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Cholesterol and mevalonate metabolism: a time course study *in vivo**

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SUMMARY

Within 30 minutes after injecting DL-mevalonate-2-C^{14} into young rats, the total body nonsaponifiable fraction contained 47.6% of the biologically active C^{14}-mevalonate. Time course data suggest that at this time utilization of tracer MVA was essentially complete. Urinary radioactivity accounted for slightly more than one-half of the dose. The metabolism of nonsaponifiable fractions of liver and gut may be related by two processes having similar but reciprocal half times. Kidney tissue was found to contain the greater part of the radioactivity previously associated with carcass nonsaponifiable fractions. A small but consistent amount of label was found in the saponifiable fractions.

The problem of cholesterol and fatty acid biosynthesis, transport, and turnover has been under investigation in this laboratory for some time (1 to 5), the larger part of these studies having been made with acetate-1-C^{14} as the labeling agent. The multiple pathways available for acetate metabolism tend to obscure the information desired on any single pathway, particularly if the time periods involved allow for recycling of the tracer.

It appears well established that mevalonic acid (MVA) is a unique and a more direct precursor of squalene and cholesterol in mammalian systems (6 to 15) than is acetate. While most of these studies have been made with homogenates, we have compared acetate and mevalonate as tracers in a liver slice system (1). In the homogenate and liver slice systems, using C^{14}-acetate and C^{14}-mevalonate as tracers, it is possible to obtain definitive information largely on biosynthetic pathways. We have shown that the *in vivo* and *in vitro* methods are not directly comparable for studies on cholesterol metabolism (16), and that both *in vivo* cholesterol transport and turnover may be important facets of cholesterol metabolism (2).

The present experiments were designed to utilize the more selective labeling of cholesterol by mevalonate in studies on cholesterol biosynthesis and turnover in the intact rat. This paper presents data on the early time course of the labeling of the nonsaponifiable fraction in normal rats injected with MVA-2-C^{14}. Included in the presentation are data on the amount of label appearing in CO_2, blood, and urine during the first 4 hours after tracer administration. A detailed analysis of the participation of the tissues of the carcass is also presented.

It is shown that the intact rat quickly utilizes tracer MVA-2-C^{14} for sterol synthesis. The studies reveal that the turnover rates of liver and gut nonsaponifiable fractions seem to be reciprocally related, the processes, moreover, having half times shorter than any of those described by others. Kidney is shown to convert a seemingly disproportionate amount of MVA-2-C^{14} to nonsaponifiable material.

METHODS AND MATERIALS

Male Sprague-Dawley rats (Northwest Rodent Co., Pullman, Wash.) were maintained on Purina Chow for at least 5 days, and were then trained to feeding (5). Animals weighed 80 to 100 g at the time of use. Young animals were utilized because of the observation of Wright and Cleland (9) that liver homogenates from young rats favored incorporation of MVA into cholesterol. In our laboratory, work *in vivo* has indicated, however, that 200-g young adult rats show essentially the same magnitude and pattern of labeling as do the 80-g animals. Following a 1-hour feeding period and a 1-hour fast, animals were injected

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intraperitoneally with 0.42 μc contained in 0.46 μmole of DL MVA-2-C^{14} (Tracerlab, Inc.). This nutritional regimen was followed to minimize the variations in lipogenesis which are in part dependent upon fasting and feeding periods (4, 5, 17). In the present studies 30 animals were used.

Following the injection of label, the animals were maintained in metabolism cages for urine collection.[1] Respiratory CO_2 was collected for various periods on five animals, using the apparatus previously described (18), which allows for total CO_2 collection and also continuously monitors the $C^{14}O_2$ activity. After the specified time interval, the animals were killed by decapitation, at which time blood was collected. Initially, the animals were separated into the four tissue fractions: liver, gut, carcass,[2] and skin. In subsequent experiments the following tissues comprising the carcass fraction were separately studied: kidney, adrenal, epididymal fat pad, brain, lung, spleen, leg muscle, diaphragm, heart, and testes.

