252. The Influence of Fasting on the Synthesis of Cholesterol, Squalene, Fatty Acids, and Ubiquinones in Liver, Small Intestine, and Kidney of Rats *in vivo*

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(17.VIII.77)

Summary

(1) The existence of a regulating site of the cholesterol synthetic pathway in rat liver located after the squalene formation is shown by a comparison of the incorporation of labelled acetate and mevalonate *in vivo* over a period from 7.5 minutes to several hours under fasting and non fasting conditions. The existence of such a regulating site becomes also evident by the influence of fasting on the incorporation of labelled squalene into the cholesterol of the liver.

(2) It is assumed that enzymes acting between acetylcoenzyme A and mevalonate, *e.g.* the HMG-coenzyme-A reductase regulate the flux of substrate in direction squalene with the tendency to keep it constant. Such an assumption is in agreement with the observed minor influence of fasting on the squalene and ubiquinone synthesis.

(3) It could be shown, that the cholesterol synthesis in the kidneys is regulated by the same mechanism as in liver, whereas such a regulation could be excluded in the small intestine.

In a previous communication results were reported pointing to a regulation site of the cholesterol synthesis in rat liver located after the squalene formation. This was shown by administrating various labelled precursors such as acetate, mevalonate and glucose and by measuring the incorporation of radioactivity *in vivo* into cholesterol and squalene of the liver after a fasting period of 16 hours [1]. We now report results obtained by measuring the incorporation of labelled precursors *in vivo* as function of the time after various fasting periods. The cholesterol and squalene syntheses were also studied in the small intestine and the kidneys in order to find out whether these substances synthesized there may interfere with observations made with liver. For the same reason the equilibration of cholesterol and squalene between these organs and blood was investigated. For obtaining more informations on the influence of fasting on the flux of substrate in the liver the incorporation of labelled acetate into the fatty acids and the ubiquinones were included in these studies.

Experimental procedures. – Animals, materials, and isolation procedure for liver are used as described previously [2]. The same isolation procedure was used for the kidneys. 10 cm of the jejunum

from each rat or the whole small intestine are removed, cleaned from peritoneal fat and intestine content, pooled, weighed and worked up by the procedure used for liver. 3–4 ml of blood are collected from each rat from the heart. After determination of the total volume the cholesterol and squalene are extracted as described for liver. The specific radioactivity of the cholesterol of liver and of the whole small intestine is determined after isolation of the cholesterol in crystalline form. The specific radioactivity of the cholesterol of blood, jejunum and kidney and that of the squalene of all tissues is calculated from the total incorporation in the corresponding fraction obtained by TLC. and the amount of substance analytically determined. The same procedure is used for determining the specific radioactivity of the ubiquinones of the liver [2]. Cholesterol was determined by colorimetry [3] and squalene by gas-chromatography after column-chromatography [1]. In addition the squalene content was determined by a comparison of spots, obtained by TLC. on silicagel with dichloromethane as solvent, after spraying with 50% sulfuric acid in methanol and heating at 120° for 10 min. Spots of 2.5, 5, 10 and 15 µg are densitometrically compared with spots obtained with 2.5, 5, 10 and 15 µl of a solution of the unsaponifiable in ethyl acetate (the total unsaponifiable of the liver and the small intestine of 3–5 rats in 2 ml, that of the kidneys in 0.5 ml). The fatty acids were determined as previously described [2].

Each value of the tables and the figures was obtained by pooling the organs of groups of 4–7 rats. Comparative studies with 5 control groups of 5 rats revealed that the specific radioactivity of the cholesterol varied within the limits of ± 5%, that of the squalene, the ubiquinones, and the fatty acids ± 15%. The small limit of error of the specific radioactivity of the cholesterol is obviously due to the reduction of the experimental variations by isolating the cholesterol in pure form.

