Lipogenesis from amino acids in perfused isolated dog skin

VICTOR R. WHEATLEY, GEORGE LIPKIN, and TAE HA WOO

Department of Dermatology, New York University School of Medicine, New York 10016

ABSTRACT Lipogenesis from amino acids has been studied in isolated perfused dog skin. Uniformly labeled alanine-^{14}C, glycine-^{14}C, isoleucine-^{14}C, leucine-^{14}C, phenylalanine-^{14}C, and valine-^{14}C are all incorporated into the cutaneous lipids, with significant incorporation into most of the isolated lipid fractions. Efficiency of lipogenesis has been expressed relative to the extent of incorporation of acetate under the same experimental conditions. This efficiency was highest for the three branched-chain amino acids.

The accuracy, uses, and limitations of the perfusion technique for the study of cutaneous lipogenesis have been evaluated.

KEY WORDS dog · skin · perfusion · lipogenesis · amino acids · branched-chain · fatty acids

IT HAS BEEN KNOWN for almost a century that the lipids of the skin are very different from those of other tissues. Some of the peculiarities of skin lipids have been useful; for example the occurrence of large amounts of lanosterol in wool wax (1) and of squalene in surface lipids of human skin (2) has proved invaluable in the elucidation of cholesterol biogenesis. Skin is also somewhat unusual in that the rate of lipogenesis in it is rather high. Srere, Chaikoff, Teitman, and Burnstein (3) recognized early the rapidity of cutaneous cholesterogenesis, which represents by no means the total lipogenesis of the skin. The bulk of the skin consists of inert fibrous tissue, of which living cells form only a part. If we correct for the inert tissue we can calculate, from the best available data (4, 5), a cutaneous lipogenesis rate in man of about 2 g of lipid per day from some 300 g of tissue. Though no more than an educated guess, this estimate does represent the order of magnitude.

Abbreviation: TLC, thin-layer chromatography.

The skin is a heterogeneous structure in which lipogenesis does not occur uniformly throughout the tissue. There are two major sites of cutaneous lipogenesis, the living cells of the epidermis and those of the sebaceous glands (6). The two sites appear to form different types of lipids. In man the epidermis is the active site of cholesterogenesis, whereas the sebaceous gland forms little or no cholesterol but actively forms squalene (6, 7). Biological and anatomical features lead us to expect such a difference. In both the epidermis and the sebaceous gland, cells attached to a basement membrane undergo division and most of the daughter cells move away from the attachment membrane into a lethal environment. In the epidermis the cells are subjected to a complex drying process which leads to the formation of the horny layer, whereas in the sebaceous gland the cell still remains enclosed in an acinus of the gland. The sebaceous cell is virtually incubated under conditions resembling those of tissue culture, and supports lipogenesis. It finally becomes so distended with lipid globules that the cell wall ruptures and the lipid, sebum, is liberated into the sebaceous duct. It is not yet clear to what extent epidermal lipogenesis is limited to the basal cells and to what extent it occurs after these cells have moved away from the basement membrane. For the sebaceous gland, however, we know that lipogenesis seems to begin only after the cell has left the basement membrane; that it appears to be a true de novo lipogenesis of both fatty acids and other lipids [there is some evidence that in the sebaceous gland acetate-^{14}C is incorporated into fatty acids, sterols, and other lipids (8)]; and that it does not involve degeneration of the nucleus or cytoplasm since these remain unchanged until the cell wall ruptures (9). In addition to these differences in cellular environment, other factors must play a role. Permeability constants of the basement membranes of the epidermis or sebaceous gland will determine the amounts of precursors available for lipogenesis. The oxygen available to the cells under-

going lipogenesis will not be the same in both sites. Finally, the sebaceous gland cells are rich in mitochondria whereas mitochondria are sparse in the epidermal cells (10).

