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Bile acids and lipids in isolated rat hepatocytes. II. Source of cholesterol used for bile acid formation, estimated by incorporation of tritium from tritiated water, and by the effect of ML-236B

Herman Jan Kempen, Margreet Vos-van Holstein, and Jan de Lange

Gaubius Institute, Health Research Division TNO, Herenstraat 5d, 2313 AD Leiden, The Netherlands

Abstract After incubation in the presence of tritiated water, incorporation of tritium into cholesterol and into different bile acids was several-fold higher using hepatocytes of cholestyramine-fed rats than that found using hepatocytes of control rats. Labeling of the trihydroxylated cholic and β-muricholic acids was markedly greater than that of dihydroxycholanoic acid. The total amount of label in all bile acids was 30% or less of that in free cholesterol, in both types of hepatocytes. In combination with the data on bile acids mass production we could calculate the average number (N^a) of tritium atoms incorporated per molecule of newly-formed bile acid. The experimental values of N^a for cholic and β-muricholic acid were compared with values of N^n or N^o, theoretically predicted if these bile acids were derived entirely from newly made or pre-existent cholesterol, respectively. It was deduced for hepatocytes of cholestyramine-fed rats that the bile acids produced in the first hour of incubation should be totally derived from pre-existent cholesterol, whereas 50% and 25% of the cholic and β-muricholic acid, respectively, produced during the second and third hours of incubation should be derived from newly synthesized cholesterol. The contribution of newly made cholesterol as substrate for bile acid production was also estimated by using ML-236B. In a concentration of 12 μM, it depressed cholesterol synthesis by 90% during 1 or 3 hours of incubation of hepatocytes of cholestyramine-fed rats. Mass production of cholic acid was depressed by 25% and that of β-muricholic acid was not inhibited at all by ML-236B during the first hour of incubation, while they were depressed by 71 and 52%, respectively, during the second plus third hours of incubation. It is concluded that *1)* in isolated hepatocytes newly made cholesterol can be a significant substrate for bile acid formation; *2)* there are separate cholesterol substrate pools for the productions of cholic or β-muricholic acid; *3)* of the total carbon flux directed into cholesterol synthesis, the major part ends up as free cholesterol and only a minor part as bile acids, even in hepatocytes with a derepressed bile acid production.—**Kempen, H. J., M. Vos-van Holstein, and J. de Lange.** Bile acids and lipids in isolated rat hepatocytes. II. Source of cholesterol used for bile acid formation, estimated by incorporation of tritium from tritiated water, and by the effect of ML-236B. *J. Lipid Res.* 1983. **24:** 316–323.

Supplementary key words cholestryamine • newly synthesized cholesterol • endogenous cholesterol

A number of authors have quantitated the rate of hepatic cholesterol synthesis by measuring incorporation of tritium label in cholesterol after giving tritiated water to the liver, or the animal (1–7). This method is based on the fact that a number of steps in the biosynthesis of cholesterol comprise saturation of carbon-carbon or carbon-oxygen double bonds, and thus lead to incorporation of hydrogen into stable carbon-hydrogen bonds. These hydrogen atoms are obtained either directly from medium water, or from NADPH in the cytosolic compartment. Taking into account the individual enzymatic steps between acetyl-CoA and cholesterol, Lakshmanan and Veech (4) arrived at a theoretical number of 22 ^{3}H-atoms incorporated from tritiated water in a molecule of cholesterol newly formed from acetyl-CoA, of which 15 would be introduced via NADPH. Experimental data, obtained by various authors for rat liver in vitro using ^{14}C-labeled substrates and tritiated water, are consistent with values between 21 and 25 ^{3}H-atoms per molecule newly-made cholesterol (5, and references given therein). This agreement between the theoretically expected number and experimental values suggests that during short-term experiments in vitro *a)* cytosolic NADPH is rapidly equilibrated with ^{3}H from tritiated water, and *b)* cytosolic acetyl-CoA is virtually the sole substrate for de novo synthesis of cholesterol (i.e., there is no substantial cholesterol formation from pre-existent precursors like mevalonate, squalene, etc.).

It is well known that newly synthesized cholesterol in the liver can serve as substrate for bile acid formation (8–12). Consequently, a bile acid molecule produced in the presence of tritiated water can possess a number of ^{3}H-atoms, the actual value of this number depending on whether this molecule is derived from newly synthesized (^{3}H-labeled) cholesterol or pre-existent (unlabeled) cholesterol (see Appendix I).

