NON-STEROL METABOLISM OF MEVALONATE IN VITRO: ARTIFACTS AND REALITIES[1]

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Received December 10, 1976

Summary. Commercial [5-^{14}C]mevalonate is shown to contain several radioactive impurities, which give artifactually high amounts of Hyamine bound, volatile acidic radioactivity when incubated with killed or living rat renal cortex slices, as compared with [5-^{14}C]mevalonate purified either by liquid-liquid partition chromatography or through the enzymically generated R-5-phospho-[5-^{14}C]mevalonate by ion-exchange chromatography. The artifactual $^{14}CO_2$ results were not diluted by incubation with increasing amounts of unlabelled mevalonate, whereas the $^{14}CO_2$ and [^{14}C]cholesterol produced by rat renal cortex slices incubated with purified [5-^{14}C]mevalonate were both diluted to the same extent by unlabelled mevalonate. It is concluded that R[5-^{14}C]mevalonate is genuinely oxidized to $^{14}CO_2$ in vitro, and that purification of substrate before its use is necessary. Production of $^{14}CO_2$ and various [^{14}C]lipids from purified [5-^{14}C]mevalonate, as a function of time and substrate concentration, by renal cortex and liver slices, is described.

It was demonstrated recently in our laboratory that MVA[2] was not used exclusively for sterol biosynthesis in vivo, but that it also took part in reactions resulting in the transfer of carbon atoms to the C_2-pool (1-3). It was shown that [5-^{14}C]MVA was a particularly useful substrate for the study of this shunting of MVA-carbon atoms to non-steroidal pathways, as it gave rapidly $^{14}CO_2$ in the breath of rat and man (2). It was also shown that the kidneys played an important role in the shunt as nephrectomy resulted in an over two-fold decrease in $^{14}CO_2$ production and an over five-fold increase in hepatic sterol synthesis from [5-^{14}C]MVA and a four-fold increase in the release into the blood of newly synthesized sterol (3,4).

The study of organ slices as an in vitro "shunting" system for biochemical characterization is the subject of this communication. In the course of these experiments, it became apparent that commercial RS[5-^{14}C]MVA was unsuitable for

[1] Supported by U.S.P.H.S. grants HD-06576 and HL-12745.

[2] MVA = mevalonate.

studying $^{14}CO_2$ production from this substrate as a measure of the shunt, as it contained radiochemical impurities which produced serious artifacts. We present data on the existence of impurities in the commercial material and on the effect of these on *in vitro* $^{14}CO_2$ measurements. We then report on *in vitro* experiments with purified [5-^{14}C]MVA.

MATERIALS AND METHODS

Calcium-free Krebs-Ringer phosphate buffer, pH 7.4, was prepared as described by Umbreit (5) with the exception that isotonic saline solution was substituted for $CaCl_2$ solution.

Substrates

The commercial RS[5-^{14}C]MVA used was purchased as the dry sodium salt from Schwartz Mann, Orangeburg, New York. The samples of impure commercial stock used were from Lot AT-1970. A specimen of pure RS[5-^{14}C]MVA was prepared from Schwartz Mann Lot AT-1825 by chromatography on a Celite-0.5 N H_2SO_4 column with $CHCl_3$ (6).

Pure R[5-^{14}C]mevalonate was prepared from Schwartz Mann Lots AT-2089 and BT-2279 by conversion of the natural enantiomer with mevalonate kinase (EC 2.7.1.36) to R-5-P-[5-^{14}C]MVA (7) followed by the separation of the product and the unreacted S[5-^{14}C]MVA by chromatography on a Bio-Rad AG 1x8 column (1.5 cmx 20 cm; carbonate form) with a linear gradient (0.01 M to 1.0 M) of triethylammonium carbonate buffer, pH 9.5 (T.S. Parker, unpublished). The isolated 5-P-MVA was hydrolyzed with alkaline phosphatase; the reaction mixture, with the phosphatase, was diluted two-fold with water and was chromatographed on the ion-exchange column as above, yielding a single symmetrical radioactive peak. The MVA fractions were pooled and were lyophilized (cf. Fig. 1B).

Unlabelled MVA was purified by Dr. Thomas Parker by crystallization from diethyl ether of the lactone (Sigma), previously dried by azeotropic distillation of water with benzene. It was then hydrolyzed with KOH and standardized by assay with MVA kinase (7). Hyamine (methyl benzethonium) hydroxide was purchased as the 1 M solution in MeOH from Sigma, St. Louis, Mo., and used directly.

