261. On the Influence of Cholesterol Feeding and of a Lipogenic Diet on the Cholesterogenesis in Rat Liver *in vivo*

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Summary

1) The existence of a main regulation site(s) of the cholesterol synthesis located after the squalene formation is demonstrated by cholesterol feeding with labelled glucose, acetate, mevalonate as precursors and by measuring the incorporation rates into cholesterol, squalene, ubiquinones, and fatty acids in rat liver *in vivo*.

2) By administration of labelled squalene *in vivo* it is shown that the isoprenic synthesis is inhibited between squalene and lanosterol but that other regulation site(s) after the lanosterol formation must exist.

3) A regulation site of the cholesterogenesis in rat liver located after the squalene synthesis is also shown after a lipogenic diet administered during 5 days. The cholesterol synthesis is thus reduced to about one third of the control rats. Another regulating site controlling the utilization of acetylcoenzyme A for the synthesis of mevalonate is also shown to exist. No indication was obtained for a regulating influence located between mevalonate and squalene.

By administering labelled glucose and mevalonate to rats and by measuring the incorporation of radioactivity into cholesterol and squalene we have shown that feeding of cholesterol inhibits the cholesterol synthesis *in vivo* by acting at a regulating site located after the squalene formation [1]. It was also demonstrated that the cholesterogenesis is inhibited at the same site after a lipogenic diet [2]. In order to get a more overall view on the metabolic changes caused by these influences *in vivo*, the synthesis of fatty acids and ubiquinones was studied in liver in addition to that of cholesterol. Besides labelled glucose and mevalonate previously used, labelled acetate, palmitate, and squalene were included in these investigations.

Results. – 1. *Influence of cholesterol feeding on food intake and relative liver weight of rats* (Table 1). Food intake and relative liver weight were not affected by the addition of 5% cholesterol to the diet prior to a feeding period of 22 hours. Such controls seemed necessary because it was observed previously that a fasting period of only a few hours reduced the relative liver weight and especially the fatty acid synthesis in the liver considerably [5]. In the present experiments no fasting effect induced by an embittering influence of the added cholesterol is observed.

Table 1. *Influence of cholesterol feeding on food intake and relative liver weight*

Group	C(10)	E(10)	C(12)	E(12)
Food intake per rat per day	17.8 g	19.6 g	18.9 g	19.1 g
Body weight/liver weight	22.0 g	22.5 g	22.1 g	22.4 g

( ) number of rats; C = control group; E = experimental group.

2. *Influence of cholesterol feeding on the cholesterol, squalene, ubiquinone, and fatty acid concentration of rat liver* (Table 2). Our results for liver cholesterol – increase to about 130% of the controls by a 5% cholesterol diet – confirm the previous findings of *Shapiro & Rodwell* [6] with this diet (after a 10 hours feeding period increase to about 150% of the controls). A similar increase of the fatty acids of liver occurs, whereas the squalene and ubiquinone concentration is not significantly affected.

Table 2. *Influence of cholesterol feeding on the concentration of cholesterol, squalene, ubiquinones, and fatty acids in rat liver (percent of controls per g liver), controls, and experimental groups consisting of 3 rats each*

Group	1	2	3	average
Cholesterol	131%	125%	143%	133%
Squalene	75%	92%	100%	89%
Ubiquinones	112%	92%	107%	104%
Fatty acids	143%	158%	133%	145%

3. *Influence of cholesterol feeding on the incorporation of radioactivity from labelled glucose + mevalonate and of labelled acetate into cholesterol, squalene, ubiquinones, and fatty acids of rat liver* (Table 3). The results demonstrate that a main regulating site of the cholesterogenesis is located after the squalene formation. This becomes evident with both [^{14}C]-glucose and [^{3}H]-mevalonate as precursors by measuring the incorporation of radioactivity into cholesterol, squalene, ubiquinones, and fatty acids. The combined administration of the two precursors, resulting in an incorporation of both labels into the same molecules and reducing thus biological and experimental variations is obviously responsible for the good agreement of the results obtained for glucose and mevalonate respectively. Previous results obtained by oral administration of labelled glucose are confirmed. Oral administration of labelled mevalonate, gave, however, somewhat different results [1]. The incorporation of radioactivity into squalene was found stimulated, that into cholesterol not affected.