Results. 1. *The influence of fasting on the content of cholesterol, squalene, fatty acids, and ubiquinones in rat tissues. Langdon & Bloch* [4] found in rat liver 25–30 µg squalene per g by its isolation as hydrochloride. We obtained somewhat higher values by gas-chromatographic and TLC.-determination *(Table 1)*. Our values for cholesterol, ubiquinones, and fatty acids are in agreement with previous results. The concentration of these substances was determined in order to find out whether an accumulation or a reduction by fasting occurs. In liver an accumulation of cholesterol, squalene, and fatty acids seems to occur after a fasting period of 24 h. From *Table 2* it is seen, however, that despite the fact that the rat had access to water a considerable weight loss results from fasting. After a fasting period of 24 h almost one third of the relative liver weight is lost, apparently mainly by dehydration. The average reduction of the body weight of 30 rats was only 5% after a fasting period of 24 h. Thus the amount of cholesterol, squalene and fatty acids remains practically constant per liver, whereas that of the ubiquinones is reduced by about one third.

Table 3 shows the good agreement of the values of specific radioactivity of liver squalene, obtained respectively by direct determination after gas-chromatographic isolation, and by calculation from the concentration of squalene in the liver and the incorporation rate of radioactivity. The indirect method for determining the specific radioactivity of squalene could therefore be considered as reliable. The blood squalene was not determined, for calculating the specific radioactivity values obtained by *Goodman* [5] were used indicating a concentration of 0.35 µg squalene per ml.

2. *The influence of fasting on the synthesis of cholesterol and squalene in rat liver from labelled acetate, mevalonate, and squalene as a function of time.* The incorporation of acetate into cholesterol is strongly reduced over a period of 7.5 min. to several hours, whereas at the same time the squalene synthesis is not diminished but even stimulated for a certain time *(Fig. 1)*. No indication was thus obtained for a reduced flux of substrate in direction squalene, despite the strong inhibition of

Table 1. *Influence of fasting on the concentration of various lipids in rat tissues*

		Liver	Kidney	Jejunum	Blood
Cholesterol	C	2.43 (16)	2.5 (5)	1.7 (4)	0.41 (5)
mg/g tissue	F	3.06 (12)	2.7 (4)	1.5 (4)	0.44 (5)
Squalene	C	42.5 (7)[a])	30 (4)[a])	20 (4)[a])	–
µg/g tissue		41.0 (32)[b])			
	F	63.0 (7)[a])	35 (4)[a])	25 (4)[a])	–
Total fatty Acids	C	21.8 (13)	–	12.5 (10)	1.1 (10)
mg/g tissue	F	32.3 (17)	–	9.5 (10)	1.0 (10)
Ubiquinones	C	128 (26)	–	–	–
µg/g tissue	F	128 (26)	–	–	–

( )= number of rats. C= controls. F= 24 h fasted. [a])= determined by TLC. [b])= determined by gas-chromatography.

Table 2. *Relative weight loss of rat liver by fasting* (each time 5 rats)

Fasting period beginning 8.30 *a.m.*	0 h	6 h	24 h	30 h
Body weight/Liver weight	23.5	28.5	36.9	37.2

Table 3. *Comparison of the specific radioactivity of liver squalene directly determined and calculated from the tissue concentration of squalene and the incorporation rate of radioactivity* (5 µCi (124 µg) of DL-[^{14}C]*mevalonate administered orally per rat at 9 a.m., 2 hours prior to killing))*

Number of rats per group	Specific radioactivity of liver squalene (dpm/µg)	
	directly determined	calculated
10	1879	1600
10	1916	1745
12	2315	2224

the cholesterol synthesis. This points to a regulating effect located after the squalene formation. This assumption is confirmed with mevalonate as precursor *(Fig. 2)*. The smaller inhibition of the cholesterol synthesis from mevalonate than from acetate by fasting must obviously be explained by the stimulation of the incorporation of mevalonate into squalene by fasting (*Fig. 1* and *2*).

As to be expected *figure 3* shows that the incorporation of labelled squalene into cholesterol is also inhibited by fasting. Compared with acetate and mevalonate the incorporation of squalene is much slower due probably to a slower transport of the squalene from the injection to the reaction site.

