# Ribonuclease Inhibition of Mevalonic Acid Utilization.* (24066)

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Mevalonic acid (MVA) is a highly active biological precursor of the "isoprenoid" structure or of structures derived from this configuration. MVA is, therefore, readily converted to squalene or cholesterol in a number of systems. During the course of studies on factors influencing incorporation of MVA into cholesterol (CHL) or non-saponifiable material (NSF) by rat liver homogenates(1) it has been observed that the capacity for MVA utilization may be abolished by a predigestion of the system with crystalline ribonuclease (RNAse). The mechanism of this inactivation has not been fully elucidated but the technic for blocking biosynthesis at the MVA stage would appear to have sufficient implications to warrant detailed publication of the technic and initial findings at this time. A preliminary report, describing the utility of the procedure to demonstrate that mevaldic acid is convertible biologically into MVA has appeared(2).

*Materials and methods.* The experimental methods involved in the CHL biosynthesis studies have been described in detail(1). In brief, liver from young rats is homogenized with a "loose" homogenizer and incubated under oxygen with labeled precursors. The digest is then saponified and a NSF prepared which may be counted as such or treated with digitonin to form CHL digitonide which is similarly counted. In the experiments involving predigestion with RNAse or other enzyme prior to the addition of $C^{14}$-MVA or $C^{14}$-acetate, the flasks receiving the precursor were given a second aeration with oxygen at the time of addition. All incubations were carried out for 4½ hours from the start. RNAse used in most of the experiments was 3X crystallized material from Worthington Biochemical Corp., Freehold, N.J. This material is described as prepared by the McDonald modification(3) of the Kunitz procedure to render it free of protease activity. In addition, absence of protease activity was confirmed in these laboratories by the use of a highly sensitive method(4†). Similar results were obtained with crystalline RNAse from Nutritional Biochem. Corp., Cleveland, Ohio. Where microbiological assays for MVA were carried out samples were prepared by removing 0.1 ml aliquots from the incubation mixtures at the conclusion of the experiments, diluting to 10 ml and heating at 100° for 10 min. Microbiological determinations of MVA were carried out with *Lactobacillus acidophilus* 4963 according to the method of Skeggs *et al.*(5). All values for MVA are expressed in terms of the DL dibenzylethylenediammonium (DBED) salt used as a standard in the microbiological assays even though the $C^{14}$-MVA actually used in the enzyme experiments was added as the sodium salt prepared by extraction of the DBED by ether from alkaline solution. Two preparations of 2-$C^{14}$-MVA were used‡, one (A) had an activity of 15,500 cpm/mg, the other (B) had an activity of 36,000 cpm/mg. The $C^{14}$-acetate used had an activity of 1,250,000 cpm/mg.

*Results.* Preliminary experiments showed that when MVA and RNAse are added at the same time to an active liver homogenate the RNAse is without effect on the activity of the CHL isolated. In subsequent experiments the effect of various enzymes has been tested by allowing the enzymes to act for varying periods of time before the labeled substrates are added. Under the conditions described (Table I) utilization of MVA as determined by counts found in the NSF or CHL or by the amount of MVA remaining by microbiological assay is only slightly impaired by a

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TABLE I. Biosynthesis of Non-Saponifiable Material and Cholesterol Together with Unmetabolized Mevalonic Acid Encountered in Experiments Involving Predigestion with 5 mg of Ribonuclease for Varying Time Intervals.

| Exp. | MVA addition, min. | NSF, cpm/mg | CHL, cpm/mg | MVA found, mg |
|---|---|---|---|---|
| 1 | 0 | | 1435 | |
| | 0 | | 1728 ( 0) | |
| | 15 | | 1805 | |
| | 15 | | 785 ( 56) | |
| | 30 | | 1422 | |
| | 30 | | 31 ( 98) | |
| 2 | 0 | | 2042 | |
| | 0 | | 2000 ( 2) | |
| | 15 | | 2582 | |
| | 15 | | 928 ( 64) | |
| | 30 | | 1960 | |
| | 30 | | 8 (100) | |
| | 60 | | 745 | |
| | 60 | | 5 (100) | |
| 3 | 0 | | 1915 | |
| | 0 | | 1865 ( 2) | |
| | 15 | | 1750 | |
| | 15 | | 986 ( 54) | |
| | 30 | | 1985 | |
| | 30 | | 0 (100) | |
| | 60 | | 1429 | |
| | 60 | | 0 (100) | |
| 4 | 0 | 440 | 1172 | .0 |
| | 0 | 481 ( 0) | 971 ( 17) | .0 ( 0) |
| | 15 | 365 | 1142 | .0 |
| | 15 | 270 ( 26) | 536 ( 53) | .3 ( 30) |
| | 30 | 358 | 725 | .0 |
| | 30 | 1 (100) | 2 (100) | 1.0 (100) |
| | 60 | 4 | 6 | 1.0 |
| | 60 | 0 | 4 | 1.0 |

1 mg 2-$C^{14}$-MVA (A) added at the times indicated followed by a second flushing with oxygen.
Figures in parentheses indicate % inhibition of either biosynthesis or utilization of precursor.

preliminary digestion for periods up to 30 min. Some liver preparations even retained essentially unchanged their capacity for NSF and CHL biosynthesis after incubation for a period of 60 min. On the other hand, complete inactivation of MVA utilization is encountered after a preliminary digestion for 30 min with 5 mg of RNAse. One-half inactivation is obtained with about 100-200 $\gamma$ per flask (Table II). Incorporation of MVA into cholesterol is not impaired by a similar preliminary digestion with DNAse. Other experiments have shown elastase and transacetylase to be inactive. Incorporation of acetate into NSF or CHL also does not occur in RNAse-treated liver (Table III). In all instances where both microbiological assays for utilized MVA and determinations of MVA incorporation into NSF or CHL have been carried out it was found that incorporation is correlated directly with MVA disappearance. Where RNAse inhibition was complete essentially quantitative recovery of unused MVA is found. MVA is found at the conclusion of an experiment only when the homogenate contained added MVA *and* added RNAse. (Table IV). Presumably in the absence of added MVA there is not enough "endogenous" MVA to be determined. Similarly in the absence of added RNAse the amount of MVA added does not exceed the capacity of the tissue for biosynthesis and the added factor is readily utilized.