We have shown previously, that rat hepatocytes pro-

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duce cholic and β-muricholic acids during in vitro incubation, and that the production rates of both bile acids are markedly increased when hepatocytes are taken from cholestyramine-fed rats (13). In the present study we have measured ^{3}H-incorporation into these bile acids and into cholesterol, after incubation of hepatocytes in the presence of tritiated water. In combination with the mass productions of these bile acids this enabled us to calculate the average number of ^{3}H-atoms incorporated per molecule of cholic or β-muricholic acid, newly formed during the incubation. From this number, and from available literature data, conclusions were drawn concerning the relative contribution of newly synthesized and pre-existent cholesterol as substrates for bile acids formation. It was inferred that a significant contribution from de novo synthesis occurred only during the second and third hour of incubation, while all bile acids produced during the first hour were derived from pre-existent cholesterol.

To estimate the contribution of these two cholesterol sources as substrate in another independent manner, we have used the compound ML-236B, a potent and selective inhibitor of the enzyme HMG-CoA reductase (14) and of cholesterogenesis (15, 16). In agreement with the above conclusions, this substance depressed the mass productions of both bile acids much stronger during the second and third hours than during the first hour of incubation.

MATERIALS AND METHODS

Sources of commercially obtained materials were as described before (12). Source and treatment of the rats, preparation of the cholestyramine-containing diet, isolation and incubation of hepatocytes, addition of a trace of ^{14}C-taurocholic acid, and extraction of lipids and bile acids were all as outlined in our previous paper (13). During the incubations tritiated water was present in a concentration of 0.25 mCi/ml.

The lipids in the chloroform layer were dried down, and dissolved in 4.5 ml of chloroform–methanol 1:2. After induction of phase separation by addition of chloroform (1.5 ml) and water (2.7 ml), the chloroform layer was collected and dried again under a stream of nitrogen. This double extraction removed the last traces of tritiated water from the lipids. One-quarter of the total lipid residue was applied on a thin layer (0.25 mm) of silica; after chromatographic separation the various lipid classes were identified by iodine staining, scraped from the plate, eluted, and dried (17). Tritium radioactivity was determined directly in free cholesterol after dissolving it in 10 ml of Insta Fluor®, by counting in a scintillation spectrometer (Packard 2425). Cholesteryl esters were saponified in 2 ml of 0.3 M KOH in 95% ethanol for 30 min at 70°C. After addition of 2 ml of water, the liberated cholesterol was extracted from the hydrolysate with 2 × 5 ml of hexane. The hexane extract was dried under a stream of nitrogen and the residue was dissolved in Insta-Fluor® to be counted for radioactivity.

The bile acids in the upper layer of the extraction were isolated using a cartridge of C-18 silica, then enzymatically deconjugated, separated by thin-layer chromatography, scraped from the plates, eluted, and dried, as described before (13). The residues were dissolved in 0.6 ml of Tris-HCl buffer, pH 9.0, also containing NAD and hydroxysteroid dehydrogenase (13). The amount of NADH generated after 60 min incubation was determined by luminometry in 10 μl of the incubation mixture (13). The remaining part of the incubation was mixed with 10 ml of Picofluor® (Packard), and counted for ^{3}H and ^{14}C radioactivity using the ^{3}H/^{14}C preset channels of a Packard 2425 scintillation counter. Counting was repeated after addition of internal standards (tritiated water and ^{14}C-taurocholate) to the counting vials, in order to assess counting efficiency for ^{3}H, spillover of ^{14}C activity in the ^{3}H-channel, and recovery of ^{14}C-taurocholate added to the hepatocyte incubation tubes. Values for these experimental parameters were around 45%, around 50%, and between 50 and 60%, respectively.

The recovery of the different bile acids, isolated from each individual incubation, was considered to equal that of ^{14}C-taurocholate. The results of mass and ^{3}H-radioactivity assays were corrected accordingly.