The synthesis of the fatty acids is drastically and much more reduced by fasting than the cholesterol synthesis *(Fig. 1)*. Under non fasting conditions their specific radioactivity remains practically constant for several hours indicating a recycling of degradation products. It must therefore be considered that possibly such degradation products may be reused for the squalene synthesis and be responsible for the long lasting remain of radioactivity in the squalene. Such an effect contributing considerably to the radioactivity of the squalene seems, however, very unlikely on the basis of a comparison of the specific radioactivities of squalene and

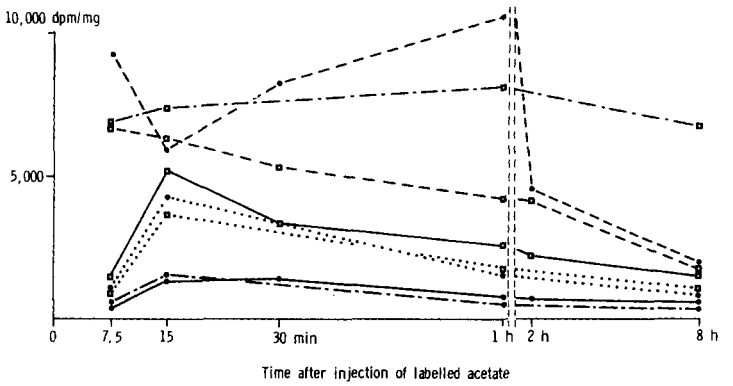

Fig. 1. *Influence of fasting on the specific radioactivity of cholesterol, squalene, fatty acids, and ubiquinones of rat liver*

30 μCi of [^{14}C]acetate in 0.1 ml H_2O are injected intraperitoneally at 8.30 *a.m.* per rat. During the experimental period the animals had access to water only. The values of each point were obtained from groups of 4–7 rats. The values of the specific radioactivity of the fatty acids are multiplied by 3 and those of the ubiquinones by 5.

Cholesterol (control) — Cholesterol (24 h fasted)
Squalene (control) — Squalene (24 h fasted)
Fatty acids (control) — Fatty acids (24 h fasted)
Ubiquinones (control) — Ubiquinones (24 h fasted)

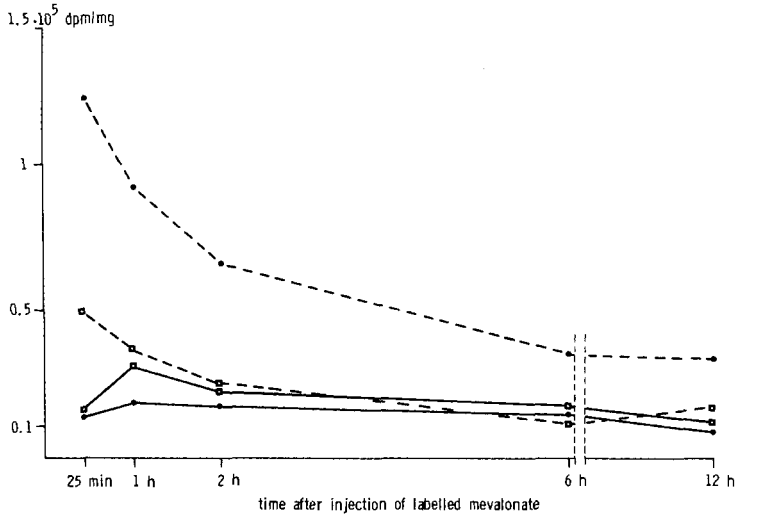

Fig. 2. *Influence of fasting on the specific radioactivity of cholesterol and squalene of rat liver after labelled mevalonate*

3 μCi of DL-[^{14}C]mevalonate in 0.1 ml H_2O were injected intraperitoneally at 8.30 *a.m.* per rat. During the experimental period the animals had access to water only. The values of each point were obtained from groups of 4–5 rats. The values of the specific radioactivity of the squalene are reduced to half.

