194. On Metabolic Degradations of Squalene, Lanosterol and Cholesterol in Rat Liver *in vivo* Evidence for Recycling of Metabolites for the Synthesis of Isoprene Compounds

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Summary

1) Radioactivity of biosynthetically labeled squalene, injected in tracer amounts in rats is incorporated to about equal parts into cholesterol and component(s) of the fatty acid fraction of the liver. The ubiquinones isolated from the liver are radioactive and show about the same specific radioactivity as the cholesterol. It appears therefore, that the squalene which escapes incorporation into cholesterol is degraded to metabolite(s) suited for the synthesis of isoprene compounds.

2) Radioactivity of injected biosynthetically and chemically labeled cholesterol is traced in the ubiquinones and the squalene of rat liver indicating that a degradation of cholesterol and a recycling of the metabolite(s) occurs.

3) A recycling of metabolite(s) of squalene and of cholesterol can explain the observation that after labeled mevalonate or acetate as precursor, radioactivity can be traced in the squalene of the liver many hours after the injection and maintains a constant value during a period of several hours.

4) Radioactivity of biosynthetically labeled lanosterol is not only incorporated into cholesterol but a considerable part of it into component(s) of the bile acid fraction of the liver. Evidence is obtained that this transformation occurs by circumventing cholesterol as intermediate.

Previous investigations have demonstrated the existence of a regulation site of cholesterol synthesis in rat liver located after squalene formation [1]. As the mechanism of this regulation is unknown, it is considered now that branching points of the reaction sequence after squalene may exist, thus enabling a change of the flux of substrates in that area of the cholesterogenesis. For the evaluation of such possibilities, labeled squalene, lanosterol and cholesterol were used *in vivo*, in order to verify the existence of degradations - until now unknown - of these compounds. For this purpose, its radioactivity was traced in the fatty acid and bile acid fractions of the liver. The possibility that degradation products may be utilized for the synthesis of isoprene compounds was also taken into consideration. Previously it was shown that rat liver can synthesize the isoprene side chains of ubiquinones

[2] [3]. As these ubiquionones can be isolated in crystalline form, they seemed to be especially suited for elucidating an eventual recycling of possible degradation products of squalene, lanosterol and cholesterol for the synthesis of isoprene compounds.

Experimental procedure

Animals. Groups of 4–5 rats of a *Wistar-Glaxo* strain with an average weight of about 200 g were used. The dose of the labeled precursors was injected intraperitoneally dissolved in 0.1 ml solvent. For removing the livers, the animals were sacrificed under slight anaesthesia.

Materials: 1-[^{14}C]-acetic acid, sodium salt (0.35 mCi/mg), DL-2-[^{14}C]-mevalonic acid lactone (5–10 mCi/mmol), DL-2-[^{3}H] mevalonic acid lactone (100–500 mCi/mmol), 1α,2α(n)-[^{3}H] cholesterol (40,000–60,000 mCi/mmol) from the *Radiochemical Centre Amersham* were used. The 1α,2α(n)-[^{3}H] cholesterol was purified with TLC. on silica gel and dichloromethane as solvent as described below. Biosynthetically labeled squalene, lanosterol and cholesterol were isolated from rat kidney and liver respectively, after administration of labeled mevalonate. Kidney is especially appropriate for obtaining labeled squalene and lanosterol as these compounds are formed there with high specific radioactivity (4). Labeled cholesterol was isolated from liver, for which purpose 30–50 µCi alkaline-treated DL-2-[^{3}H] mevalonic acid lactone was injected in adult female rats, in each case after a fasting period of 24 h and sacrificed 1 h after the injections. From a group of 16 rats 52 mg of crystalline cholesterol with a specific radioactivity of 170,000 dpm/mg was obtained. Squalene and lanosterol were isolated from the unsaponifiable of the kidney by column chromatography as described previously [1] and further purified by TLC. as mentioned below. From 16 rats 63 million dpm as squalene and 30 million dpm as lanosterol were isolated. Both compounds, not isolated as pure substances, have a specific radioactivity of at least 20 million dpm/mg. Labeled squalene was identified by gas chromatography and mass spectroscopy, labeled lanosterol by crystallisation with cold lanosterol.