This divergence results probably from the different mevalonate doses used. Substrate doses (248 µg/rat) were administered orally [1] whereas tracer doses (0,16 µg/rat) were injected intraperitoneally (this paper). As shown previously [5] artefacts must be considered with substrate doses of mevalonate, probably due to the fact that mevalonate is not a normal constituent, or only present in very minor amounts, in blood and tissues. The incorporation of labelled acetate into cholesterol, squalene, ubiquinones, and fatty acids is reduced. The reduction into cholesterol is,

however, much more pronounced than into squalene and ubiquinones, resulting in about the same inhibiting effect between squalene and cholesterol as after labelled glucose + mevalonate as precursors.

4. *Influence of cholesterol feeding on the incorporation of labelled squalene into cholesterol and lanosterol of rat liver* (Fig. 1). The incorporation of squalene into cholesterol is slowly rising and significantly inhibited by cholesterol feeding. The radioactivity of squalene in the liver is faster declining in rats on the cholesterol diet

Table 3. *Influence of cholesterol feeding on the specific radioactivity (dpm/mg) and the incorporation rates of radioactivity per g liver (inc. rate)*[a]) *of cholesterol, squalene, ubiquinones, and fatty acids of rat liver after labelled glucose + mevalonate, and acetate (groups of 3 rats)*

Precursor		lab. glucose (24 μCi, 1.45 mg/rat)				lab. mevalonate (10^6 dpm, 0.16 μg/rat)				lab. acetate (60 μCi, 88 μg/rat)
Time after inj. of precursors (min)		18	31	51	average	18	31	51	average	25
Cholesterol	dpm/mg E	20.7	29	29		525	819	893		141
	dpm/mg C	66.5	102	119		1780	2467	3119		1698
	inc. rates E/C	0.42	0.36	0.35	0.38	0.38	0.42	0.42	0.41	0.09
Squalene	dpm/mg E	433	536	306		49400	57076	31766		1918
	dpm/mg C	225	311	282		36649	21490	22340		6443
	inc. rates E/C	1.45	1.58	1.09	1.37	1.01	2.44	1.43	1.63	0.29
Ubiquinones	dpm/mg E					218	491	344		250
	dpm/mg C					330	313	193		734
	inc. rates E/C					0.59	1.67	1.83	1.36	0.34
Fatty acids	dpm/mg E	114	94	63		21	20.5	17.5		1084
	dpm/mg C	148	126	71		27.5	19.5	16.1		2289
	inc. rates E/C	1.10	1.18	1.18	1.15	1.09	1.67	1.43	1.40	0.36

C = control group; E = experimental group.

a) The incorporation rates are calculated from the specific radioactivities of E and C respectively and the concentration of the substances in the liver.

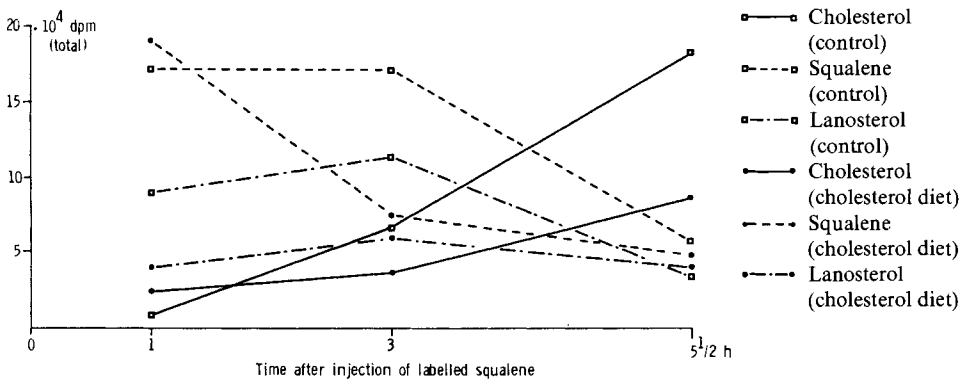

Fig. 1. *Influence of Cholesterol Feeding on the Incorporation of Labelled Squalene into the Cholesterol and Lanosterol of Rat Liver*

Dose: $1.5 \cdot 10^6$ dpm labelled squalene per rat. Each point represents the average value of three rats. The values of lanosterol are multiplied by two.

indicating a stimulation of the degradation of squalene. The conversion of squalene to lanosterol is also strongly inhibited by the cholesterol diet confirming the inhibition site located after the squalene formation. Former incorporation experiments with labelled mevalonate seemed to indicate that the inhibition of the cholesterol synthesis by cholesterol feeding is only located after the lanosterol formation, because the incorporation of the radioactivity into squalene went parallel with that into lanosterol [1]. The now reported results do not support this assumption. As in the meantime it was shown that a recycling of degradation products of squalene and cholesterol for the synthesis of isoprene compounds occurs and that lanosterol can be metabolized by circumventing cholesterol [3], it must be considered that regulating influences at these sites may be responsible for that discrepancy. The observations that the initial inhibition of the conversion of squalene to lanosterol by cholesterol feeding is reduced later and is no more evident 5.5 hours after the injection of squalene indicate that such regulating sites exist.