Calculations

The water content of a 2.5-ml incubation was calculated to equal (in mg) 2525-33-50-(mg cell dry weight), where 2525 represents the total weight, 33 the dry mineral weight, 50 the weight of the added albumin (all in mg), per incubation. The specific activity of ^{3}H in water (SA_w) was then obtained using the formula:

$$SA_w = \frac{(\text{mCi } ^3\text{H/incubation}) \times 2.22 \times 10^9}{(\text{mg water/incubation}) \times (10^6/18) \times 2} \text{ dpm/ng-atom H.}$$

In our experiments, a value between 5 and 6 dpm/ng-atom H resulted. Division of the found radioactivity (dpm) in cholesterol or bile acids by SA_w yielded ng-atoms ^{3}H incorporated. Finally, the average number (N^a) of ^{3}H atoms introduced in a molecule of bile acid synthesized during incubation was calculated by using the formula:

$$N^a = \frac{\text{ng-atom } ^3\text{H incorporated in bile acid}}{\text{nanomoles of bile acid produced}}.$$

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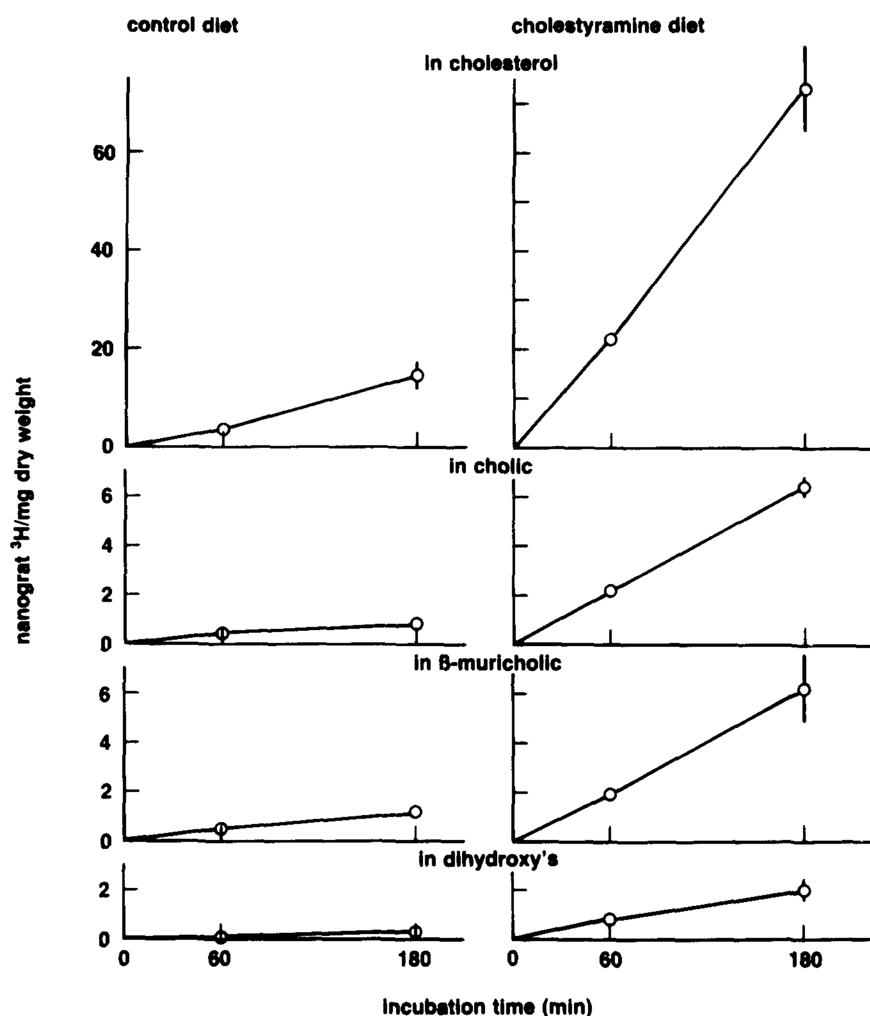

Fig. 1. Incorporation of tritium from tritiated water into cholesterol and in cholic, β-muricholic, and chenodeoxycholic acids after 60 or 180 min of incubation of hepatocytes, isolated from control (n = 6) or cholestyramine-fed (n = 8) rats. Data represent means ± 1 SEM.

RESULTS

Effects of cholestyramine on tritium incorporation from tritiated water in cholesterol and bile acids

During incubation ^{3}H was incorporated into free cholesterol at a rate that was five-fold higher in hepatocytes of cholestyramine-fed rats than in those of control rats (**Fig. 1,** upper panels). Incorporation of ^{3}H in the cholesterol moiety of the cholesteryl ester fraction was undetectably low in hepatocytes of control rats, while in hepatocytes of cholestyramine-fed rats it amounted to 1.7 and 3.2% of the label found in free cholesterol, after 1 and 3 hr of incubation, respectively (not shown).