Isolation procedures. The pooled livers or kidneys of the groups of rats are homogenized with the 5 fold volume of methanol/chloroform 1:1 and extracted under reflux during 1 h at 70°. The total filtrate is concentrated almost to dryness distillation i.V. and saponified with 15% KOH-solution in methanol/water 1:1 under reflux during 45 min at 85°. About 1 ml of this solution per g organ is used. The total saponified material is extracted 3 times with low boiling petroleum ether. Using in each case, a volume 7 times greater than the weight of the organs. Emulsions formed after shaking are completely destroyed by adding a few ml of ethanol several times. The washed and dehydrated (Na_2SO_4) combined extracts are evaporated to dryness. The residue deriving from 30-40 g liver is dissolved in 2 ml ethyl acetate. For the purpose of tracing squalene four TLC. plates of silica gel $60F_{254}$, 20×20 cm layer thickness, 0.25 mm and 2 mm *(Merck)* were used. 0.2 ml of the extract and 400 µg cold squalene dissolved in 0.1 ml are applied as a band of 15 cm length on a thin plate and chromatographed during 50 min with dichloromethane as solvent. A part of the plate is cut off, sprayed with 50% sulfuric acid in ethanol and heated at 110°. The Rf-value for squalene under these conditions is about 0.85 whereas those for lanosterol and cholesterol are 0.3 and 0.15 respectively. In order to determine the radioactivity, the squalene zone is scraped off and eluted thrice with about 10 ml of ethyl acetate on a glass filter funnel, evaporated to dryness and the residue dissolved in 15 ml scintillation fluid. For the isolation of the cholesterol and the ubiquinones 1 ml of the extract is applied as a band on a thick plate and chromatographed with dichloromethane during 1.5 h. As the ubiquinones are not stable on a dry plate, the chromatography was carried out immediately after the application of the extract and the ubiquinones extracted immediately after completion of the chromatographic separation. As reported previously liver contains a mixture of ubiquinone Q_9 and Q_{10} [3]. The TLC. system employed does not separate the two compounds; they appear as a single small intensive yellow coloured zone with an Rf-value of about 0.7. The *ca.* 0.5 cm broad zone is scraped off. After extraction with ethyl acetate the ubiquinone content is measured spectrophotometrically at 436 nm. About 100 µg were thus isolated per g liver. An aliquot is used for measuring the radioactivity. For the crystallisation of ubiquinone the combined extracts of 16 rat livers were chromatographed on six thick plates after extraction and evaporation to dryness. The residue is dissolved in 2 ml ethanol, filtered, heated and concentrated to about 0.5 ml by a nitrogen stream. The crystals formed overnight in the refrigerator are isolated and washed with cold ethanol. After drying, 1 mg crystalline substance is obtained. Its radio-

activity is measured in the liquid scintillation counter. For the isolation of cholesterol the corresponding zones are removed and extracted with ethylacetate. The residue obtained after evaporation to dryness is dissolved in about 3 ml boiling methanol, filtered and kept in the refrigerator overnight. The crystals are separated on a filter and thoroughly washed. From 4 rat livers 10–15 mg cholesterol are thus isolated and used for measuring the radioactivity.

After isolation of the unsaponifiable from the saponified liver extracts, the residue is acidified and extracted 2 times with ether using the same volumes as for the extraction with petroleum ether. The washed and dehydrated extract is evaporated to dryness. This material in so-called fatty acid and bile acid fractions was separated by TLC. With the mixture petroleum ether/ether/acetic acid 80:20:1, fatty acids migrate in such a way that palmitic acid shows an Rf-value of about 0.4 after 1 h. Bile acids do not migrate in this system. By extraction of the start zone with ethyl acetate the socalled bile acid fraction was obtained. A separation of this fraction was performed with a mixture of cyclohexane/ethyl acetate/acetic acid 35:15:15 giving Rf-values of about 0.2 for cholic acid, 0.4 for deoxycholic acid and 0.55 for lithocholic acid after 1 hour.

Results. - 1. *Incorporation of radioactivity of* DL-2-[^{14}C] *mevalonate and biosynthetically labeled squalene into the cholesterol and the ubiquinones of rat liver.* 16 rats in groups of 4 were injected intraperitoneally with a mixture of 3.2 million dpm DL-2-[^{14}C] mevalonate and 1.6 million dpm of biosynthetically labeled [^{3}H]-squalene dissolved in 0.1 ml of 50% ethanol at 8.30 a.m. The rats of the 4 groups were sacrificed 1, 2, 6 and 12 hours after the injections. From the pooled unsaponifiable material 46.5 mg cholesterol and 1.0 mg ubiquinones were isolated in crystalline form. *Table 1* shows that not only the radioactivity of mevalonate but also that of squalene is incorporated into the ubiquinones. The specific radioactivity of the cholesterol and the ubiquinones is about the same after mevalonate as precursor, showing that its turnover rates must be similar. After labeled squalene the specific radioactivity of the cholesterol is only slightly higher than that of the ubiquinones, indicating that a considerable part of the squalene is not used for the synthesis of cholesterol but degraded to a metabolite which is in term employed for the ubiquinone synthesis.