5. *Influence of the lipogenic diet on the concentration of glucose, glycogen, cholesterol, squalene, ubiquinones, and fatty acids in rat liver* (Table 4). Experimental groups were fasted for 16 hours (overnight) after 5 days on lipogenic diet. The rats of the control and the experimental groups received a load dose of 1 g of glucose dissolved in 2 ml water by stomach tube 2 hours prior to slaughtering.

Table 4 shows that glucose, glycogen and cholesterol are significantly increased in liver of the rats kept on the lipogenic diet, fatty acids are little affected and the ubiquinones and squalene content not significantly modified.

6. *Influence of the lipogenic diet on the incorporation of labelled acetate into fatty acids and of labelled glucose into fatty acids and glycogen of rat liver.* Previously it was shown with liver slices that a lipogenic diet enhances the incorporation of acetate into fatty acids about two fold and that of glucose into fatty acids about ten fold [7]. *Table 5* shows that these results are confirmed *in vivo*. The accumulation of glucose and glycogen in the liver *(Table 4)* and the increased incorporation of labelled glucose into glycogen is in agreement with stimulation of liver enzymes induced by a glucose rich diet [8].

7. *Influence of the lipogenic diet on the incorporation of radioactivity of labelled glucose, acetate and mevalonate, palmitate and squalene into isoprene compounds of the liver.* Previously reported results on the inhibition of the incorporation of labelled acetate into cholesterol by a lipogenic diet are confirmed *(Table 6)*. From the observation that the incorporation of mevalonate is not affected it was concluded that the inhibition site is exclusively located between acetylcoenzyme A and mevalonate [9]. The evaluation of the synthesis of other isoprene compounds such as

Table 4. *Influence of the lipogenic diet on the concentration of glucose, glycogen, and cholesterol in rat liver (mg/g)*

	Glucose	Glycogen	Cholesterol	Ubiquinones	Squalene	Fatty acids
Control group	10.9 (6)	11.0 (6)	2.55 (8)	100% (9)	100% (9)	100% (18)
Exper. group	17.8 (7)	22.0 (7)	3.74 (7)	107% (9)	98% (9)	120% (18)
Significance	$p<0.001$	$p<0.001$	$p<0.001$			

( ) number of rats.

Table 5. *Influence of the lipogenic diet on the incorporation of labelled glucose into fatty acids and glycogen, and of labelled acetate into fatty acids of rat liver (dpm/g liver)*

Group (6 rats each)	labelled glucose[a]) (oral) into		labelled acetate[b]) (i.p.) into
	fatty acids	glycogen	fatty acids
Experimental (E)	39300	118000	305000
Control (C)	2870	46500	107000
E/C	13.7	2.5	2.9

[a]) Both controls and experimental groups were fasted over night (16 hours). 5 μCi of ^{14}C-glucose together with a load dose of 1 g glucose in 2 ml H_2O were given to each rat by stomach tube 2 hours prior to slaughtering.

[b]) 23 μCi of ^{14}C-acetate (i.p.) in 0.1 ml water 2 hours prior to slaughtering.

Table 6. *Influence of the lipogenic diet on the specific radioactivity (dpm/mg) and the incorporation rates of radioactivity per g liver (inc. rate)[a]) of cholesterol, squalene and ubiquinones of rat liver after labelled mevalonate, glucose, palmitate, and acetate (groups of 3 rats)*

