). Having shown that in the 3*S*-[5-D_1]mevalonolactone (unattacked by enzymes) the axial, pro-*R*, hydrogen atom was replaced by deuterium (cf. Fig. 5) one could logically conclude (even without isolation and examination of the 3*R*-isomer) that it was the 3*R*-isomer which contained a deuterium atom in the equatorial position (pro-*R*) and a protium atom in the axial (pro-*S*) position. Second, the same conclusion is reached from the fact that the optical activities of the specimens of 3-methyl[5-D_1]pent-2-eno-5-lactones made by the dehydration of 3*S*-[5-D_1]- and 3*RS*-[5-D_1]mevalonolactones were indistinguishable.

The physiological role of mevaldate reductase is as yet undefined, nor is its natural substrate known. The lack of any detectable discrimination by this enzyme between the 3*R*- and 3*S*-isomers of mevaldate makes us doubt that mevaldic acid is its true substrate. The further fact that the hydride ion is added from the *re*-face of the carbonyl group in both the 3*R*- and 3*S*-isomers suggests strongly that the two enantiomers occupy the same site on the enzyme, only the carboxyl and aldehydo groups being

involved in the binding, and that the spatial relation of NADH to mevaldate on the enzyme is the same, irrespective of the stereochemistry of the substrate at C-3. It seems doubtful that mevaldate reductase might play a physiological role in polyisoprenoid biosynthesis, as free mevaldic acid is thought not to be an intermediate in the reduction of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonate (*14*, *15*). In spite of these uncertainties surrounding the role of this enzyme, it is a useful catalyst as it permits the easy preparations of mevalonate labeled stereospecifically at C-5 with a hydrogen isotope for the study of problems of polyisoprenoid biosynthesis.

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, whether isolated from yeast or rat liver and well established as the physiological generator of mevalonate, is known to be also an "A"-side specific enzyme transferring the pro-*R* hydrogen atom from C-4 of the dihydronicotinamide ring of NADPH to the substrate in each of the two reductive steps of mevalonate formation (*16*, *17*). It was shown further with the yeast and liver enzyme as well, that reduction of the mevaldate–coenzyme A thiohemiacetal, which may well be the intermediate between HMG-CoA and mevalonate, with [4*R*-4-$^3H_1$]NADPH generates the [5*S*-5-$^3H_1$]mevalonate (*18*, *19*). The reduction of the thiohemiacetal is a reaction analogous to the reduction of mevaldate by mevaldate reductase; the opposite stereospecificities of the reactions catalyzed by the two enzymes suggest different, possibly unrelated, evolutionary origins for the two reductases. Indeed, Beedle et al. (*20*) have shown recently that mevaldate reductase reduces with NADPH not only mevaldate but also 4-cyanobenzaldehyde and pyridine-3-carboxaldehyde and have suggested that this enzyme should be reclassified as a general aldehyde reductase (EC 1.1.1.2).

Our observations also indirectly shed some light on the mechanism of action of mevalonate kinase and intestinal alkaline phosphatase. Since the absolute configuration of the 3*R*-[5-D_1]mevalonolactone, isolated after the hydrolysis by phosphatase of 5-phosphomevalonate, was the same at C-5 as in the "native" 3*R*-[5-D_1]mevalonolactone, two possibilities could be entertained: (a) Neither mevalonate kinase nor intestinal alkaline phosphatase caused an inversion at C-5 or (b) each caused an inversion (two inversions = retention of configuration). We have evidence from experiments made with [5-^{18}O]mevalonate and [1-^{18}O]farnesyl pyrophosphate (to be reported) that hydrolysis of a phosphate, or pyrophosphate ester of a primary alcohol with intestinal alkaline phosphatase, results in the cleavage of the O–P bond and hence is unlikely to cause inversion of configuration. It follows then that phosphorylation of mevalonate by mevalonate kinase and ATP should not cause inversion of configuration at C-5 either. Our experiments prove the conclusions of Donninger and Popják (*4*) to have been correct and, also, the assumptions which led to those conclusions to have been correct; besides, we have extended the observations to the 3*S*-[5-D_1]mevalonate. We are also relieved to note that all the conclusions drawn from experiments on the stereochemistry of polyisoprenoid biosynthesis made with the aid of [5-D_1]- or [5-$^3H_1$]-mevalonate, generated by the reduction of mevaldate with mevaldate reductase and [D]NADH or [^{3}H]NADH, must be correct.

ACKNOWLEDGMENTS

We thank Professor Duilio Arigoni of the Eidgenössische Technische Hochschule, Zürich, Switzerland, for drawing our attention to the doctoral dissertation of P. A. von Mühll and the use of nmr

for the solution of our problem. We thank members of the Department of Chemistry at UCLA for giving us ready access to their instruments and, particularly, Professor Frank Anet, for the use of the Varian HA-100 nmr spectrometer and the Carey 140 spectropolarimeter, and Professor Donald Cram for the use of his Perkin–Elmer spectropolarimeter. Our special thanks go to Dr. Kai Fang who took all our nmr spectra, to Dr. Siu-May Wong for frequent discussions and to Professor J. W. Cornforth, F.R.S., for his advice with the interpretation of the nmr spectra of the deuterio-mevalonolactones. Professor Cornforth also pointed out to us an erroneous notation, now corrected, for the Cotton effects in the original version of this paper.

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