Cholesterol (control) — Squalene (control)
Cholesterol (24 h fasted) — Squalene (24 h fasted)

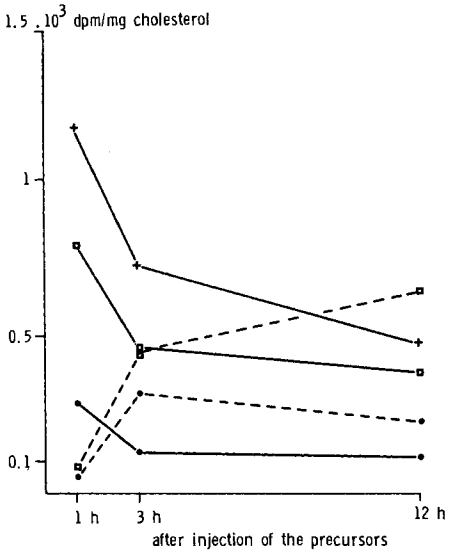

Fig. 3. *Influence of fasting on the incorporation of labelled acetate and squalene into liver cholesterol* 30 μCi[^{14}C]acetate, 3 μCi DL-[^{14}C]mevalonate in 0.1 ml H_2O, $1.6 \cdot 10^6$ dpm-[^{3}H]squalene in 0.1 ml 50% ethanol are injected intraperitoneally at 8.30 *a.m.* per rat. During the experimental period the animals had access to water only. The values of each point were obtained from groups of 4–5 rats. The values of the specific radioactivity of cholesterol after squalene and after mevalonate as precursors are reduced to one fifth.

▫——▫ control (after acetate)
•——• 24 hours fasted (after acetate)
▫- - -▫ control (after squalene)
•- - -• 24 hours fasted (after squalene)
+——+ control (after mevalonate)

fatty acids: under fasting conditions and 8 h after the injection of labelled acetate the specific radioactivity of squalene is about 20 times higher than that of the fatty acids.

The synthesis of the ubiquinones is very little affected by fasting *(Fig. 1)*. This observation again is not compatible with the assumption of a main rate limiting function of the HMG-coenzyme-A reductase. Otherwise an inhibition of the ubiquinones synthesis by fasting similar to that of the cholesterol synthesis should be observed, or an independent pathway for the synthesis of ubiquinones, outgoing from β-hydroxy-β-methylglutaryl-coenzyme A must exist.

3. *The influence of various fasting periods on the synthesis of cholesterol, squalene and fatty acids from labelled acetate in liver* (Fig. 4 and 5). In liver the cholesterol synthesis is steadily reduced with the increase of the fasting period. The fatty acid synthesis behaves accordingly. The inhibition of the fatty acid synthesis is, however, much stronger than that of cholesterol. Considering the specific radioactivities of the cholesterol and of the fatty acids and the concentrations of the substances in the tissue the total incorporation of acetate into fatty acids is many times higher than into cholesterol. It can be understood therefore that due to the drastic reduction of the fatty acid synthesis by fasting and despite an inhibition of enzymes necessary for the synthesis of squalene [6], the flux of substrate in direction squalene must not necessarily be reduced.

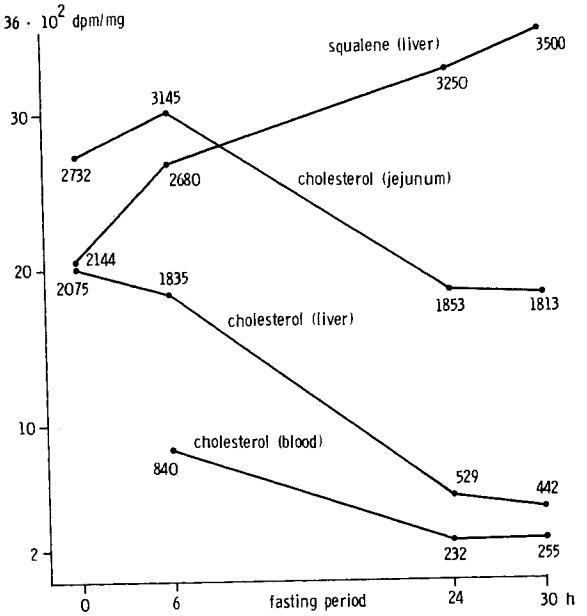

Fig. 4. *Influence of different fasting periods on the specific radioactivity of cholesterol and squalene after labelled acetate*

20 µCi[^{14}C]acetate in 0.1 ml H_2O were injected intraperitoneally per rat the first time at 8.30 *a.m.* one hour prior to killing; the indicated fasting periods correspond with the following injection times always one hour prior to killing. The values of each point were obtained by groups of 5 rats.