Tritium was found to be incorporated in cholic, β-muricholic, and dihydroxycholanoic acids (Fig. 1, lower panels). The amount of label in the former two bile acids was similar, and clearly higher than that in the latter. Hepatocytes from cholestyramine-fed rats incorporated several-fold higher amounts of tritium in these three bile acids than cells from control rats. Furthermore, with hepatocytes of cholestyramine-fed rats, the rate of ^{3}H incorporation in bile acids was constant during the entire length of the 3-hr incubation period, whereas it fell off after the first hour of incubation of the control cells.

Total incorporation of tritium in all three bile acids was several-fold lower than in free cholesterol, after 1 hr or 3 hr incubation of both types of hepatocytes.

Number of tritium atoms incorporated per molecule of newly formed bile acid

From these data, together with the results of the bile acid mass determinations, it is possible to calculate the average number (N^a) of ^{3}H atoms incorporated per molecule of newly synthesized cholic or β-muricholic acid. No net production of dihydroxycholanoic acid

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TABLE 1. Incorporation of tritium[a] in cholic and β-muricholic acids (R^a), mass productions[b] of these bile acids (P^a), and calculated values of N^a ($=R^a/P^a$) for incubations of hepatocytes of cholestyramine-fed rats

Parameter	Incubation Period	Cholic Acid	β-Muricholic Acid
R^a	0–60 min	2.45 ± 0.68	2.82 ± 0.50
	60–180 min	4.29 ± 0.54	6.51 ± 2.41
P^a	0–60 min	0.99 ± 0.21	0.90 ± 0.08
	60–180 min	0.43 ± 0.12[c]	0.84 ± 0.34[c]
N^a	0–60 min	2.6 ± 1.0	3.1 ± 0.5
	60–180 min	10.4 ± 1.8	7.9 ± 1.0

[a] In ng-atoms of tritium incorporated/mg dry cell weight.
[b] In nmol of bile acid produced/mg dry cell weight.
[c] Significantly lower production rate than in first hour ($P < 0.01$).
Data are means ± 1 SD for four separate experiments.

occurred during the hepatocyte incubations (13), prohibiting the calculation of N^a for this bile acid.

Calculation of N^a for the bile acids produced by hepatocytes of normally fed rats was omitted in view of the relatively large error in the low rates of ^3H-incorporation and mass production. The calculation of N^a for cholic and β-muricholic acids, produced by hepatocytes of cholestyramine-fed rats is shown in **Table 1.** The values of N^a are found to be about 3 for the two bile acids produced during the first hour of incubation, but rise to about 10 for cholic acid and about 8 for β-muricholic acid, produced during the second plus third hours of incubation.

Cholesterol source for bile acid production, as deduced from the values of N^a

The experimental values of N^a, arrived at in the previous section for hepatocytes of cholestyramine-fed rats, can be compared with theoretical values N^n and N^o, to be expected if the bile acids would be synthesized entirely from newly made (N^n) or pre-existent (N^o) cholesterol. These theoretical values have been deduced from pertinent literature dealing with the number and location of tritium atoms in newly synthesized cholesterol (4, 5) and with the various steps involved in the conversion of cholesterol into bile acids (18–23) (see Appendix I). For N^n we have arrived at a value of 18 or 20 and for N^o at a value of 3 or 4 ^3H-atoms per molecule of newly formed cholic or β-muricholic acid, respectively. The experimental values of N^a for both bile acids, produced during the first hour of incubation, are even below the theoretical values of N^o (Table 1), which suggests that bile acids are derived only from pre-existent cholesterol in that period.

In contrast, the experimental values of N^a for both bile acids during the second and third hour of incubation are between the theoretical values of N^o and N^n. This indicates that during this incubation period a certain fraction of both cholic and β-muricholic acid is derived from newly made cholesterol. The size of this fraction (x) can be calculated using equations (4) and (5), deduced in Appendix II. As shown in **Table 2,** mean values of 0.50 and 0.25 are obtained for the fraction of cholic and β-muricholic acid, respectively, derived from newly made cholesterol. The difference between these two values is statistically significant. The absolute amounts of the two bile acids, produced from either newly synthesized or pre-existent cholesterol, are also given in Table 2.

