Vol. 83, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 14, 1978 Pages 857-862

THE DEGRADATION OF LABELLED LANOSTEROL BY HOMOGENATE OF RAT LIVER: EVIDENCE FOR THE FORMATION OF LITHOCHOLIC ACID FROM LANOSTEROL WITHOUT CHOLESTEROL AS INTERMEDIATE

Oswald and Verena Wiss

Department of Biochemistry
University of Basle
Vesalgasse 1, CH-4051 Basle, Switzerland

Received May 31,1978

SUMMARY: In contrast to labelled cholesterol, labelled lanosterol is rapidly degraded by rat liver homogenate leading to metabolites which behave like bile acids by characterization with thin layer chromatography. Lithocholic acid could be identified as a minor component of that mixture. The same composition of metabolites could be traced after injection of labelled cholesterol in rat liver in vivo.

Previously it could be shown that lanosterol can be degraded by rat liver in vivo to metabolites behaving like bile acids after saponification of the lipid extract and by characterization with thin layer chromatography. This transformation obviously occured without cholesterol as intermediate (1).

MATERIALS AND METHODS: Rats of a Wistar-Glaxo strain weighing between 200-250 g were used. Materials and Methods of the in vivo experiments were described previously (1,3). In addition 4-[^{14}C] cholesterol (57 mCi/mmol) was purchased from Radiochemical Centre Amersham and lithocholic acid from Calbiochem, San Diego, Calif. The homogenates were prepared from two livers by addition of the double volume of 0.1 M potassium phosphate buffer, pH 7.4. 50 ml of it were incubated in a 100 ml Erlenmeyer flask placed in a water bath of 38°C with electromagnetic stirring. Labelled lanosterol and cholesterol, respectively, dissolved in 0.5 ml ethanol were added after temperature adjustment. Samples of 6 ml were taken at various time intervals and added to 100 ml of a mixture of chloroform and methanol (1:1) and extracted under reflux at 70°C during 30 min. After concentration of the filtrate to about 10 ml, 4 ml 30 % KOH in methanol were added for saponification under reflux at 85°C during 45 min. The unsaponifiable was extracted three times with 100 ml low boiling petroleum ether. Emulsions were destroyed by adding a few ml of ethanol. The washed and with Na_2SO_4 dehydrated extract was evaporated to dryness and the residue dissolved in 1 ml ethyl acetate. 0.1 ml of it was used for measuring the radioactivity (using the isotope dilution method, carried out with parallel samples, it could be shown that more than 95 % of the radioactivity corresponded to the unchanged lanosterol and cholesterol, respectively). The resi-

0006-291X/78/0833-0857$01.00/0

Copyright © 1978 by Academic Press, Inc.
All rights of reproduction in any form reserved.

due obtained after extraction of the unsaponifiable was acidified and extracted two times with 100 ml ether. The washed and dehydrated extract was evaporated to dryness and the residue dissolved in 1 ml ethyl acetate (A). 0.1 ml of A is applied as a 10 cm long and 0.5 cm broad band on a thin layer plate (silica gel) of 0.25 mm thickness and chromatographed during 1 hr with a mixture of high boiling petroleum ether, ether, acetic acid, 90:10:1 (B) as solvent. In order to obtain the bile acid fraction a 1 cm broad band of the start zone was scraped off and extracted three times with about 10 ml ethyl acetate. The dry residue was used for measuring the radioactivity. For further characterization of the bile acid fraction, the dry residue obtained by the same procedure was dissolved in 0.2 ml ethyl acetate and separated by thin layer chromatography with a mixture of cyclohexane, ethyl acetate, acetic acid, 30:15:15 as solvent during 30 min. 70-80 % of the radioactivity were found to be located in a 1 cm broad zone with a Rf value of 0.7, made visible by spraying with 50 % sulfuric acid in methanol and by heating at 120°C on a part of the plate not used for extraction. This zone was clearly separated from those of cholic, deoxycholic, and chenodeoxycholic acids, shown by addition of these substances to the materials being chromatographed. For the identification of the lithocholic acid by the isotope dilution method, 0.8 ml of A were applied on a thin layer plate with 2 mm thick silica gel and chromatographed during 2 hr with B as solvent. A 1 cm broad band, scraped off from the start zone, was extracted as described above. Depending on the number of crystallizations intended, 50-110 mg of lithocholic acid were added to the residue and dissolved in ethyl acetate (1 ml per 10 mg lithocholic acid). The volume of the hot solution was reduced to about one third by a nitrogen stream. After cooling during about 1 hr in the deep freezer, about 70 % of the added lithocholic acid were obtained as crystals which were extensively washed with cold ethyl acetate before drying. For measuring the radioactivity, about 10 mg of the crystals were dissolved in 1 ml ethanol before adding 15 ml of the scintillation fluid.