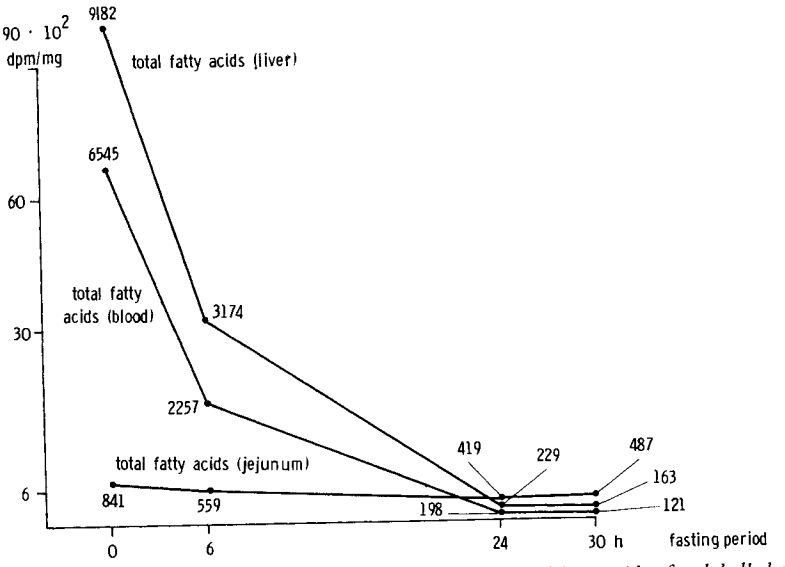

Fig. 5. *Influence of different fasting periods on the specific radioactivity of fatty acids after labelled acetate* (experimental conditions see *Fig. 4*)

The slight stimulation of the squalene synthesis observed 1 h after the injection of acetate *(Fig. 1)* is confirmed. This effect is increasing with the fasting time *(Fig. 4)*.

4. *The influence of fasting on the cholesterol, squalene, and fatty acid synthesis in the small intestine of rats.* Previous observations [7] of only a slight reduction of the cholesterol synthesis from acetate by fasting are confirmed *(Fig. 4)*. The fatty acid synthesis is similarly affected *(Fig. 5)*. The incorporation of mevalonate into cholesterol and squalene is not significantly influenced by fasting *(Table 4)*. Thus no indications are obtained for a regulating effect of the cholesterol synthesis located between squalene and cholesterol. The incorporation rate of labelled acetate 1 h after the injection was for both the whole small intestine and the jejunum alone the same per g tissue namely about 90% of that observed per g liver. With tissue slices the incorporation rate of labelled acetate after a 2 h incubation period was for the ileum 64% and for the jejunum only 8.5% of the corresponding liver values [7].

5. *The influence of fasting on the cholesterol and squalene synthesis in rat kidneys.* The cholesterol and especially the squalene synthesis from labelled mevalonate is much higher in the kidneys than in the liver (*Table 5* and *Fig. 2*). Thus previous results [8] are confirmed. The cholesterol synthesis from acetate is, however, in the kidneys much smaller than in the liver, whereas the squalene synthesis from acetate is about the same in both organs per g tissue *(Table 5, Fig. 1)*. As mevalonate is possibly not a normal constituent of the blood, the enormous squalene synthesis from mevalonate may reflect an accumulation of injected mevalonate in kidney prior to excretion.

In the kidneys the cholesterol synthesis from acetate is strongly inhibited by fasting without a corresponding inhibition of the squalene synthesis, whereas the squalene synthesis from mevalonate is stimulated and cholesterol synthesis inhibited *(Table 5)*. Thus the kidneys show the same pattern of the regulation of the cholesterol synthesis as the liver. These results are not in agreement with observations made with tissue slices on the acetate incorporation. Only an insignificant inhibition of the cholesterol synthesis similar to that occurring in the small intestine was observed under fasting conditions [7].