Precursor		10^6 dpm (0.16 μg) [2-^{3}H]-mevalonate i.p. per rat				10 μCi (29 μg) [6-^{14}C]-glucose together with a load of 2 g glucose in 2 ml water orally/rat	13.5 μCi (44.2 μg) [1-^{14}C]-palmitic acid together with a load of 2 g glucose in 2 ml water orally/rat	23 μCi (33 μg) [1-^{14}C]-sodium acetate in 0.1 ml water i.p. per rat
Time after administration (min)		23	44	75	average	120	120	120
Cholesterol	dpm/mg E	908	1491	2279		9.5	476	252
	dpm/mg C	1164	1755	1848		64.6	520	2560
	inc. rates E/C (A)	0.78	0.84	1.23	0.95	0.2	0.13	0.14
Squalene	dpm/mg E	36410	3015	2588		4924	1030	1315
	dpm/mg C	14520	1329	891		6254	2425	3181
	inc. rates E/C (B)	2.5	2.3	2.9	2.6	0.83	0.42	0.37
Ubiquinones	dpm/mg	1096	1368	904				
	dpm/mg	300	640	533				
	inc. rates E/C	2.1	1.8	2.6	2.2			
	B/A				2.7	4.1	3.2	2.7

C = control group, E = experimental group.

[a]) The incorporation rates are calculated from the specific radioactivity of E and C respectively and the concentrations of the substances in the rat liver.

squalene and ubiquinones *(Table 6)* reveals, however, that a main inhibition site is located after the squalene formation. While the incorporation of mevalonate into cholesterol is not significantly reduced, that into squalene and ubiquinones is greatly enhanced *(Table 6)*. The incorporation of radioactivity from glucose into squalene is about the same in both control and experimental animals whereas that into cholesterol is strongly reduced in the experimental animals. The incorporation of radioactive palmitate and acetate is reduced into squalene and, much more, into cholesterol

by the lipogenic diet. The inhibiting effect located between squalene and cholesterol is similar for all the used precursors, resulting in a reduction of the cholesterol synthesis to about one third of that of the controls.

From figures of *Table 6* the approximate relation of the incorporation of ^{14}C into cholesterol from glucose on the one hand and palmitate on the other can be calculated: In both control and experimental rats about two thirds of the ^{14}C derive from glucose and one third from palmitate.

The existence of a regulating site(s) after the squalene is also evident from the results obtained with labelled squalene *(Fig. 2)*. A significant reduction of its incorporation into cholesterol by the lipogenic diet is observed, whereas its use for the ubiquinone synthesis is stimulated. The time dependence of these effects shows that they cannot be explained by a single regulating influence. Possible regulation sites between squalene and lanosterol, lanosterol and cholesterol, and regulating influences by recycling of squalene and cholesterol degradation products must be considered [3].

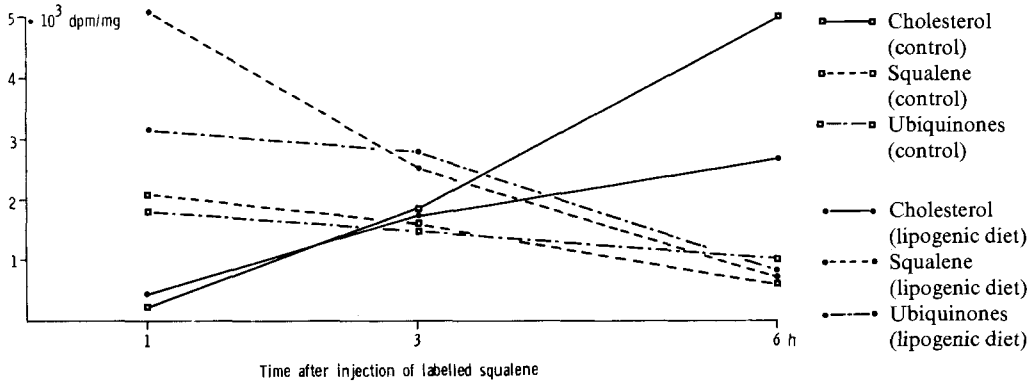

Fig. 2. *Influence of a Lipogenic Diet on the Specific Radioactivity of Cholesterol, Squalene, and Ubiquinones of Rat Liver after Labelled Squalene*

Dose: $1.5 \cdot 10^6$ dpm biosynthetically labelled squalene in 0.1 ml 50% ethanol per rat by i.p. injection. Each point represents the value of a group of 3 rats. The specific radioactivity of the cholesterol of the rats on the lipogenic diet was multiplied by the factor resulting from the accumulation of cholesterol in the liver for enabling a direct comparison of the incorporation rates between control and experimental rats. Such a correction was not necessary for the squalene and ubiquinones, because their concentration in the liver is not influenced by the lipogenic diet.