Organ Slices

Adult male Sprague-Dawley rats, 250 to 400 g were killed by decapitation after mild ether anesthesia. The liver and kidneys were quickly removed and placed in iced buffer. Slices, 0.3 mmx0.3 mm prisms, were made from renal cortex and liver with the mechanical chopper described by McIlwain and Biddle (8). The slices were washed from debris in a centrifuge tube with ice-cold buffer, until the supernatant became clear. Aliquots from a stirred suspension of slices from a known weight of tissue were then measured into reaction vessels with an automatic pipette which had a wide orifice. Lipid analyses for total unsaponifiables, squalene and saponifiables were performed as described by Popják (7).

RESULTS

Paper Chromatography of [5-^{14}C]MVA Samples

Fig. 1 shows typical paper radiochromatograms obtained from commercial RS[5-^{14}C]MVA and from the kinase-purified R[5-^{14}C]MVA. The commercial prepara-

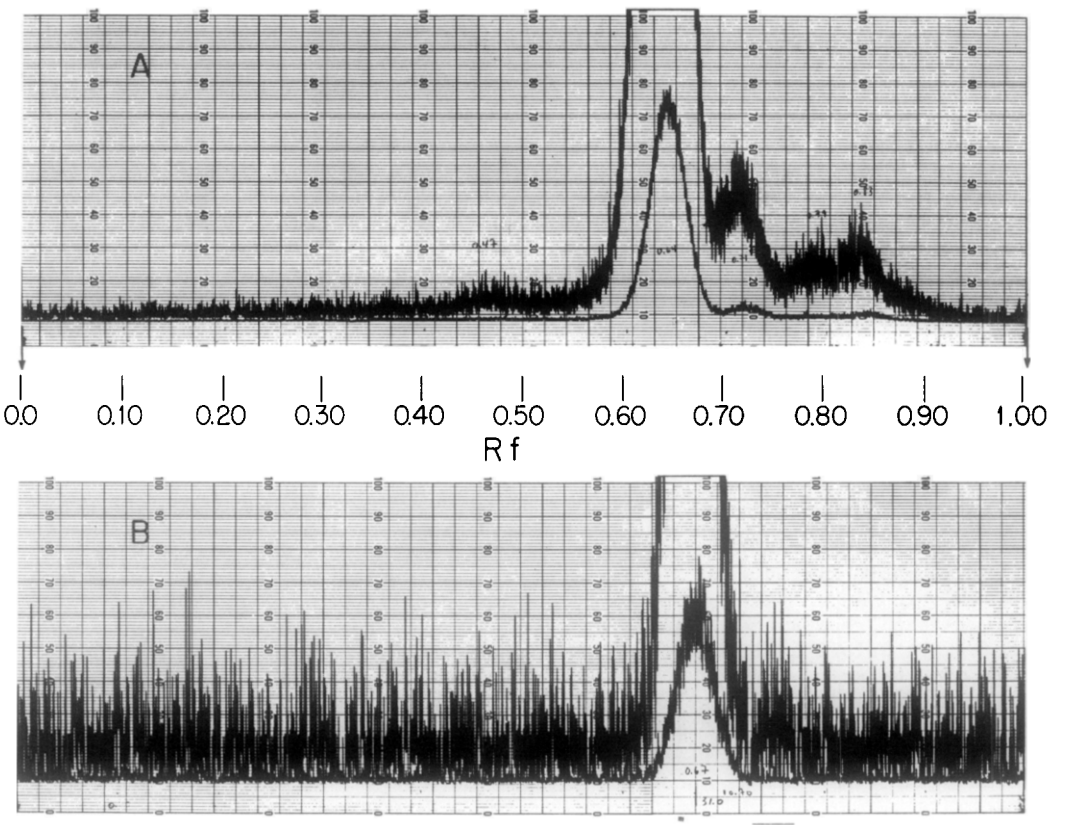


Figure 1. Paper chromatograms of [5-^{14}C]MVA. Sodium RS[5-^{14}C]MVA, Schwartz Mann lot #AT-1970, 0.39 μCi, was chromatographed on a 50x5 cm strip of Whatman 3 MM paper with n-propanol:conc. NH_4OH:H_2O (6:3:1, v/v). The paper was scanned at full scale ranges of $3x10^3$ and $3x10^4$ cpm on a Packard Model 7201 Radiochromatogram Scanner. B. R[5-^{14}C]MVA, 35 nCi, prepared as described under Materials and Methods, was chromatographed and analyzed as described above. Full scale ranges $3x10^2$, $3x10^3$ cpm.

tion contained at least four radioactive impurities, which were absent from the specimen of the pure R-MVA. A 0.2% impurity could have been detected. Chromatograms of the celite-purified RS-MVA (not shown) failed to reveal any of the impurities.