Cholesterol source for bile acid production, as deduced from the effect of ML-236B

As an independent check of the methodology described above, we have studied the effect of ML-236B, a potent inhibitor of cholesterol synthesis (14–16), in the same experiments with hepatocytes of cholestyramine-fed rats as described in the previous section.

As shown in **Fig. 2,** ML-236B depressed tritium incorporation into free cholesterol by 88% during the first hour, and by 91% during the second and third hours of incubation, when present in a concentration of 12 μM. Tritium incorporation into fatty acids was not affected by the drug (not shown).

The effect of this treatment on the mass production

TABLE 2. Fractions of total cholic and β-muricholic acids production derived from newly made cholesterol (x), and absolute productions[a] of these bile acids from newly made (P^n) or pre-existent (P^o) cholesterol in hepatocytes of cholestyramine-fed rats, as deduced[b] from the values of N^a

Incubation Period	Cholic Acid			β-Muricholic Acid		
	x	P^n	P^o	x	P^n	P^o
0–60 min	0	0	0.99 ± 0.21	0	0	0.90 ± 0.08
60–180 min	0.50 ± 0.12	0.20 ± 0.02	0.23 ± 0.12	0.25 ± 0.06[c]	0.20 ± 0.02	0.64 ± 0.29

[a] In nmol produced/mg dry weight.
[b] First, the values of x were calculated by substituting the values of N^a into equations *4* or *5* of Appendix II; next, P^n was calculated as $x \cdot P^a$ and P^o as $(1 - x) \cdot P^a$.
[c] Significantly lower than for cholic acid ($P < 0.01$).
Data represent means ± 1 SD for the same four experiments as given in Table 1.

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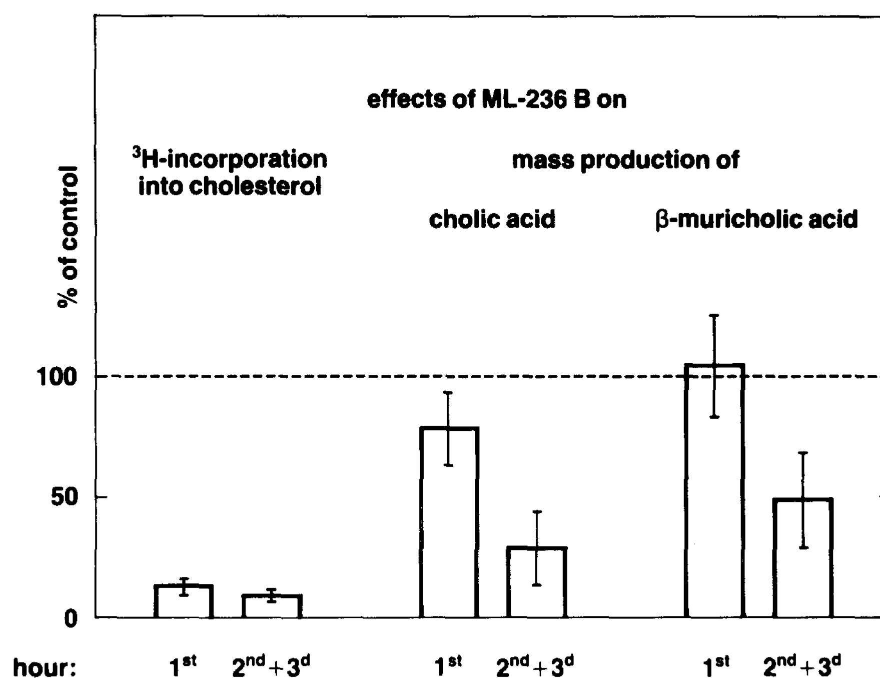

Fig. 2. Percentile effects of ML-236B (12 μM) on the tritium incorporation into free cholesterol (left), and on the mass productions of cholic and β-muricholic acids (right), during the first hour or second and third hours of incubation. Data represent means ± 1 SD, and refer to the same four experiments as shown in the Tables.

of bile acids is also shown in Fig. 2. During the first hour of incubation, ML-236B inhibited the production of cholic acid by 21% and had no effect on that of β-muricholic acid. In contrast, the productions of these two bile acids were decreased by 72% and 51%, respectively, during the second and third hours of incubation, the difference between these two values being statistically significant.