Discussion. – The incorporation of radioactivity from glucose and mevalonate into squalene is little influenced by cholesterol feeding, the same is observed for the synthesis of ubiquinones and fatty acids from mevalonate. The incorporation of radioactivity of glucose and of mevalonate into cholesterol is, however, strongly reduced, demonstrating the inhibiting effect of cholesterol feeding located after the squalene formation. As the observed effects after glucose + mevalonate as precursors are practically the same, it seems therefore that cholesterol feeding has no influence on the pathway from glucose to squalene. It was, however, demonstrated

that enzymes acting between acetylcoenzyme A and mevalonate are inhibited by cholesterol feeding [6], [10], [11]. Results on the incorporation of labelled acetate into squalene are in agreement with an inhibition located in that part of the cholesterol synthetic pathway, as a reduction of the squalene and the ubiquinone synthesis from acetate by cholesterol feeding is observed *in vivo*. Nevertheless the inhibition of the cholesterol synthesis between squalene and cholesterol is also evident after acetate as precursor, the synthesis of cholesterol being much more reduced than that of squalene and ubiquinones. Cholesterol feeding affects not only the cholesterol but also the fatty acid synthesis. The incorporation of acetate into fatty acids is similarly reduced as that into squalene. In addition a considerable accumulation of other lipids besides cholesterol in the liver is observed. It may be assumed therefore that the difference in the incorporation pattern between labelled glucose and labelled acetate reflects a reduced turnover of fatty acids by cholesterol feeding with the consequence of a preferred use of acetylcoenzyme A deriving from glucose for the cholesterogenesis. The identical effect of cholesterol feeding on the incorporation of glucose and mevalonate into squalene would thus be accidental. As an alternative it can be considered that the utilization of acetate for the synthesis of acetylcoenzyme A is reduced by cholesterol feeding, *e.g.* by inhibiting the acetylthiokinase. This assumption is in agreement with the almost identical effect of cholesterol feeding on the inhibition of the squalene, fatty acids, and ubiquinones synthesis from acetate. If an inhibition of enzymes acting between acetylcoenzyme A and mevalonate, *e.g.* the β-hydroxy-β-methyl-glutaryl(HMG)-coenzyme-A reductase did represent the predominant effect of cholesterol feeding on the lipid metabolism, even a stimulation of the fatty acid synthesis from acetate could be expected by recycling of acetylcoenzyme A formed by degradation of HMG-coenzyme A. The existence of a regulation site of the cholesterol synthesis could also be demonstrated by administering labelled squalene *in vivo*. Cholesterol feeding leeds to a significant reduction of the lanosterol and cholesterol synthesis from squalene. The radioactivity of squalene of the liver is more reapidly declining in the experimental group than in the control group. The radioactivity of lanosterol, one hour after the injection, much higher in the control group, becomes about equal in the control and the experimental group four and half hours later, indicating that in addition to the inhibition between squalene and lanosterol other regulating sites after the lanosterol must exist.

The hypothesis of the predominant role of the HMG-coenzyme-A reductase for regulating the cholesterol synthesis and the exclusion of other enzymes in the synthetic pathway is mainly based on *in vitro* experiments. First indications were obtained by comparing the incorporation of labelled acetate and mevalonate into cholesterol by liver homogenate under normal conditions and after cholesterol feeding [12], [13]. Comparison of the incorporation of labelled acetate into β-hydroxy-β-methylglutarate and into mevalonate with liver enzyme preparation leads to the conclusion that the inhibition of the HMG-coenzyme-A reductase is responsible for the inhibition of the cholesterol synthesis by dietary cholesterol [10]. Good correlations were found between cholesterol content of liver, reduction of the incorporation of acetate into cholesterol *in vitro* and the activity of HMG-coenzyme-A reductase after cholesterol feeding [6]. The rate limiting function of HMG-coenzyme A for the cholesterol synthesis has been put to doubt due to the observa-

tion that this enzyme is obviously not located on a branch point of the reaction sequence. It is assumed that the cytosolic thiolase of liver can reduce the flux of substrate but its inhibition by cholesterol feeding is unsufficient to account for the total inhibition of the cholesterol synthesis [11].

The discrepancy between results obtained *in vivo* and *in vitro* may have various reasons. *In vivo* it could be shown that the inhibition of the cholesterol synthesis located after the squalene formation with mevalonate as precursor becomes only evident if tracer doses are used or if with substrate doses both the incorporation of mevalonate into squalene and cholesterol is compared. *In vitro* [13] substrate doses were used and only the incorporation of mevalonate into cholesterol measured. The preparation of tissue for *in vitro* evaluation may induce artefacts differing in the control and in the experimental animals. The strongly reduced cholesterol synthesis occurring *in vivo* in kidney of rat by fasting [5] could not be demonstrated with tissue slices [14]. If an enzyme acts on a branch point of a reaction its inhibition may not necessarily lead to a reduced flux of substrate if another enzyme which competes for the same substrate is more inhibited. An example for such a situation has previously been described [15].

