The Biosynthesis of Squalene and Sterols by the Adipose Tissue of Rat, Sheep and Man

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1. The subcutaneous and omental adipose tissue of man, the epididymal fat pads of the rat and the fat tail of the Syrian sheep incorporate mevalonic acid into non-saponifiable lipids. 2. Time studies showed that the rates of decarboxylation of mevalonic acid and synthesis of non-saponifiable lipids slightly decline after 20 min. but subsequently remain linear for 6 hr. 3. About one-half of the incorporated radioactivity in the non-saponifiable lipids was in squalene, 20% in lanosterol and cholesterol, and the remainder in unidentified substances.

Previous studies (Vaughan, 1961) have shown that oxidative and synthetic pathways are operative in adipose tissue. However, the capacity of this tissue to synthesize sterols and other non-saponifiable lipids remained hitherto unknown. In this study, mevalonic acid, an efficient precursor of these compounds, was incubated with four types of adipose tissue, namely omental and subcutaneous human fat, the epididymal fat pads of the rat and the fat tail of the Syrian sheep. Evidence for the biosynthesis of squalene, cholesterol and lanosterol is provided.

MATERIALS AND METHODS

Tissue. Biopsy specimens of omental and abdominal subcutaneous human fat were kindly provided by Dr K. Nahra and Dr F. Suidan from the hospital of the American University of Beirut, Lebanon. The epididymal fat pads of the male rats (Wistar strain) were isolated and finely minced with a pair of scissors. Biopsy specimens were obtained from the fat tail of the Syrian sheep, *Ovis aries* var. *crassicornus*, under anaesthesia, and similarly minced.

Chemicals. DL-[¹⁴C]mevalonolactone was obtained from The Radiochemical Centre, Amersham, Bucks. Squalene (extracted from whale oil), farnesol, nerolidol and geraniol were obtained from L. Light and Co. Ltd., Colnbrook, Bucks. Cholesterol, lanosterol and related sterols were obtained from Mann Research Laboratories, New York, N.Y., U.S.A. Alumina suitable for chromatography was obtained from E. Merck A.-G., Darmstadt, Germany. Buffers were prepared as described by Durr & Cortas (1964).

Assay. The activity of the tissues was assayed first by the measurement of radioactivity in the CO₂ liberated from DL-[¹⁴C]mevalonolactone as described by Durr & Shwayri (1964). The second assay involved the incubation of DL-[¹⁴C]mevalonolactone with the tissue. The reaction mixture was saponified for 24 hr. with 10% (w/v) KOH in methanol-water (7:3, v/v) by using 5 ml./g. of tissue. The saponification mixture was concentrated to one-fifth of its

volume, and the aqueous layer was exhaustively extracted with peroxide-free diethyl ether. The ether layer was washed with water until the washings were neutral and free of radioactivity. The ether layer was then dried over anhydrous Na₂SO₄; a sample was plated at infinite thinness, and the radioactivity was determined in a thin-window counter (Nuclear-Chicago Corp.) with a sensitivity of 6×10^5 counts/min./μC, and having a background counting rate of 18 counts/min. The results of duplicate incubations agreed within 2%.

Chromatography of non-saponifiable liquids. Squalene was separated from sterols and related compounds by chromatography on deactivated alumina as described by Schneider, Clayton & Bloch (1957). Cholesterol and lanosterol were separated by chromatography on deactivated alumina as described by Salokangas, Rilling & Samuels (1964).

Crystallizations. Squalene was crystallized as the hexahydrochloride as described by Heilborn, Kam & Owens (1926). I have found that farnesol, nerolidol and geraniol do not crystallize under these conditions. Cholesterol and lanosterol were crystallized from hot methanol-acetone (1:1, v/v), and their digitonides were precipitated and purified as described by Sperry & Webb (1950). Melting points were determined on a hot-stage apparatus (Fisher-Johns).

Thin-layer chromatography. Small samples of the non-saponifiable lipids were chromatographed on silica gel with ethyl acetate-hexane (1:1, v/v) as the solvent. The spots were developed by spraying the plate with conc. H₂SO₄ and heating at 100°.

Colorimetric determinations. Cholesterol was determined by the Liebermann-Burchard test (Sackett, 1925). Lanosterol was similarly determined except that the wine-red colour was read at 540 mμ after 1 hr.

RESULTS

Stoichiometry and rate of mevalonolactone metabolism. Results in Table 1 show that when various

types of adipose tissue were incubated with DL-[2-¹⁴C]mevalonolactone no radioactivity was detected in the respired carbon dioxide. However, a significant amount of radioactivity appeared in the non-saponifiable lipids. On the other hand, when

Table 1. *Stoichiometry of mevalonolactone metabolism by adipose tissue*

The incubation mixture contained 3.2 μ moles of DL-[¹⁴C]mevalonolactone (1300 000 counts/min.), 200 μ moles of glucose, 250 μ moles of phosphate buffer, pH 7.0, and 2 g. of tissue, in a final volume of 2.8 ml. Incubation was for 3 hr. at 37°.