6. *Equilibration of lipids between liver, kidney, small intestine, and blood.* A possible exchange of metabolites between various organs mediated by the blood must be considered when interpreting incorporation experiments carried out *in vivo*. An equilibration of lipids is well known between liver and blood but not between intestine and kidney. The decline of the specific radioactivity of blood cholesterol and blood fatty acids follows exactly that of liver cholesterol *(Fig. 4)* and liver fatty acids *(Fig. 5)*, indicating an equilibration between liver and blood somewhat easier for fatty acids than for cholesterol. A significant secretion of cholesterol from small intestine into the blood can be excluded, as otherwise the specific radioactivity of blood cholesterol would show a slower decline.

From *Table 6* it can be seen that the specific radioactivity of blood squalene parallels approximatively that of the liver but not that of the kidneys, which is about 100 times higher. A transport of even a very minor amount of squalene from the kidneys to the liver can be excluded, otherwise the specific radioactivity

Table 4. *Influence of fasting on the incorporation of labelled mevalonate into the cholesterol and the squalene of the small intestine of rats* (3 μCi of DL-[^{14}C]-mevalonate dissolved in 0.1 ml H_2O injected intraperitoneally at 8.30 *a.m.* per rat one hour prior to killing)

		Specific radioactivity dpm/mg Time after injection of mevalonate	
		25 min	5.5 h
Cholesterol	control (4)	389	2282
	24 h fasted (4)	389	1933
Squalene	control (4)	20779	5536
	24 h fasted (4)	17984	4227

( ) = number of rats.

Table 5. *Influence of fasting on the incorporation of labelled acetate and mevalonate into the cholesterol and squalene in rat kidneys* (30 μCi[^{14}C]-acetate in 0.1 ml H_2O and 10.6 μCi DL-[^{3}H]mevalonate in 0.2 ml H_2O injected at 8.30 *a.m.* i.p. per rat)

		Specific radioactivity dpm/mg			
		Time after injection of acetate		Time after injection of mevalonate	
		15 min	2 hours	1 hour	2 hours
Cholesterol	C	229 (4)	316 (7)	34400 (5)	75300 (5)
	F	47 (4)	77 (7)	31700 (5)	29000 (5)
Squalene	C	11593 (4)	4695 (7)	$9.5 \cdot 10^6$ (5)	$10.2 \cdot 10^6$ (5)
	F	5887 (4)	7319 (7)	$20 \cdot 10^6$ (5)	$13.3 \cdot 10^6$ (5)

C = controls. F = 24 h fasted. ( ) = number of rats.

Table 6. *Equilibration of squalene between liver, kidney, and blood of rats* (10.6 μCi DL-[^{3}H]mevalonate dissolved in 0.2 ml H_2O injected intraperitoneally at 8.30 *a.m.* per rat one hour prior to killing)

	Specific radioactivity of squalene (dpm/μg)		
	Liver	Blood	Kidney
Control (5)	$90 \cdot 10^3$	$75 \cdot 10^3$	$95 \cdot 10^5$
24 h fasted (5)	$183 \cdot 10^3$	$97 \cdot 10^3$	$200 \cdot 10^5$

( ) = number of rats.

of the blood squalene would be higher than that of the liver, as more as the total amount of squalene of the liver is about 100 times and that of the kidneys about 15 times higher than that of the circulating blood. The possibility that the labelled squalene synthesized in the small intestine is transported to the liver and influences the results there obtained can also be excluded due to the fact that the specific radioactivity of the liver squalene is about 5–10 times higher than that of the small intestine squalene observed under equal conditions (*Fig. 2* (liver), *Table 4* (small intestine)).

Discussion. – Previous results [1] on the influence of fasting on the squalene and cholesterol synthesis from labelled acetate and mevalonate are confirmed by measuring the incorporation as a function of time. Between 7.5 min and 8 h after the injection of acetate no reduction of the incorporation of radioactivity into squalene by fasting was observed despite a strong reduction of cholesterol synthesis *(Fig. 1)*. An inhibiting effect of fasting after the squalene formation must therefore be assumed. This assumption is confirmed with mevalonate as precursor. The outstanding effect of fasting is the strongly enhanced incorporation of mevalonate into squalene which compensates considerably the inhibiting effect located between squalene and cholesterol *(Fig. 2)*. Fasting reduces also the incorporation of labelled squalene into cholesterol confirming again the inhibition between squalene and cholesterol. The slow rise of the incorporation of squalene into cholesterol as a function of time must probably be explained by a slow transport of squalene from the injection to the reaction site.