The amounts of cholic and β-muricholic acid, produced from newly made cholesterol in the absence of ML-236B, were calculated from these data by assuming that *1*) these productions are depressed by ML-236B to the same degree as the tritium incorporation into cholesterol, and *2*) that the drug does not affect the conversion of pre-existent cholesterol into bile acid (see Appendix III).

As shown in **Table 3,** these calculations indicate that about one-quarter of the cholic acid, and none of the β-muricholic acid, produced during the first hour of incubation, is derived from newly made cholesterol, whereas 76% of the cholic acid and 56% of the β-muricholic acid produced during the second and third hours of incubation are derived from this source. The difference between these values for the two bile acids is statistically significant. Moreover, these values are significantly higher than the corresponding ones, arrived at in the previous section (compare the values of x in Table 2 with those in Table 3).

TABLE 3. Productions of cholic and β-muricholic acids from newly made cholesterol (P^n), or pre-existent (P^o) cholesterol, both absolute[a] and as fraction of the total mass production (x), by hepatocytes of cholestyramine-fed rats, as deduced[b] from the effect of ML-236B on the tritium incorporation into cholesterol and on the total bile acid mass productions

Incubation Time	Cholic Acid			β-Muricholic Acid		
	P^n	P^o	x	P^n	P^o	x
0–60 min	0.25 ± 0.19	0.74 ± 0.11	0.24 ± 0.15	0.06 ± 0.07	0.85 ± 0.14	0.07 ± 0.08
60–180 min	0.34 ± 0.16	0.09 ± 0.06	0.76 ± 0.18	0.50 ± 0.36	0.34 ± 0.23	0.56 ± 0.22[c]

[a] In nmol/mg dry weight.
[b] First, P^n is calculated as outlined in Appendix III; then P^o is found as $P^a - P^n$, and x as P^n/P^a.
[c] Significantly lower than x_{cholic} ($P < 0.05$).
Data represent mean ± 1 SD for the same 4 experiments as presented in Tables 1 and 2.

DISCUSSION

In this paper we have estimated by two different methods to what extent the bile acids produced by hepatocytes of cholestyramine-fed rats are synthesized entirely *de novo* or are derived from pre-existent cholesterol as substrate. Both methods have indicated that the relative importance of de novo synthesis is considerably greater for the bile acids produced during the second and third hours than for those produced during the first hour of incubation. The two methods also agree in finding an increased absolute production rate of β-muricholate from newly made cholesterol during the second and third hours of incubation, as compared to that in the first hour. Further, according to both procedures, the absolute production rates of both cholic and β-muricholic acids out of pre-existent cholesterol are strongly decreased after the first hour. The latter evidence suggests that at the start of the incubation a limited amount of pre-existent cholesterol is available for bile acid production, which is largely consumed already after 1 hr of incubation. This cholesterol, carried over from the in vivo situation, may come from plasma lipoprotein material taken up by the hepatocytes immediately before the rat was killed.

Finally, the two methods both lead to the conclusion that the fraction of cholic acid production that is derived from newly made cholesterol is greater than that of β-muricholic acid. This is in line with evidence reported by others for more intact rat liver preparations, that cholic and chenodeoxycholic (9, 10, 12) or cholic and β-muricholic (24) acids originate from partly separate substrate pools, containing newly made and pre-existent cholesterol in different proportions.

In our opinion, the large degree of qualitative agreement between the two methods gives mutual assurance about the validity of each of the two approaches. Notwithstanding this qualitative consistency between both methods, some differences are apparent as well. First, the percentage of the production of bile acids being derived from newly synthesized cholesterol is higher according to the experiments with ML-236B than as deduced from the values of N^a and equations *4* and *5* of Appendix II. Further, the former method indicates that production of cholic acid out of newly made cholesterol takes place already in the first hour, while the latter method does not. The question then arises, which of the two approaches leads to the most realistic conclusions.

Using quite different approaches, Long et al. (11) found a mean value of 48% for the percent of biliary acidic sterols derived from newly made cholesterol in rats having a bile fistula for 9–11 hr, while Björkhem and Lewenhaupt (12) report a value of 50% for the percent of cholic acid derived from this source in rats with fistula existing for 48 hr. Based on these data it is reasonable to expect that hepatocytes of cholestyramine-fed rats are able to produce a certain amount of bile acids from newly made cholesterol during the first hour of incubation as well. Accordingly, we think that the approach with ML-236B gives truer information than the approach based on substitution of N^a into the equations of Appendix II.