To assess the effect the impurities might have on *in vitro* assays of $^{14}CO_2$ production from [5-^{14}C]MVA samples, incubations with each of the three [5-^{14}C]MVA preparations (unpurified, celite-purified, and kinase-purified) were done at 100 μM R[5-^{14}C]MVA with acid-killed (control) and with surviving (ex-

Table I. Hyamine bound radioactivity observed in incubations of rat renal cortical slices with three preparations of [5-^{14}C]MVA.

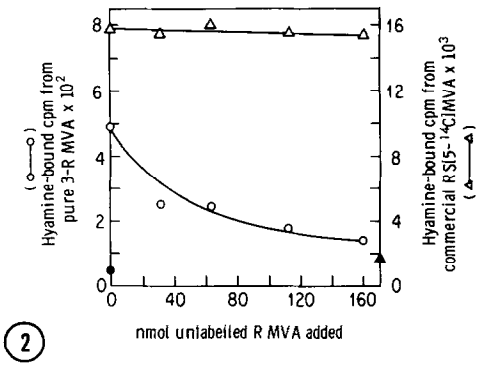
Mevalonate preparation	$^{14}CO_2$ controls[a] dpm/μCi of R-MVA/incubation	Total ^{14}C in incubation μCi	V_{CO_2}[b] nmol R-MVA to CO_2 per g tissue per hour
Unpurified commercial RS[5-^{14}C]MVA	2189	2.0	12.98
Celite-purified RS[5-^{14}C]MVA	70	2.7	1.41
MVA kinase-purified R[5-^{14}C]MVA	42	1.0	2.08

[a] $^{14}CO_2$ controls: reaction vessels were 20x150 mm glass tubes sealed with rubber stoppers fitted with plastic center wells containing 0.3 ml 1 M Hyamine hydroxide in MeOH. Incubations of 200 mg rat renal cortex slices in 2 ml calcium-free Krebs-Ringer phosphate buffer (pH 7.4) received 0.25 ml 2N H_2SO_4 immediately before addition of each MVA preparation sufficient to bring the final concentration to 100 μM R[5-^{14}C]MVA. Vessels, gassed 30 sec with 95/5 O_2/CO_2, before adding rubber stopper, were maintained at 37° with shaking at 160 oscillations/min for 30 min. Then Hyamine cups were placed directly in 10 ml Econofluor scintillation fluid for counting. Values were expressed as dpm in Hyamine per μCi of theoretically available R[5-^{14}C]MVA.

[b] V_{CO_2} measurements. Hyamine bound radioactivity ("$^{14}CO_2$") was measured in incubations as for controls, except that the H_2SO_4 was added after 20 min of incubation; shaking was continued for an additional 30 min, then the Hyamine cups were counted as above. Corrections were made for the control values, and values are expressed per g wet weight of tissue per hour of incubation, assuming that the specific activity of added R-MVA is essentially unchanged by endogenous pools.

perimental) rat renal cortex slices. The [5-^{14}C]MVA preparations purified by either method yielded similar results both for $^{14}CO_2$ controls and for measured rates of $^{14}CO_2$ formation by tissue (Table I). The impure commercial material, on the other hand, gave control values thirty- to fifty-times greater, and tissue-dependent $^{14}CO_2$ formation six- to nine-times greater than those observed with the purified [5-^{14}C]MVA preparations. These results suggested the presence in the commercial [^{14}C]MVA preparation of an acidic, volatile, non-mevalonate impurity oxidizable to CO_2 by the tissues.