All tissues were saponified, and lipid fractions separated as described earlier using an aliquot technique (17). The amounts of fatty acid, the saponifiable fraction, were determined gravimetrically. The nonsaponifiable material was determined colorimetrically[3] by the method of Zlatkis *et al*. (19), using cholesterol as the standard. In our hands these fractions gave essentially identical values when either the Zlatkis color or the Liebermann-Burchard color was used.

The lipid fractions were radioassayed as infinitely thin samples with a Nuclear-D47 Micromil gas flow counter. The radioactivity of the blood and urine samples was assayed as liquid samples (20). The radioassay values were converted to their infinitely thick $BaCO_3$ equivalents, using factors determined in this laboratory. The total activity of the blood was calculated, using 8% of the body weight of the rat as the total blood volume.

[1] It was found during the course of these studies that the urinary excretion of radioactivity is a good indication of a successful intraperitoneal injection. If the injection is made incorrectly, i.e., into the gut or into some other tissue mass, there is a delayed urinary excretion of activity. The occasional animals which excreted less than 40% of the labeled dose in the first hour were not used for the present series because nonsaponifiable fraction labeling in these animals directly paralleled the excreted dose up to a maximum of 23% incorporation.

[2] Residual tissue of the entire animal after removal of liver, gut, and skin. The carcass fraction also contains the blood collected at the time of decapitation.

[3] In this paper the word cholesterol is used to describe the material colorimetrically determined in nonsaponifiable fractions. In our hands the colorimetric determination and radioassay of other nonsaponifiable fractions of liver, gut, carcass, and skin fractions, one and two hours after giving mevalonate-2-C^{14} to intact animals, indicate that better than 90% of the C^{14} is in the form of digitonin precipitable, color-reacting sterol. Since our present interests are in cholesterol or related sterols, the use of digitonin was omitted.

RESULTS

In the upper portion of Table 1 are recorded, as cholesterol, the amounts of nonsaponifiable material found in the major tissues studied, and in the lower portion the values for the various tissues of the carcass fraction. Carcass tissue contains 55% of the animal's total cholesterol, while the gut, skin, and liver contains 23%, 16%, and 6%, respectively. It is of interest that the amount of cholesterol per gram of tissue is relatively constant in the four fractions cited above. Brain and adrenal tissue each have an appreciably higher cholesterol concentration than other tissues. The amounts of cholesterol in the various fractions are similar to those found by Chevallier (21).

TABLE 1. CHOLESTEROL CONTENT OF TISSUE AND DISTRIBUTION OF BODY CHOLESTEROL

Fraction	No. of Animals	Tissue Weight	Cholesterol		Total Body Cholesterol
		g	*mg/g*	*mg*	*per cent*
Liver	16	4.5	2.6* ± 0.6	11.7	6
Gut	16	21.4	2.0 ± 0.6	42.8	23
Skin	17	10.3	2.9 ± 0.9	29.9	16
Total carcass†	17	51.7	2.0 ± 0.3	103.4	55
Kidney	7	0.88	4.3* ± 0.4	3.8	2.0
Brain	4	1.70	13.1 ± 2.9	22.3	11.9
Adrenals	8	0.02	34.5 ± 11.0	0.7	0.4
Diaphragm	3	0.32	0.73‡	0.2	0.1
Leg muscle	3	1.00§	0.85	0.9	0.5
Testes	2	0.84	1.90	1.9	0.9
Epididymal fat pads	2	0.80	0.51	0.4	0.2
Lung	2	0.64	4.50	2.9	1.5
Spleen	2	0.31	3.81	1.2	0.6
Heart	3	0.34	1.33	0.5	0.2

* Mean value ± standard deviation.
† Residual tissue of the whole animal after removal of the liver, gut, and skin.
‡ Mean value.
§ One gram of muscle tissue was used. The total muscle and bone mass accounts for the remaining cholesterol listed above as being present in the carcass fraction.