RESULTS AND DISCUSSION: Based on available data it is assumed that the significance of lanosterol in the metabolism is limited to its function as an intermediate in the cholesterol synthetic pathway. Table 1 and Fig. 1 show, however, that lanosterol is degraded by liver homogenate leading mainly to so far unidentified components of the bile acid fraction which were characterized by thin layer chromatography with two different solvent systems (see under Materials and Methods). A minor portion of it could be identified as lithocholic acid (Table 3, Fig. 2). Metabolites with the same properties are found after injection of labelled cholesterol in rat liver (Table 2 and 3) but not after incubation of labelled chol-

TABLE 1. Degradation of labelled lanosterol and cholesterol by homogenate of rat liver

Experiment	lanosterol: dpm·10^3			dpm·10^3 recovered in bile acid fraction
	30 sec	60 min	difference	
1	217	185	32	33
2	214	185	29	37
3	259	194	65	38
4	327	264	63	51
average	254	207	47	40
	cholesterol: dpm·10^3			dpm·10^3 recovered in bile acid fraction
1	233	229	4	2.1
2	201	201	0	3.3
3	226	222	4	4.2
average	220	220	2.7	3.2

$3.5 \cdot 10^5$ dpm (approx. 0.6-1 µg) biosynthetic [^{3}H]lanosterol and $3.5 \cdot 10^5$ dpm (0.14 µg) 4-[^{14}C]cholesterol were added as a mixture per sample.

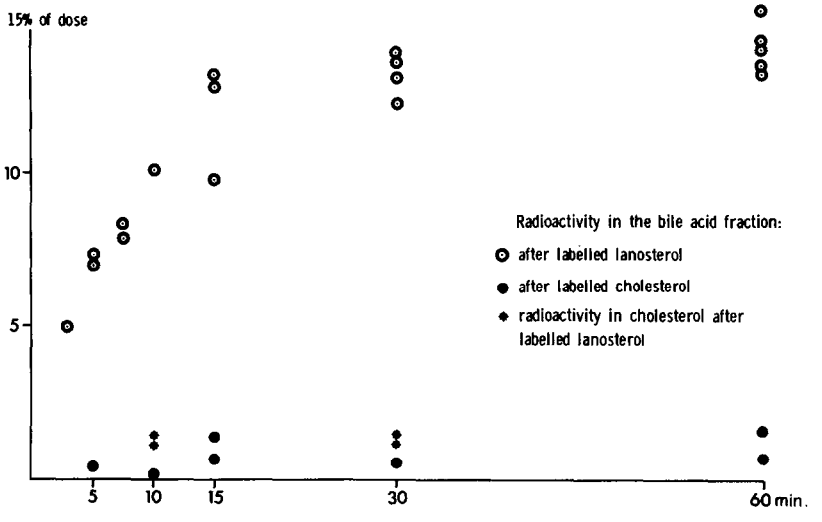

Fig. 1. Incorporation of radioactivity from labelled lanosterol and cholesterol into the bile acid fraction of homogenate of rat liver. $3.5 \cdot 10^5$ dpm biosynthetic [^{3}H]lanosterol and $3 \cdot 10^5$ 4-[^{14}C]cholesterol were added as a mixture per sample. Blanks corresponding to about 1 % of the dose were substracted.

TABLE 2. Comparison of the incorporation of radioactivity from cholesterol (in vivo) and lanosterol (in vitro) into bile acid fractions of rat liver

from cholesterol in vivo[1)]		
time after infection of lab. cholesterol	dpm/g liver	% of labelled cholesterol of liver
7.5 min	1040	2.17
21 min	2490	7.56
32 min	1340	5.13
from lanosterol in vitro[2)]		
time of incubation	dpm/g liver	% of labelled lanosterol
7.5 min	13600	8.10
15 min	20000	11.9
30 min	22000	13.1

1) $6.7 \cdot 10^6$ dpm 1α,2α(n) [^{3}H] cholesterol dissolved in 0.1 ml ethanol were injected intraperitoneally at 8.30 a.m. per rat. The livers of 2 rats were pooled.
2) The radioactivity of lanosterol was determined 30 sec after its addition to the liver homogenate.

esterol with liver homogenate (Table 1, Fig. 1 and 2).