Further evidence that there are non-mevalonate, oxidizable impurities in



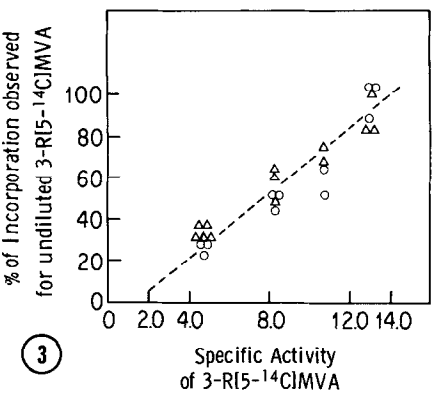


Figure 2 (on left). Dilution of $^{14}CO_2$ production by unlabelled MVA. Rat renal cortical slices were incubated for 20 min as described in Table I except that each incubation contained either 1 μCi of kinase-purified R[5-^{14}C]MVA (○) or 2 μCi of impure RS[5-^{14}C]MVA (△). Controls are shown with closed symbols on the appropriate ordinates. Values plotted represent the mean of duplicate incubations, done with 30 to 160 nmol of added, unlabelled purified MVA. Concentrations of R[5-^{14}C]MVA ranged from 38 μM (no unlabelled MVA added) to 118 μM (160 nmol unlabelled MVA added). Scale for R-preparation on left side, impure RS on right.

Figure 3 (on right). Formation of $^{14}CO_2$ and [^{14}C]cholesterol from kinase-purified R[5-^{14}C]MVA in incubations of rat renal cortical slices as a function of the specific activity of the substrate. Incubations were carried out as described in Table I, except that the concentration of R[5-^{14}C]MVA was maintained at 100 μM while the specific activity (S.A.) was varied from 13.0 to 4.83 Ci/mol. The cholesterol (non-squalene) fractions were eluted from alumina columns (7) directly into scintillation vials, and after evaporation of the solvent, 10 ml Econofluor was added for counting. The experimental points were done in triplicate, and values are plotted as percentage of the ^{14}C-incorporation into CO_2 and cholesterol observed for the undiluted R[5-^{14}C]MVA incubations, i.e. (cpm $^{14}CO_2$ or [^{14}C]cholesterol at S.A. "x"/cpm $^{14}CO_2$ of [^{14}C]cholesterol at S.A. 13.0) x 100. The dotted line indicates the plot derived from a least-squares analysis of the data. $^{14}CO_2$ (○); [^{14}C]cholesterol (△).

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the commercial material came from attempts to dilute the $^{14}CO_2$ formed from [5-^{14}C]MVA by purified, unlabelled MVA which had been recrystallized as the lactone and then hydrolyzed and standardized. Fig. 2 shows the Hyamine-bound radioactivity ("$^{14}CO_2$") obtained when renal cortical slices were incubated with 1 μCi of kinase-purified R[5-^{14}C]MVA or 2 μCi of impure RS[5-^{14}C]MVA and increasing amounts of unlabelled MVA. The undiluted impure substrate gave 14,000 dpm as compared to 450 dpm for the undiluted kinase-purified substrate. The $^{14}CO_2$ production from the impure material was not diluted by the unlabelled MVA, whereas that from purified R[5-^{14}C]MVA was. To confirm the true nature of [5-^{14}C]MVA as substrate for $^{14}CO_2$ production, and to control for concentration

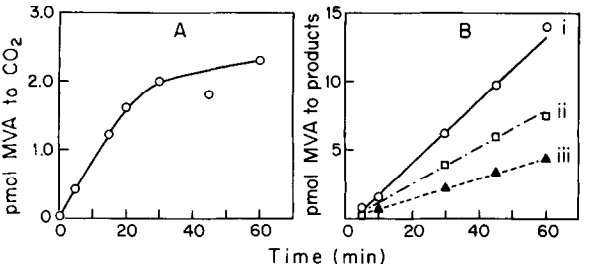

Figure 4. Time-dependent formation of $^{14}CO_2$ and [^{14}C]lipids from [5-^{14}C]MVA in incubations of rat renal cortical slices. Incubations were carried out as described in Table I. The celite-purified RS[5-^{14}C]MVA was used as substrate, at a final concentration of 2 μM (1 μM R). At the indicated times, incubations were acidified with 0.25 ml 2 N H_2SO_4 and were analysed for labelled CO_2 and lipids. Panel A: $^{14}CO_2$ production; panel B: labelled (i) total unsaponifiable lipids (○); (ii) squalene (□); and (iii) saponifiable lipids (▲).

effects, the experiment described in Fig. 3 was performed, in which renal cortical slices were incubated with kinase-purified R[5-^{14}C]MVA and increasing amounts of unlabelled purified MVA, holding the total concentrations of R-MVA constant at 100 μM. $^{14}CO_2$ and [^{14}C]cholesterol formation showed essentially identical dependence on the specific activity of R[5-^{14}C]MVA, confirming that mevalonate was the source of $^{14}CO_2$.