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Table 2 presents information on the total amounts of the administered dose found in various fractions through the 4-hour time period. The molecular species containing the radioactivity of the blood and urine were not characterized, but it is possible that the greater part of the activity in the urine is the biologically inactive isomer of MVA. The first observation of interest from Table 2 is that in 30 minutes nonsaponifiable fraction label reached 95% of the maximum which was found at the 1-hour postinjection time.

TABLE 2. RECOVERY OF INJECTED MEVALONATE-2-C^{14} RADIOACTIVITY*

Time	No. of Animals	Fractions					Total Recovery
		CO_2†	Nonsaponifiable‡	Saponifiable‡	Urine§	Blood§	
15 min	3	0.46	15.9	2.5	16.0	7.0	41.9
30 min	3	1.44	22.7	3.0	23.3	5.0	55.4
1 hour	3	3.50	23.8	3.8	52.5	2.5	85.8
2 hours	8	6.40	21.4	2.3	59.6	2.4	92.3
3 hours	3	8.00	21.9	3.3	58.6	2.5	94.3
4 hours	5	9.00	18.0	3.6	57.0	2.7	90.4

* Per cent of the total dose of DL MVA-2-C^{14}.

† The CO_2 data were obtained from a total of 5 animals, the serial samples being so spaced in time as to yield a minimum of three values for each time interval.

‡ Sum of activities of all tissues, i.e., total animal activity.

§ Total radioactivity of the urine collected after injection, and of the total blood volume.

It appears that about one-fourth of the tracer dose (or about one-half of the biologically active isomer) is the maximum which can be incorporated into the nonsaponifiable fraction under the present conditions. Of the total dose, 3.8% is directed to the saponifiable fraction, and again in this fraction an early maximum is reached. Urinary excretion of C^{14} activity is 90% complete within the first hour, paralleled by a decrease in radioactivity of the blood to a low level at this time. At the 15- and 30-minute time intervals, total recovery of activity is only 41.9% and 55.4%, respectively. It is assumed that the activity unaccounted for in this total is as yet unused MVA per se. We have shown previously (1) that MVA remains in an aqueous phase, normally discarded in our fractionation procedure, and does not appear either in our saponifiable or nonsaponifiable fractions. By the end of the first hour, 86% of the injected radioactivity was recovered. The excretion of $C^{14}O_2$, as indicated in Table 1, is shown to increase during the 4 hours and differences between these figures, each normalized to periods of 1 hour, reveal that a maximum rate was obtained at 1 hour, the subsequent intervals showing a decreased rate of $C^{14}O_2$ excretion.

Table 3 presents data on the radioactivity found in the nonsaponifiable fractions of liver, gut, carcass, skin, and brain over a 24-hour period following injection. The radioactivity of liver, carcass, and skin fractions reached maximum values within a short time. The brain tissue of these intact animals is relatively inert as regards lipid synthesis, even in these young animals. There was a loss of nonsaponifiable fraction activity from the liver after the early maximum, and there was a concomitant increase in the labeling of the corresponding fraction of gut. If the information concerning liver

TABLE 3. TIME COURSE OF NONSAPONIFIABLE FRACTION LABELING FROM MVA-2-C^{14}

Time*	No. of Animals	Incorporation into Nonsaponifiable Lipid				
		Liver	Gut	Carcass	Skin	Brain
		per cent	*per cent*	*per cent*	*per cent*	*per cent*
15 min	3	4.0† (3.3–4.7)	1.1 (0.8–1.7)	10.1 (8.9–11.2)	0.4 (0.3–0.4)	0.04
30 min	3	4.9 (4.6–5.2)	1.3 (1.1–2.9)	16.0 (15.2–16.7)	0.7 (0.5–0.8)	0.03
1 hour	3	4.6 (4.1–5.7)	1.8 (1.5–2.0)	17.1 (16.3–17.9)	0.6 (0.6)	
2 hours	4	2.3 (1.8–2.5)	2.6 (2.4–2.7)	17.4 (16.7–18.5)	0.5 (0.5–0.6)	
3 hours	3	2.5 (1.9–3.1)	2.9 (2.4–3.4)	16.0 (14.9–17.0)	0.5 (0.5)	0.05
4 hours	3	1.8 (1.2–2.4)	3.0 (2.9–3.1)	13.0 (9.5–16.5)	0.5 (0.5)	0.06
24 hours	2	1.1 (0.9–1.5)	2.6 (2.4–2.8)	16.6 (16.5–16.6)	0.6	

* Time animal was sacrificed following injection.