Until now it was not possible to demonstrate the formation of lithocholic acid from cholesterol with enzymes of animal origin. It is postulated that lithocholic acid is formed from chenodeoxycholic acid by removing the hydroxy group in the 7 position. Such a reaction could, however, only be demonstrated with enzymes of microbiological origin (2).

Our data suggest that lanosterol represents a branch point of the reaction sequence of the cholesterogenesis and that lithocholic acid is formed without cholesterol as intermediate. Previously it was shown that cholesterol can be degraded in vivo to metabolites suited for the synthesis if isoprene compounds such as squalene and ubiquinones (1). It seems, therefore,

TABLE 3. Identification of lithocholic acid from bile acid fractions by isotope dilution

crystallization No	mg recovered	mg measured	dpm(total) recovered	% of total radioact.
from cholesterol (in vivo)				
1	77.3	7.4	3380	5.63
2	51.1	8.3	1270	2.12
3	25.8	7.4	1170	1.95
4	9.3	9.3	1200	2.00
from lanosterol (in vitro)				
1	80.0	6.4	4090	4.54
2	58.2	8.3	1420	1.58
3	28.1	8.4	1250	1.39
4	11.3	11.3	1180	1.31

For obtaining the bile acid fraction the rest of the samples not used for analysis from experiments reported in Table 2 were pooled and worked up as described under Materials and Methods. The bile acid fraction ($60 \cdot 10^3$ dpm) obtained from labelled cholesterol (in vivo) was mixed with 104.5 mg lithocholic acid, the bile acid fraction ($90 \cdot 10^3$ dpm) from labelled lanosterol (in vitro) with 107.5 mg lithocholic acid.

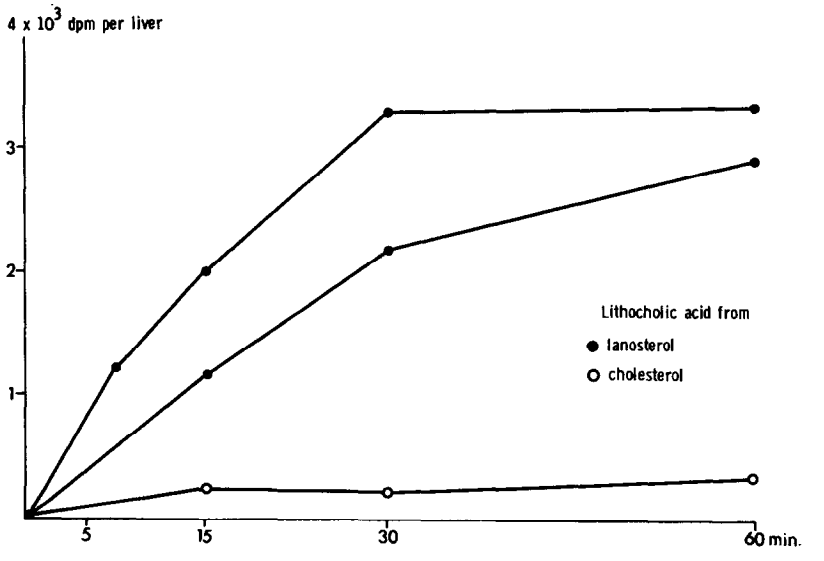

Fig. 2. Incorporation of radioactivity from labelled lanosterol and cholesterol into lithocholic acid by homogenate of rat liver. $3 \cdot 10^5$ dpm biosynthetic [^{3}H] lanosterol and $3 \cdot 10^5$ dpm 4-[^{14}C]cholesterol were added as a mixture per sample. The radioactivity of lithocholic acid is calculated from the substance recovered b the second crystallization.

that the transformation of cholesterol to lithocholic acid observed in vivo but not in vitro occurs via lanosterol as intermediate.

Evidence as previously has been obtained, a regulation site of the cholesterogenesis is located between lanosterol and cholesterol (3). The existence of a branch point in the reaction sequence after lanosterol is an essential prerequisite for the existence of such a regulation site.

ACKNOWLEDGEMENT: We thank Miss U. Olbrecht for the excellent care of the animals and F. Hoffmann-La Roche & Co. Ltd. Basle for financial support.

REFERENCES:

1. Wiss, O. and Wiss, V. (1977) Helv. Chim. Acta 60, 1961-1966
2. Danielson, H., and Sjöval, J. (1975) Ann. Rev. Biochem. 44, 233-253
3. Wiss, O. (1976) Biochem. Biophys. Res. Comm. 68, 353-357