We have indeed obtained an experimental indication that the tritium incorporation in bile acids in reality does not take place according to theory set out in Appendix I. The experimental values of N^a for both cholic and β-muricholic acids (Table 1), produced during the first hour of incubation, are even below the theoretically predicted values for N^o. If this is not the result of an incomplete equilibration of the cytosolic NADPH with tritiated water, this would suggest that the present knowledge of the processes of cholesterol and/or bile acid biosynthesis is incorrect or incomplete.

A possible reason to distrust the conclusions reached by using ML-236B is the finding, reported by Endo et al. (25), that treatment of rats for 8 days with a daily dose of 250 mg/kg of ML-236B causes a decrease in cholesterol-7α-hydroxylase activity in their liver microsomes. However, Bensch, Ingebritsen, and Diller (26) have not found a decrease in the activity of this enzyme in microsomes isolated 3 hr after a single injection of

TABLE 4. Comparison of absolute *de novo* syntheses of cholesterol (P_c^n)[a] and of bile acids[b], the latter being estimated by either the tritium incorporation method (P_1^n) or the ML-236B method (P_2^n)

Incubation Period	P_c^n	P_1^n	$P_1^n/P_c^n \cdot 100\%$	P_2^n	$P_2^n/P_c^n \cdot 100\%$
0–60 min	1.01 ± 0.25	0	0	0.31 ± 0.25	31 ± 25
60–180 min	1.93 ± 0.28	0.40 ± 0.06	21 ± 6	0.84 ± 0.50	48 ± 28

[a] Calculated as ng-atoms of tritium incorporated into cholesterol/mg dry cell weight/22.
[b] Calculated as $P_{cholic} + P_{muricholic}$, from Table 2 ($P_1^n$) or from Table 3 ($P_2^n$).
Data are means ± 1 SD for the same four experiments as shown in Table 1; values of P^n are given in nmol/mg dry cell weight.

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50 mg/kg of ML-236B, favoring the assumption that there is no effect on this enzyme in our hepatocytes during a 3-hr incubation either.

Finally, our data allow a comparison between the absolute de novo syntheses of cholesterol and of bile acids. As shown in **Table 4,** the accumulation of bile acids synthesized de novo, as estimated by the tritium incorporation method, is about 20% of the accumulation of newly made free cholesterol (calculated from the tritium incorporation into free cholesterol), during the second and third hours of incubation. Using the ML-236B method this value would amount to 48%.

We conclude that of the total flux of carbon from acetyl-CoA towards cholesterol the major part ends up in the form of free cholesterol, but a substantial part can appear in the form of bile acids as well, as evidenced for the case of hepatocytes of cholestyramine-fed rats after the first hour of incubation. It remains to be seen whether this distribution in end products of sterol synthesis can be influenced by hormones, drugs, or added bile acids.

APPENDIX I

The number (N) of ^3H atoms incorporated in a molecule of cholic or β-muricholic acid, synthesized during incubation of hepatocytes in tritiated water, has been deduced from the literature in the following manner.

For the case that the bile acids are made from cholesterol, newly synthesized during the incubation, we have started from the number (twenty-two) and location of ^3H-atoms in such cholesterol, arrived at by Lakshmanan and Veech (Fig. 7 in ref. 4). Furthermore, it is assumed that $NADP^3H$ in the cytosol is in equilibrium with tritiated water (5). During conversion of this cholesterol into bile acids, the following changes take place:[1]

a) loss of one tritium from position 7α, due to cholesterol-7α-hydroxylase (18);

b) loss of one tritium from position 3α, due to dehydrogenation of 7α-hydroxycholesterol into Δ^5-cholesten-7α-ol-3-one;

c) loss of one tritium atom from position 4α (transferred for 4–12% to position 6α, which is neglected) without incorporation of tritium from tritiated water, due to isomerization of Δ^5-cholesten-7α-ol-3-one to its Δ^4-isomer (19);

d) loss of one tritium from position 12α, due to 12α-hydroxylase (analogous to step *a)*;

e) introduction of two tritium atoms at positions 4β and 5, respectively, due to saturation of the Δ^4-ethylenic bond in the Δ^4-cholesten-7α-ol-3-one (20, 21);

f) introduction of one tritium at position 3, due to reduction of the oxo-function at this position (20).