2. *Incorporation of radioactivity of biosynthetically labeled lanosterol and cholesterol and of 1α,2α (n)-*[^{3}H] *cholesterol of rat liver.* 3 groups of 4 rats each were injected with 1 million dpm labeled lanosterol, 1 mg of biosynthetically labeled cholesterol (170,000 dmp/mg) and 1 mg of 1α 2α (n)-[^{3}H] cholesterol (0.5 μCi/mg) respectively at 8.30 a.m. 3 hours later, the rats were sacrificed. The cholesterol was isolated from the unsaponifiable in crystalline form. The ubiquinones were obtained in solution and rechromatographed 2 times in order to check that the specific radioactivity remains constant. From *Table 2* it is seen that from labeled lanosterol and cholesterol radioactivity is incorporated into the ubiquinones.

3. *Incorporation of the radioactivity of biosynthetically labeled lanosterol and cholesterol and 1α,2α (n)-*[^{3}H] *cholesterol into squalene.* The unsaponifiable of the

Table 1. *Specific radioactivities* (dpm/μmol) *of cholesterol and ubiquinones of the liver after* 2-[^{14}C] DL-*mevalonate and biosynthetically labeled squalene as precursors*

	^{14}C	^{3}H
Cholesterol	880	497
Ubiquinones	885	351

same group of rats described above was used. Squalene was isolated by TLC. The squalene zone was extracted and rechromatographed to make sure that the radioactivity is not lost. *Table 2* demonstrates that the radioactivity from both lanosterol and cholesterol is incorporated into squalene. The squalene content of 10 pooled rat livers was determined by GC. A value of 37 μg/g liver was obtained (unpublished results) in contrast to about 25 μg/g liver found previously [5]. On the basis of 37 μg/g, the specific radioactivity of the squalene is calculated. Surprisingly this value is considerably higher than the specific radioactivity of isolated cholesterol present at the same time in the liver.

Table 2. *Specific radioactivities* (dpm/μmol) *of cholesterol, ubiquinones and squalene of the liver after labeled lanosterol and cholesterol as precursors*

	After biosynth. labeled lanosterol	After biosynth. labeled cholesterol	After 1α,2α (n)-[^{3}H] cholesterol
Cholesterol	520	347	16701
Ubiquinones	225	278	1615
Squalene	258	1007	2302

4. *Degradation of biosynthetically labeled squalene, lanosterol and cholesterol and of 1α,2α (n)-*[^{3}H] *cholesterol to constituents of the fatty acid and bile acid fraction of the liver.* The saponified material of the same groups of rats described above were used. The ether extracts obtained after having removed the unsaponifiable yielded 0.7–1 g dried material per group of rats. Separation into a so-called fatty acid and bile acid fraction was performed as described above. *Table 3* shows the incorporation rates of these fractions in comparison with the total radioactivity of the cholesterol. About 50% of the radioactivity of squalene escapes incorporation into cholesterol and is mainly located in a broad zone above palmitic acid of the fatty acid fraction obtained after TLC.-separation with a mixture of petroleum ether, ether and acetic acid. Almost one third of the radioactivity of lanosterol escapes incorporation into cholesterol and is mainly found in the bile acid fraction. TLC. with cyclohexane/ethyl acetate/acetic acid leads to distinct accumulation of the radioactivity located between lithocholic and deoxycholic acid. After both biosynthetically labeled cholesterol and 1α,2α (n)-[^{3}H] cholesterol only a small portion of the radioactivity (less than 1/30) is located in the bile fraction compared with radioactivity traced at the same time in the cholesterol.

5. *Comparison of the incorporation of ^{14}C-labeled acetate and ^{14}C-labeled* DL-*mevalonate into cholesterol and squalene after different time intervals.* For the incorporation of acetate and mevalonate eight groups of 4 rats were used. 12 μCi

Table 3. *Incorporation of the radioactivity* (total dpm) *of labeled lanosterol and cholesterol into constituents of the fatty acid and bile acid fractions of the liver*

	After biosynth. labeled squalene	After biosynth. labeled lanosterol	After biosynth. labeled cholesterol	After 1α,2α (n)-[^{3}H] cholesterol
Cholesterol	92627	107520	69350	3452480
Fatty acid fraction	74366	29000	860	11956
Bile acid fraction	6283	43732	2212	43296

acetate and 1.4 μCi DL-mevalonate were injected at 8.30 a.m. The rats were sacrificed 1, 3, 6 and 12 hours after the injections and were not fed during that period.