As already reported [2] the decline of the radioactivity of cholesterol and squalene after acetate and mevalonate as precursors is levelling off and remains practically constant over a period of several hours. The observed type of decline would not occur by a regulary continuous degradation or transformation of these metabolites. An influx of labelled cholesterol and squalene into the liver from other tissues was therefore considered, but could be excluded at least for the kidneys and the small intestine by comparing the specific radioactivities of the squalene and the cholesterol in the liver, the kidneys, the small intestine, and the blood (*Fig. 4* and *5; Tables 4* and *5*). A reasonable explanation especially for the long persistence of the radioactivity in the liver squalene is offered by the already reported observation of a recycling of degradation products of squalene and cholesterol for the synthesis of isoprene compounds [2].

Under non fasting conditions the capacity of the liver for synthesis is much higher for fatty acids than for cholesterol. The reduction of the synthesis by fasting, however, is much more pronounced for fatty acids than for cholesterol. Both pathways are located in the cytosol of the liver cells in contrast to the degradation of acetate *via* acetylcoenzyme A localized in the mitochondria. The inhibition of the acetylcoenzyme-A carboxylase, the prominent effect of fasting on the fatty acid synthesis, may lead to a preference of the use of acetylcoenzyme A in direction mevalonate. Inhibition of enzymes acting between acetylcoenzyme A and mevalonate, *e.g.* β-hydroxy-β-methylglutaryl (HMG)-coenzyme-A reductase may be responsible for the more or less constant flux of substrate in direction squalene under fasting conditions. The unimpaired synthesis of the ubiquinones by fasting can thus also be explained.

The assumption of the main rate-limiting function of the HMG-coenzyme-A reductase in the cholesterol synthetic pathway in the liver and the exclusion of enzymes for such a function acting after the mevalonate formation rely mainly on the comparison of the incorporation of various precursors into cholesterol by liver enzyme preparations [9]. Metabolic transformations observed *in vivo* are, however, not necessarily in parallel with observations made *in vitro*. *In vitro* the incorporation of labelled acetate into squalene and cholesterol by liver homogenate centrifuged with $5000 \times g$ was found reduced to less than 5% of the controls

after a 24 h fasting period [9], whereas *in vivo* under the same conditions the cholesterol synthesis from acetate was reduced to about 30% and that of squalene was not affected or even stimulated. The incorporation of mevalonate into cholesterol is little affected *in vivo*, whereas *in vitro* a reduction to about 25% of the controls is observed [9]. The incorporation of squalene into cholesterol is found to be reduced both *in vivo* and *in vitro* by fasting, significantly more, however, *in vivo*. Various reasons can be responsible for such discrepancies. It is conceivable that such a drastic intervention as a 24 h fasting period resulting in a relative weight loss of about one third of the liver induces changes in the tissue with different consequences on enzyme activities respectively *in vivo* and *in vitro*. Artefacts produced by the isolation of enzymes may thus be different in tissues of fasted and non fasted animals.

If mevalonate is used as precursor *in vivo* the inhibiting effect of fasting on the cholesterol synthesis located after the squalene formation becomes only evident if both the incorporation of mevalonate into squalene and into cholesterol are examined, because the cholesterol synthesis from mevalonate is little affected, the squalene synthesis, however, strongly stimulated. Injected *in vivo* mevalonate and acetate behave quite differently. The most prominent effect is the enormous incorporation of mevalonate into the kidney squalene, being about 100 times higher than in the liver squalene, whereas with acetate as precursor the incorporation rates into both squalene of liver and of kidney are about the same. An accumulation of injected mevalonate in the kidney may be responsible for that phenomenon. A certain accumulation of injected mevalonate may also occur in the liver and the kidney by fasting, possibly by an impaired excretion. The stimulation of the mevalonate incorporation can be explained by such an effect.

The authors thank Miss *U. Olbrecht* for the excellent care of the animals and *F. Hoffmann-La Roche & Co. Ltd.* Basle for financial support.

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