The existence of a regulating mechanism of the cholesterogenesis located after the squalene in the reaction sequence is also demonstrated with labelled mevalonate, glucose, acetate, and palmitate as precursors by measuring the incorporation of its radioactivity into cholesterol, squalene, and ubiquinones, influenced by a lipogenic diet in rat liver *in vivo*. Such a regulating mechanism seems to be of general significance as it was also observed after fasting and after dietary cholesterol [1], [2], [5]. For its evaluation in details further investigations are needed, as more as various regulation sites between squalene and cholesterol must be considered [3].

The observed differences between the utilization of labelled glucose, palmitate, and acetate for the squalene synthesis under lipogenic conditions must obviously be caused by a different use either of these precursors for the acetylcoenzyme A formation or of the acetylcoenzyme A for the squalene synthesis. The lipogenic diet stimulates the incorporation of ^{14}C from glucose into fatty acids about ten fold, that from acetate about three fold. As the squalene synthesis from glucose is not significantly affected, that from acetate, however, reduced to about one third, it seems that the lipogenic diet affects similarly the utilization of both glucose and acetate for both the fatty acid and the squalene synthesis. Whether the reduction of the flux of substrate from acetylcoenzyme A in direction squalene is reduced due to the stimulation of the fatty acid synthesis limiting thus the availability of acetylcoenzyme A for the squalene synthesis or whether an inhibition of enzymes located between acetylcoenzyme A and mevalonate or whether both effects are responsible remains an open question. An inhibiting influence between mevalonate and squalene does not seem to occur, as the incorporation of mevalonate into squalene is even stimulated by the lipogenic diet. Despite the reduced synthesis of the cholesterol of the liver by the lipogenic diet an accumulation of cholesterol in the liver is observed. It seems therefore that the bile acid formation or the degradation of cholesterol to other metabolites is also inhibited.

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Experimental procedures. – *Animals and materials.* Rats of a *Wistar-Glaxo* strain with an average weight of 173 g were used. Half of them obtained a diet enriched with 5% cholesterol added to the ground stock diet *(Nafag).* The control rats were fed with the same ground stock diet without added cholesterol. The rats had access to that food from 10 *a.m.* until 8 *a.m.* of the following day. The lipogenic diet fed during five days had the following composition: 70% glucose, 24% vitamin-free casein, 5% minerals and 1% vitamin mixture. The diet of the controls consisted of 60% cereals, 18% plant proteins, 4% minerals and 1% vitamin mixture. D-[U-^{14}C]-glucose (2,99 mCi/mmol), [1-^{14}C]-palmitic acid (17,7 mCi/mmol) and DL-[2-^{3}H]-mevalonic lactone (375 mCi/mmol) (previously hydrolized by alkaline treatment and neutralized) from the *Radiochemical Centre Amersham* were mixed and administered intraperitoneally dissolved in 0.1 ml water per dose. [1-^{14}C]-acetic acid sodium salt (57 mCi/mmol) from *F. Hoffmann-La Roche & Co. Ltd.* Basle, was dissolved in 0.1 ml water and biosynthetically labelled squalene obtained as previously described [3] was dissolved in 0.1 ml 50% ethanol per dose and administered by intraperitoneal injection at 8.30 *a.m.* For the determination of liver glycogen and its radioactivity 5 ml of liver homogenate diluted 1:4 are used. Glycogen was determined colorimetrically [4], its radioactivity was measured in the precipitate of glycogen obtained after the alkaline digestion. The liver glucose was determined enzymatically with the diagnostica kit of *F. Hoffmann-La Roche & Co. Ltd.* Basle.

Isolation procedures. The pooled livers of three rats were worked up as described previously [3]. About 10 mg of crystalline cholesterol were isolated, whose specific radioactivities of ^{14}C and ^{3}H were measured. The isolated fatty acids were weighed and dissolved in 10 ml of ethyl acetate; 0,1–0,2 ml were used for measuring the radioactivities. The radioactivities of squalene, lanosterol, and ubiquinones were determined after isolation of the substances by TLC. on silica gel with dichloromethane as solvent. The concentration of cholesterol, squalene, and ubiquinones was measured as previously described [3, 5].

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