† Mean (upper figure) and range of values (figures in parentheses).

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and gut (Table 3) is plotted as in Figure 1, biphasic reciprocal curves appear to be present in these fractions. The liver fraction has a rapid component with a half life ($t^1/_2$) of 1.3 hours, and a slower component with a $t^1/_2$ of 5.0 hours. The gut fraction is resolvable into two components that have half lives of 1.5 and 8.0 hours.

Because of the cellular and metabolic heterogeneity of the carcass fraction, it was felt desirable to define more precisely the participation of the different tissues. Table 4 records the values obtained on the distribution

TABLE 4. DISTRIBUTION OF LABELED NONSAPONIFIABLE RADIOACTIVITY 2 HOURS POSTINJECTION

Fraction	No. of Animals	Total*	Fraction	No. of Animals	Total*
		per cent			per cent
Kidney	4	64.0† (46.0–77.0)	Lung	3	0.7 (0.5–1.0)
Carcass‡	4	10.6 (7.0–15.4)	Spleen	3	0.5 (0.3–0.8)
Gut	4	12.1 (11.4–12.6)	Diaphragm	3	0.2 (0.1–0.3)
Liver	4	10.7 (8.4–11.7)	Heart	2	0.2 (0.1–0.3)
Skin	4	2.5 (2.3–2.5)	Testes	3	0.2 (0.1–0.3)
Adrenal	2	0.2 (0.1–0.2)	Muscle§	4	0.2 (0.1–0.5)
Epididymal fat pads	3	0.2 (0.2–0.3)			

* Per cent of the animals' total nonsaponifiable radioactivity found in the indicated tissues.
† Mean (upper figure) and range of values (figures in parentheses).
‡ Residual muscle mass and bone structure after removal of the other tissues listed.
§ One gram of tissue.

of labeled nonsaponifiable material 2 hours after mevalonate injection. The results for the liver, gut, and skin fractions agree well with the previous data for animals 2-hour postinjection (Table 3). A most interesting finding is the relative amount of radioactivity found in the nonsaponifiable fraction of kidney tissue. The other fractions of the carcass contribute relatively little of the radioactivity of the nonsaponifiable fraction when compared to the kidney.

With the finding that at 2 hours the kidney contained the greater part of the radioactivity of the carcass fraction, it appeared desirable to define more precisely the role of the kidney in cholesterologenesis. To this end, a special time course study was made to follow

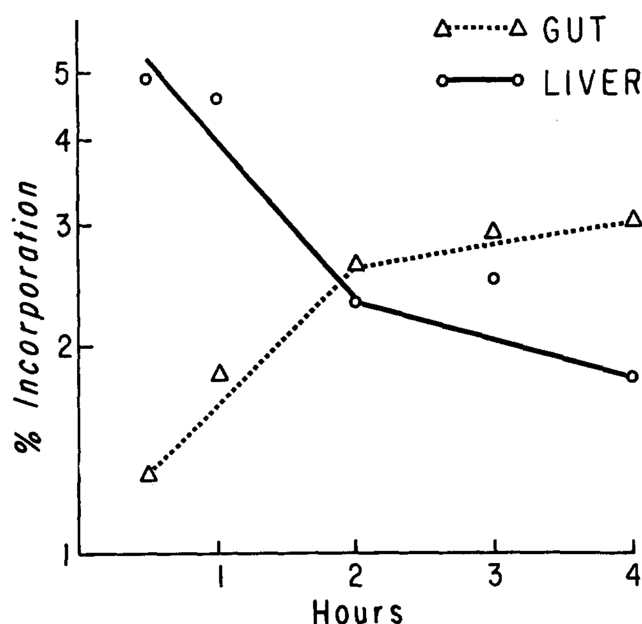

FIG. 1. Turnover of nonsaponifiable fractions of liver and gut. The logarithms of the per cent incorporation of MVA-2-C^{14} into nonsaponifiable fractions have been plotted with respect to time after injection. The straight line functions were obtained by the method of least squares. The half life of the fast component of liver is 1.3 hours, and of the slower component 5.0 hours. The more rapid component of gut tissue has a half life of 1.5 hours, and the slower component one of 8.0 hours.