	Radioactivity recovered in fraction (counts/min.)			
	DL-[1- ¹⁴ C]- Mevalonolactone		DL-[2- ¹⁴ C]- Mevalonolactone	
	CO ₂	Non-saponifiable lipids	CO ₂	Non-saponifiable lipids
Adipose tissue				
Human subcutaneous	7 680	0	0	4 800
Human omental	5 400	0	0	3 250
Rat epididymal pad	10 920	0	70	4 335
Sheep fat tail	87 600	0	210	55 250

Fig. 1. Rates of CO₂ liberation and non-saponifiable lipid synthesis from mevalonolactone. The incubation mixture contained 0.8 μ mole (700 000 counts/min.) of each of DL-[1-¹⁴C]mevalonolactone and DL-[2-¹⁴C]mevalonolactone, 1 g. of sheep fat tail, 100 μ moles of phosphate buffer, pH 7.5, and 200 μ moles of glucose, in a final volume of 3 ml. Incubation was at 37°. ●, Rate of synthesis of non-saponifiable lipids at 30 min. intervals; ○, rate of CO₂ liberation at 30 min. intervals; △, rate of CO₂ liberation at 10 min. intervals.

DL-[1-¹⁴C]mevalonolactone was used, the label appeared in carbon dioxide but not in the non-saponifiable lipids. These results are in agreement with the known mechanism for the metabolism of mevalonolactone (Popják & Cornforth, 1960).

Fig. 1 shows that the rate of carbon dioxide production paralleled that for the synthesis of non-saponifiable lipids by the fat tail of the Syrian sheep when both were simultaneously measured at 30 min. intervals. In an independent run the rates of carbon dioxide production and non-saponifiable lipid synthesis were linear for at least 6 hr. after the initial decline. Similar results were obtained with the epididymal fat pads of the rat.

Concentration of mevalonolactone. The effect of the concentration of mevalonolactone on the rate of carbon dioxide liberation from this substrate by the fat tail of the sheep and the epididymal fat pad of the rat is shown in Fig. 2. The rate of carbon dioxide production increases with increasing concentrations of mevalonolactone and slowly approaches zero-order kinetics.

Nature of the non-saponifiable lipids. Preliminary purification of the non-saponifiable lipids on deactivated alumina sharply resolved the hydrocarbon fraction from that of the sterol and other substances.

Identification of squalene. A hydrocarbon fraction of sheep non-saponifiable lipids (18 740 counts/min.)

Fig. 2. Dependence of CO₂ liberation on the concentration of mevalonolactone. The incubation mixture contained 1.5 g. of tissue, DL-[1-¹⁴C]mevalonolactone having a specific activity of 1150 000 counts/min./ μ mole, 200 μ moles of glucose and 250 μ moles of phosphate buffer, in a final volume of 3 ml. Incubations were for 3 hr. at 37°. ●, Sheep fat tail, pH of buffer being 7.5; ○, rat epididymal fat pads, pH of buffer being 6.6.

was mixed with 0.4 ml. (334 mg.) of authentic whale squalene as carrier and was further chromatographed on deactivated alumina. The radioactivity was quantitatively recovered in the squalene peak. Squalene was subsequently crystallized as the hexahydrochloride derivative, and purified to a constant specific activity (28, 33, 33 and 33.4 counts/min./mg.). This is equivalent to a theoretical yield of 17100 counts/min. in squalene. Hence squalene represents about 92% of the hydrocarbon fraction.

The hydrocarbon fraction of the non-saponifiable lipids of the epididymal fat pad of the rat and the subcutaneous adipose tissue of man were subjected to the same treatment. Squalene accounted for 91 and 67% of the hydrocarbon fraction of the adipose tissue from rat and man respectively. Thin-layer chromatography of the hydrocarbon fraction of the various adipose tissues before adding carrier squalene showed a single orange spot (R_f 0.86). Authentic squalene gave a similar spot.