The figures from *Table 4* show that the specific radioactivity of cholesterol deriving from mevalonate declines rapidly within the first hours and reaches a constant level between 6 and 12 hours after the injection. Practically the same is observed after acetate as precursor. The decline of the specific activity of the cholesterol, however, is somewhat delayed. Surprisingly, the radioactivity of squalene shows the same pattern both after mevalonate as after acetate as precursors. Thus, the ratio of the specific radioactivities of cholesterol and of squalene remains practically constant over a period of 11 hours.

Table 4. *Specific radioactivities* (dpm/μmol) *of squalene and cholesterol after injections of* [1-^{14}C] *acetic acid and* DL-[2-^{14}C] *mevalonate*

Hours after injections	Squalene after acetate	Cholesterol after acetate	Squalene after mevalonate	Cholesterol after mevalonate
1	647	209	11653	1438
3	647	173	5146	860
6	384	127	4809	590
12	367	107	4148	615

Discussion. – Previously it was shown that injected labeled mevalonate is very rapidly incorporated into the squalene of the rat liver and that its radioactivity rapidly declines and is accumulated in the cholesterol [6]. Results reported here show that the decline of the radioactivity of squalene stops subsequently and remains constant at least over the period of 3–12 hours after injection of the labeled mevalonate. The same type of decline of the radioactivity of cholesterol is observed after acetate, but is somewhat delayed. The following findings can explain this behaviour. A branching of the reaction sequence of the cholesterogenesis after squalene obviously occurs, evidenced by the incorporation of a considerable part of its radioactivity in component(s) of the fatty acid fraction. The incorporation of radioactivity of labeled squalene into the ubiquinones of the liver confirms this assumption and shows in addition that a degradation product of squalene may be used for the synthesis of isoprene compounds. A recycling of squalene for the cholesterol synthesis must therefore be considered. Furthermore, it is observed that radioactivity of labeled cholesterol is incorporated into the ubiquinones and the squalene of the liver. The degradation of cholesterol leading to a metabolite suited for the synthesis of the ubiquinones must be considerable since the specific radioactivity of the isolated ubiquinones is not much lower than that of the cholesterol present at the same time in the liver. The incorporation of radioactivity of labeled cholesterol into squalene shows that a recycling of a cholesterol degradation product for the cholesterol synthesis obviously occurs. The injection of biosynthetically labeled cholesterol leads to a specific radioactivity of squalene about three times higher than that of the cholesterol present in the liver. It appears that this observation can only be explained by the assumption that more than one metabolic pool of cholesterol in liver exists and that injected cholesterol undergoes

preferentially a degradation. The differences of the specific radioactivities of the squalene and ubiquinones after biosynthetically labeled cholesterol on the one hand and ring A labeled cholesterol on the other hand indicate a different use of different parts of the cholesterol molecule for recycling.

A branching of the reaction sequence of the cholesterol synthetic pathway seems also to exist after the lanosterol formation. After the injection of biosynthetically labeled lanosterol a considerable part of the radioactivity, about half of that incorporated into cholesterol, is traced in the bile acid fraction. Radioactivity was also found in this fraction after injection of biosynthetically and ring A labeled cholesterol. By comparing the incorporation rates after lanosterol and cholesterol as precursor, it seems obvious that lanosterol is metabolized to a sterol acid type of compound by circumventing the cholesterol.

From the above results it can be concluded that three points of attack can be considered for regulating influences in the cholesterol synthetic pathway after squalene, namely degradation of squalene, of lanosterol and of cholesterol itself and finally recycling of metabolites for the cholesterol synthesis.

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REFERENCES

[1] *O. Wiss*, Biochem. biophys. Res. Commun. *68*, 353 (1976).
[2] *U. Gloor & O. Wiss*, Experientia *14*, 410 (1958).
[3] *U. Gloor, O. Schindler & O. Wiss*, Helv. *43*, 2089 (1960).
[4] *K. H. Hellstrom, M. D. Siperstein, L. A. Bricker & L. J. Luby*, J. clin. Invest. *52*, 1303 (1973).
[5] *R. G. Langdon & K. Bloch*, J. biol. Chemistry *200*, 129 (1953).
[6] *D. S. Goodman, J. Avigan & D. Steinberg*, J. biol. Chemistry *238*, 1287 (1963).