the metabolism of the nonsaponifiable fraction in kidney tissue. From the data of Table 5 it is clear that at

TABLE 5. TIME COURSE DATA ON TOTAL CARCASS AND ON KIDNEY TISSUE

Time	No. of Animals	Incorporation of MVA-2-C^{14} into Carcass		Carcass Activity Found in Kidney	
		Nonsaponifiable Fraction	Saponifiable Fraction	Nonsaponifiable C^{14}	Saponifiable C^{14}
		per cent	per cent	per cent	per cent
15 min	2	12.5* (11.8–13.3)	0.9†	79.1	36.0
1 hour	2	17.9 (17.2–18.6)	1.5†	75.2	39.5
2 hours	4	13.9 (9.9–16.6)	0.7 (0.31–1.21)	65.0	29.2
3 hours	1	15.6 (15.6)	0.9†	71.0	27.3
4 hours	2	13.0 (12.8–13.2)	0.6 (0.5–0.6)	72.2	16.7

* Mean value (upper figure) and range of values (figures in parentheses).
† Single determinations.

the 15-minute time the kidney contains some 79% of the radioactivity generally attributed to the carcass nonsaponifiable fraction. This radioactivity remained high during the 4-hour period of study. Some 40% of the total carcass saponifiable fraction was also found in kidney tissue at the 1-hour time interval.

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DISCUSSION

The distribution of cholesterol in four rat tissues of liver, gut, carcass, and skin has been followed and reported from this laboratory in previous publications (2 to 5). The present investigation has shown the total carcass to be a metabolically heterogeneous system. The adrenal gland is shown to contain the highest concentration of color-producing sterol. The brain contains by far the largest total quantity of sterol. Liver, gut, skin, kidney, testes, lung, spleen, and heart contain lesser amounts of cholesterol per gram of tissue, but of this last group, kidney contains the highest. In contrast to the organ structures, muscle contains only small amounts of cholesterol per gram of tissue.

The information presented in Table 2 shows that 30 minutes after the intraperitoneal injection of MVA-2-C^{14}, 22.7% of the C^{14} dose is found in the nonsaponifiable fraction, 3% in the saponifiable fraction, 23.3% in the urine, 5% in the blood, and 1.44% in the CO_2, giving a total recovery of 55.4% of the injected dose. In the next 30 minutes the animals seemingly utilized all the available MVA. In other words, labeling of the nonsaponifiable fraction from MVA would appear to be essentially complete after 30 minutes, so that any major change in the radioactivity of this fraction should be due to transport or metabolism. It is also likely that the major part of the label was now in cholesterol fractions because calculations made, as before (1), reveal that the $C^{14}O_2$ activity at 1 hour represents the conversion of 0.0081 μmole of L-mevalonate to CO_2. Considering the radioactivity of the nonsaponifiable fraction at this time to represent true cholesterol activity, one would expect the release of some 0.01 μmole of $C^{14}O_2$ by the demethylation of lanosterol to cholesterol. The similarity in magnitude of these observed and calculated figures is of interest.

It was originally thought that the radioactivity in the saponifiable fraction might consist of intermediates in cholesterol synthesis (22), i.e., farnesyl pyrophosphate, but the maintenance of this activity over the 4-hour period would tend to rule this out. If the activity was present in obligate precursors of cholesterol, there would probably have been diminished amounts of label remaining in the saponifiable fraction after 30 minutes. It can be concluded that this activity constitutes substances not on the direct cholesterol biosynthesis pathway, and perhaps similar to the acid described by Ogilvie and Langdon (23, 24). Elwood *et al.* (1) and Popják (22) have previously recorded activity of saponifiable components from MVA-2-C^{14} labeling.