Identification and quantitative estimation of lanosterol and cholesterol. The sterol fraction (14000 counts/min.) from sheep non-saponifiable lipids was resolved by column chromatography into two fractions containing 11820 and 2740 counts/min. respectively. Fraction I (11820 counts/min.) was recovered with the peak of lanosterol. However, thin-layer chromatography of a sample gave two spots (R_f 0.73 and 0.76) having a grey and an orange colour respectively. Authentic lanosterol gave an orange spot (R_f 0.76). Crystalline lanosterol (120 mg.) was added as carrier to the lanosterol fraction and recrystallized from hot methanol-acetone (1:1, v/v) repeatedly to constant specific activity (14, 16, 18 and 17 counts/min./mg.), thereby giving a theoretical yield of 2040 counts/min. in lanosterol. This was further purified as the digitonide and recrystallized to constant specific activity (3.4, 3.8 and 3.5 counts/min./mg.), which is equivalent to a total of 1630 counts/min. Hence lanosterol accounted for about 13% of the total sterol fraction. When cholesterol was added to another sample of fraction I and crystallized, no radioactivity was detected in cholesterol. The more polar fraction (II) was recovered with the peak of cholesterol. Thin-layer chromatography of a sample of fraction II showed three violet spots (R_f 0.6, 0.63 and 0.66). Authentic cholesterol gave one violet spot (R_f 0.63). Crystalline cholesterol (120 mg.) was added to fraction II and recrystallized as described for lanosterol. It was calculated that 8% of the radioactivity in fraction II was in cholesterol. Addition of crystalline lanosterol to another sample of fraction II and recrystallization gave no radioactive lanosterol. It may be noted here that, when the non-saponifiable lipid fractions from the various tissues were treated with digitonin before the addition of carriers, the radioactivity in the digitonin-

precipitable sterols was 10–20% of the total non-saponifiable lipids, which could be accounted for in lanosterol and cholesterol. Fractions I and II, when freed of cholesterol, and lanosterol still gave a faint positive Liebermann–Burchard test but no precipitate was obtained with digitonin. Attempts to identify the remaining compounds were not successful. Separate additions of dihydrocholesterol, Δ^7 -dehydrocholesterol, Δ^4 -cholesten-3-one, cholestan-3-one and desmosterol as carriers and subsequent recrystallizations did not account for more than 1% of the radioactivity in the non-saponifiable lipids. The sterol fractions from the adipose tissue of rat and man showed a similar pattern.

DISCUSSION

Data presented in this paper provide evidence for the incorporation of mevalonic acid into the non-saponifiable lipids of omental and subcutaneous adipose tissue of man, epididymal fat pad of rat and the fat tail of the Syrian sheep. The radioactivity in the non-saponifiable lipids derived from DL-[2- 14 C]mevalonolactone was fractionated into squalene, cholesterol, lanosterol and unidentified substances. Whereas squalene was the major component of the non-saponifiable lipids, lanosterol and cholesterol accounted for approx. 10% of the total radioactivity in non-saponifiable lipids. The remaining fraction could not be accounted for as Δ^7 -dehydrocholesterol, dihydrocholesterol, Δ^4 -cholesten-3-one, cholestan-3-one or desmosterol. Ptak (1964) has extracted two highly polar types of steroids from the brown fat of the rat. These were not identified, but one of them is apparently related to corticosterone and deoxycorticosterone. The presence of several compounds accompanying lanosterol and cholesterol has been also observed in testicular tissues (Tsai, Ying & Gaylor, 1964; Salokangas *et al.* 1964). The accumulation of squalene observed in my studies is not typical of adipose tissue only. A massive accumulation of squalene was also observed when mevalonic acid was incubated with testicular tissues from rat (Tsai *et al.* 1964; Salokangas *et al.* 1964).

It was also shown that, when DL-[1- 14 C]mevalonolactone was incubated with adipose tissue, the respired carbon dioxide was radioactive but not the non-saponifiable lipids. However, the distribution of radioactivity was reversed when DL-[2- 14 C]mevalonolactone was used, thereby suggesting that mevalonic acid is decarboxylated, presumably via the mechanisms operative in other mammalian tissues and bacteria (Popják & Cornforth, 1960). Durr & Shwayri (1964) have shown that for every mole of labelled carbon dioxide liberated from [1- 14 C]mevalonolactone, 1 g. atom of labelled carbon was incorporated from [2- 14 C]mevalonolactone into

the non-saponifiable lipids of *Lactobacillus plantarum*. This stoichiometry in the metabolism of mevalonolactone by lactobacilli could not be demonstrated in adipose tissue. Frequently the radioactivity in the non-saponifiable lipids derived from [2-¹⁴C]mevalonolactone ranged from 40 to 85% of that in the carbon dioxide liberated from [1-¹⁴C]mevalonolactone. Preliminary studies indicate that the balance of radioactivity could be quantitatively accounted for in a water-soluble fraction containing at least two acids. It is possible that this fraction may contain a mixture of terpenic acids and alcohols, the biosynthesis of which occurs in rat liver (Ogilvie & Langdon, 1959; Popják, Lowe, Moore, Brown & Smith, 1959), ascites tumour (Schroepfer & Gore, 1963) and testis (Tsai *et al.* 1964). Popják, Horning, Bucher & Cornforth (1959) have shown that the metabolism of mevalonolactone can be significantly diverted to the synthesis of terpenic acids rather than squalene by withholding ascorbic acid from the incubation medium. Gosselin & Lynen (1964) have reported the synthesis of a polar lipid via pathways independent of those between farnesyl pyrophosphate and squalene.

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