In further experiments we used the celite-purified RS[5-^{14}C]MVA at a final concentration of 1 μM R (2 μM RS) in an attempt to approximate the "physiologic" level, which has been reported in rat blood to be about 0.4 μM (9). Fig. 4 shows that $^{14}CO_2$ production was measurable and was linear for about 30 min. Total labelled unsaponifiable lipids, squalene and saponifiable lipids increased linearly with time to 60 min. The initial rates of conversion of [5-^{14}C]MVA to labelled CO_2, total unsaponifiables, squalene and saponifiable lipids were 30, 84, 52 and 27 pmol$xg^{-1}xh^{-1}$, respectively. Thus, there was a significant diversion of MVA carbon from sterol synthesis at this low substrate level. In other experiments not shown here, the $^{14}CO_2$ production from [5-^{14}C]MVA, measured at 20 min, was linear with weight of rat renal cortical slices between 84 and 330 mg. In a similar experiment done with liver slices, the initial rates of formation of labelled CO_2, unsaponifiable lipids and squalene were 34, 673 and

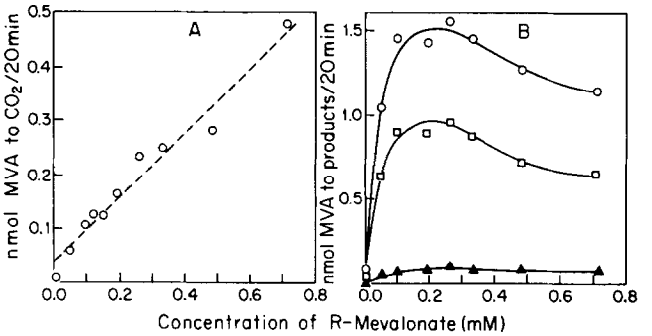

Figure 5. Relation between concentration of mevalonate and formation of products in incubations of rat renal cortical slices. The 20-minute incubations were done as described in Table I, with varying concentrations of the celite-purified RS[5-^{14}C]MVA. To conserve labelled substrate, increasing amounts of unlabelled MVA were added to achieve high concentrations of substrate. Thus, the specific activity of MVA here decreased from 12.3 to 2.36 Ci/mol as R-MVA concentration increased from 1.0 to 711 μM. Panel A: $^{14}CO_2$ formation, with dotted line being least-squares plot. Panel B: unsaponifiable lipids (○), squalene (□) and saponifiable lipids (▲).

645 pmolxg^{-1}xh^{-1}, respectively. While the initial rates of $^{14}CO_2$ production at this concentration of [5-^{14}C]MVA were similar for liver and renal cortex, the initial rate of unsaponifiable lipid synthesis was higher in liver than in kidney. This pattern is a reversal of that seen in vivo (2-4,9-11), where squalene and sterol synthesis from injected MVA is several times greater in kidney than in liver.

Next, renal cortical slices were incubated with increasing substrate concentrations from 1 μM to 711 μM R-MVA. Fig. 5 shows that $^{14}CO_2$ production increased linearly with increasing concentrations of MVA. The decrease in the rate of squalene synthesis at increasing MVA levels bears a remarkable resemblance to the inhibition of squalene synthesis at high concentrations of farnesyl pyrophosphate noted by Agnew (12). The rate of labelled saponifiable lipid production also reached a maximum at 250 μM R-MVA. Due to the low level of labelling of these "fatty acids", we were unable to identify them individually.

DISCUSSION

It is clear that R[5-^{14}C]MVA is a genuine substrate for oxidation to $^{14}CO_2$ in vitro just as it was demonstrated to be in vivo by Fogelman et al., who used

celite-purified [5-^{14}C]MVA (2). It is also clear that purification of commercial [5-^{14}C]MVA is necessary for use in studies of the MVA "shunt" in vitro. The V_{CO_2} observed with the impure [5-^{14}C]MVA in rat renal cortical slices (Table I) was quite similar to that reported by Righetti et al. (13) who did not report whether or not they purified the [5-^{14}C]MVA, which came from the same commercial source as our specimens. One is hence forced to interpret their results, and any results on $^{14}CO_2$ production from unpurified [5-^{14}C]MVA with caution. Experiments in progress in laboratories associated with ours (B.L. Johnson, personal communication) reveal that even in vivo, significant differences in $^{14}CO_2$ production from purified and unpurified [5-^{14}C]MVA are observed.

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