For the formation of chenodeoxycholic acid, step *d* does not occur; while during the conversion of chenodeoxycholic to β-muricholic acid, the next steps take place:

g) introduction of one tritium at position 7, due to reduction of the intermediary oxo-function at this position (22, 23);

h) finally, the oxidative side-chain shortening leads to the loss of three tritium atoms.

Steps *a)* through *h)* result in the following values for N:

$$N^n_{cholic} = 18 \quad \text{and} \quad N^n_{\beta\text{-muri}} = 20.$$

For the other case that these bile acid molecules are derived from pre-existent (unlabeled) cholesterol, the theoretically expected values for N would amount to (under operation of steps *e*, *f*, and *g)*:

$$N^o_{cholic} = 3 \quad \text{and} \quad N^o_{\beta\text{-muri}} = 4.$$

[1] Tritium atoms bound to oxygen (in steps *f* and *g*) are considered to be labile and to be lost during subsequent workup.

APPENDIX II

For a given amount of a bile acid produced in the presence of tritiated water, we define:

R^a = radioactivity (ng-atom ^3H) actually introduced in bile acid.
P^a = actual mass production (nmol) of bile acid.
$N^a = R^a/P^a$.
R^n = radioactivity in bile acid derived from newly made cholesterol.
R^o = radioactivity in bile acid derived from pre-existent (old) cholesterol.
x = fraction of P^a derived from newly made cholesterol.
P^n = mass production of bile acid from newly made cholesterol = $x \cdot P^a$.
P^o = mass production of bile acid from pre-existent cholesterol = $(1 - x) \cdot P^a$.
N^n = number of ^3H-atoms incorporated in a molecule of bile acid derived from newly made cholesterol = R^n/P^n.
N^o = number of ^3H-atoms incorporated in a molecule of bile acid derived from pre-existent cholesterol = R^o/P^o.

The following relations hold:

$$R^a = R^n + R^o = \frac{R^n}{P^n} \cdot P^n + \frac{R^o}{P^o} \cdot P^o = N^n \cdot P^n + N^o \cdot P^o. \qquad \text{Eq. } 1)$$

Substitution of $P^n = x \cdot P^a$ and $P^o = (1 - x) \cdot P^a$ in Eq. *1* yields:

$$R^a = N^n \cdot x \cdot P^a + N^o \cdot (1 - x) \cdot P^a. \qquad \text{Eq. } 2)$$

Division of both sides of Eq. *2* by P^a yields:

$$N^a = x \cdot N^n + (1 - x) \cdot N^o. \qquad \text{Eq. } 3)$$

From equation *3*, and the values for N^n and N^o arrived at in Appendix I, it follows that:

$$x_{cholic} = (N^a_{cholic} - 3)/15. \qquad \text{Eq. } 4)$$

$$x_{muri} = (N^a_{muri} - 4)/16. \qquad \text{Eq. } 5)$$

APPENDIX III

For hepatocyte incubations carried out in the presence of tritiated water, and in the absence or presence of ML-236B, we define:

P_- = production of bile acid in the absence of ML-236B.
P_+ = production of bile acid in the presence of ML-236B.
y = (tritium radioactivity in free cholesterol found in the presence of ML-236B)/(tritium radioactivity in free cholesterol in the absence of ML-236B).

The following relations hold: (for meaning of other symbols: see Appendix II):

$$P_-^a = P_-^n + P_-^o. \qquad \text{Eq. } 6)$$

$$P_+^a = P_+^n + P_+^o. \qquad \text{Eq. } 7)$$

If it is assumed that $P_+^n = y \cdot P_-^n$ Eq. *8*, and that $P_+^o = P_-^o$ Eq. *9*, it follows from Eq. *7*, Eq. *8*, and Eq. *9*, that

$$P_+^a = y \cdot P_-^n + P_-^o. \qquad \text{Eq. } 10)$$

Combination of equations Eq. *6* and Eq. *10* then gives:

$$P_-^a - P_+^a = (1 - y) \cdot P_-^n, \quad \text{or } P_-^n = \frac{P_-^a - P_+^a}{1 - y}.$$

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