*Discussion.* Bucher and McGarrahan previously concluded(6) that incorporation of acetate into CHL by fractionated rat liver homogenates is not influenced by small amounts of RNAse added to incubation mixtures at the same time as labeled precursor. As pointed out earlier in this paper, RNAse is without significant effect unless given a "head start" before precursor is added.

Some comment is in order concerning the amount of RNAse required to produce the effects reported here. Comparison of the RNAse required to inactivate certain protein-synthesizing enzyme systems with that required to inactivate the CHL-synthesizing system indicates that the requirement in the present system is quite high. The nature of the substrate involved in the present experi-

TABLE II. Biosynthesis of Non-Saponifiable Material and Cholesterol Together with Unmetabolized Mevalonic Acid Encountered in Experiments Involving Predigestion with Various Amounts of Ribonuclease.

| RNAse, mg | NSF, cpm/mg | CHL, cpm/mg | MVA found, mg |
|---|---|---|---|
| 0 | 1084 ( 0) | 2557 ( 0) | .0 ( 0) |
| .5 | 298 (72) | 1040 (60) | .31 ( 62) |
| 1.0 | 192 (83) | 620 (76) | .45 ( 90) |
| 2.0 | 130 (88) | 385 (86) | .45 ( 90) |
| 5.0 | 36 (97) | 94 (97) | .63 (126) |

.5 mg 2-$C^{14}$-MVA (B) added after 30 min. from start of experiment followed by a second flushing with oxygen.
Figures in parentheses indicate % inhibition of either biosynthesis or utilization of precursor.

ments, however, is unknown and it is entirely conceivable that the degradation of an "atypical RNA" or other RNAse-sensitive compound may require somewhat more enzyme than required for degradation of a more "typical" RNA.

In searching for an explanation for the phenomenon here described, the possibility has been considered that the inactivation obtained with RNAse may result from interference with the structure of tissue particles and consequent disorganization of the enzyme systems that they contain. Although a number of studies have emphasized the significance of the microsomes in CHL biosynthesis the microsomes do not appear essential for squalene biosynthesis(7,8,9). It may be pointed out in addition that pre-digestion with RNAse has been reported to be without effect on the glucose-6-phosphatase, esterase, triacetic acid lactonase, adenosine - 5′ - phosphatase, acid phosphatase, adenosine triphosphatase, and succinoxidase activity of isolated rat liver microsomes(10). Mitochondria do not appear to be essential for either squalene or CHL biosynthesis(7,8,9).

The possibility was considered that the effects observed with RNAse are due to inhibitory compounds formed from RNA by the enzyme. In a separate experiment an amount of commercial RNA equivalent to the RNA calculated to be present in the liver homogenate used was digested separately with RNAse and then added to homogenate along with MVA. No inhibitory effect was observed.

Some consideration should be given to a mechanism whereby RNAse functions only indirectly in inhibition of NSF and CHL biosynthesis, for example, by interfering with various supporting energy or reductive mechanisms that appear to be essential for conversion of MVA to NSF or CHL.

TABLE III. Biosynthesis of Non-Saponifiable Material and Cholesterol from Mevalonate or Acetate Together with Unmetabolized Mevalonic Acid Encountered in Experiments Involving Predigestion with Ribonuclease.

| Exp. | MVA (mg) | Acetate (mg) | RNAse (mg) | NSF (cpm/mg) | CHL (cpm/mg) | MVA found, mg |
|---|---|---|---|---|---|---|
| 1 | .5 | | | 351 | 489 | 0 |
| | .5 | | 5 | 118 | 95 | .47 |
| 2 | | 1.0 | | 2857 | 7315 | 0 |
| | | 1.0 | 5 | 16 | 7 | 0 |

.5 mg 2-$C^{14}$-MVA (B) or 1.0 mg $C^{14}$-acetate was added 30 min. from start of each exp. followed by a second flushing with oxygen.

TABLE IV. Biosynthesis of Non-Saponifiable Material and Cholesterol Together with Unmetabolized Mevalonic Acid Encountered in Experiments Involving Predigestion with Ribonuclease.

| Exp. | MVA (mg) | RNAse (mg) | NSF (cpm/mg) | CHL (cpm/mg) | MVA found, mg |
|---|---|---|---|---|---|
| 1 | .5 | | 259 | 1380 | 0 |
| | .5 | 5 | 23 | 120 | .64 |
| 2 | | | | | 0 |
| | | 5 | | | 0 |
| | .5 | | 369 | 930 | 0 |
| | .5 | 5 | 135 | 61 | .50 |

2-$C^{14}$-MVA (B) where used was added 30 min. from start of each exp. followed by a second flushing with oxygen.

Obviously further work is required to provide a satisfactory explanation for the experimental data obtained. Pending further elucidation of the mechanism of RNAse inactivation, the technic described stands as a convenient and useful procedure for blocking isoprenoid biosynthesis at the MVA stage.

*Summary.* Utilization of mevalonic acid as determined by incorporation of the 2-$C^{14}$-labeled compound into non-saponifiable material or into cholesterol or by disappearance of microbiological activity attributable to mevalonic acid in rat liver homogenates was found to be abolished by a preliminary digestion with ribonuclease.

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