The total radioactivity of the blood diminishes rapidly during the first 30 minutes, probably reflecting the combination of rapid tissue uptake, rapid synthetic reactions, and also urinary clearance during the same period. The activity in the blood during the first half hour probably results chiefly from both D- and L-MVA. The information reported in Table 3 on the amount of label in the various tissue nonsaponifiable fractions may represent the results of both synthesis and mobilization of lipid. The radioactivity present in the liver declines at a rapid rate during the first 2 hours, the liver losing approximately one-half of its radioactivity in this period. This loss appears to be caused by at least two processes, the more rapid one having a half life of about 1.3 hours, and the slower one a half life of 5.0 hours. In other time course studies (2), there were shown to be two components of decay, one with a half life of about 15 hours and a slower component of 8.9 days. The two rapid components described in the present study were, of course, not detectable in the previous long-term experiment. When the two studies are considered together, the metabolism of nonsaponifiable lipid in the liver may be described as the resultant of a minimum of four processes, having half times of 1.3, 5.0, 15, and 210 hours.

The gut is seen to increase in labeled nonsaponifiable radioactivity during the 4-hour interval. Figure 1 shows a biphasic-type curve for this response, the half life of the fast component being 1.5 hours and that of the slow component being 8.0 hours. Although it is not possible to equate arithmetically the radioactivity present in the gut and liver tissues, it would seem likely that at least a portion of the newly synthesized sterol was not in equilibrium with total liver sterol, and was, in fact, selectively secreted from the liver. The 5.8% of the tracer dose present in nonsaponifiable material in the gut at the 15-minute time interval would seem to indicate that appreciable cholesterol synthesis had occurred. As shown previously in this laboratory (25), the use of the intraperitoneal route of tracer acetate administration resulted in gut tissue containing more of the label than from the intravenous route of dosage. As yet, it is not clear if this results from a local utilization of tracer before systemic distribution is effected, or whether it is caused by selective distribution to certain tissues.

The skin appears to synthesize a small amount of nonsaponifiable material during the initial time when MVA is available to it. However, no further increase or decrease is seen in the activity during the time of observation. In the previous studies (2), nonsaponifiable radioactivity of skin increased in the first 4-day period. This slower response may not be detectable in the short time of the present experiments. The brain

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was quite inert as regards synthesis from MVA, or in storage of newly formed nonsaponifiable material. The activity reported here for brain may actually represent residual blood activity.

It is clear that the kidney is the tissue of the carcass which is responsible for the maximum nonsaponifiable labeling from MVA in these rats, since 79% of the carcass nonsaponifiable activity was found in the kidney at the end of 15 minutes, and this activity remained essentially constant during the 4-hour period under investigation.

These findings on the kidney tissue should not be directly interpreted as representing an unduly great synthesis of nonsaponifiable material. The intact kidney may collect, and thus concentrate, the labeled MVA, and in this fashion increase the amount of C^{14} diverted to biosynthesis in this tissue. Similar findings in the saponifiable fraction, i.e., 36% of the activity in the total acidic material found in the kidney at the 15-minute time interval, would also indicate the increased availability of label to kidney tissue. Unfortunately, without knowledge of the specific activity of the precursor pools of the individual tissues, it is not possible to compare directly the rates of synthesis of various tissues. In other *in vitro* studies (unpublished data), active labeling of nonsaponifiable and saponifiable components from mevalonic acid in kidney tissue was confirmed.

From previous work in our laboratory (2) it can be calculated that 15.8%, 35.2%, 34.9%, and 14.1% of the labeled nonsaponifiable material was in the liver, gut, carcass, and skin, respectively, 2 hours following injection of acetate-1-C^{14}. The present work lists values of 11.2%, 13.2%, 77%, and 2.7% for similar fractions labeled by MVA-2-C^{14}. It becomes evident that the labeling pattern of body lipid is grossly different when MVA-2-C^{14} is the precursor than when acetate-1-C^{14} is used.

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