Studies of cutaneous lipogenesis have employed skin slices from man (6, 11) and rat (12), rat preputial glands (13), or a tumor of this gland (14), all under in vitro conditions. Other studies have used separated fractions from mouse skin (15). The considerations given above lead us to question the interpretation of data obtained in vitro. Basic data obtained under in vivo conditions are needed for a point of reference. Hepatic and cutaneous lipogenesis are difficult to study separately in the intact animal; hence a skin perfusion model is required. Such a perfusion technique has been described for the dog by Kjaersgaard (16), and simplified by Adachi and Chow (17). This method is suitable for the study of cutaneous lipogenesis, and preliminary reports based on its use have already been published (8, 18). The technique possesses, however, certain inherent limitations; therefore we have paid particular attention, in the present study, to the reproducibility and value of the method.

In this study, the subcutaneous fat was removed from the skin and discarded. It is not a true part of the skin, and lipogenesis in this tissue should be considered separately.

MATERIALS AND METHODS

Materials

Uniformly labeled sodium acetate-^{14}C, L-alanine-^{14}C, glycine-^{14}C, L-isoleucine-^{14}C, L-leucine-^{14}C, L-phenylalanine-^{14}C, and L-valine-^{14}C; glycine-1-^{14}C and glycine-2-^{14}C were all obtained from Nuclear-Chicago Corporation, Des Plaines, Ill. These sample compounds, manufactured at the Radioactive Chemical Centre, Amersham, England, were provided together with assay data. They were used without further purification. Stated specific activity varied from 5 to 174 mc/mmole and purity was stated to be not less than 98%. One-dimensional TLC (chloroform–methanol–ammonia 2:2:1) followed by radioautography indicated that the purity was acceptable.

Perfusion of Dog Skin

Dogs, weighing at least 20 kg, were anesthetized with Nembutal (sodium pentobarbital, Abbott Laboratories, North Chicago, Ill.) and the skin flap of the thigh was isolated by the surgical procedure of Adachi and Chow (17). The two shunts, with three-way stopcocks, were inserted as described; one joins the femoral artery to the saphenous artery, the other the femoral vein to the saphenous vein. Ligatures were placed as described; in addition, however, we frequently encountered more than one cutaneous branch from the saphenous artery. When branches led away from the isolated skin flap, they also were ligated.

Oxygenated blood, 30 ml, was removed from the femoral artery and heparinized, and the appropriate precursor (dissolved in 1–4 ml of saline) was added. In order to avoid possible physiological effects of the precursor itself, we added no additional carrier material. In early experiments three dogs received 20 μc and one 5 μc of acetate-^{14}C, but in all subsequent experiments the amount of radioactive precursor was standardized at 10 μc. The blood was perfused through the skin flap, via the three-way stopcock on the arterial side, at a rate of 1 ml/min. The effluent perfused blood was collected via the three-way stopcock on the venous side, and was not allowed to return to the main circulation through the femoral vein. Some 90% of the perfused radioactivity remained in the blood after perfusion. After the entire blood sample had entered the skin, 1 ml of methylene blue was perfused through the flap so that the precise area of perfusion was indicated. The flap was then excised.

Extraction and Fractionation of Lipids

The excised skin was freed from subcutaneous fat, cut into small pieces, and freeze-dried overnight. The dried skin was ground in a Wiley mill through a 20 mesh screen, and then dried in a desiccator under vacuum to constant weight. The lipids were extracted by refluxing with acetone in a Soxhlet apparatus for 5 hr, purified by passage through chloroform, thoroughly dried, and weighed. The lipid sample was dissolved in ether and the solution was washed with a 0.5% solution of the appropriate amino acid in 0.1 N HCl, in order to remove any unchanged radioactive amino acid. Washing was continued until the specific activity of the lipids reached a constant level.

The lipids were separated into free fatty acids and neutral lipids by alkali extraction (2). Aliquots from both fractions were set aside for measurement of radioactivity. The isolated free fatty acids were invariably contaminated with neutral lipids. They were purified by passage of their solution in hexane through a 10 g column of Amberlite IRA-400 ion exchange resin [previously brought into the OH^- form with NaOH and washed (19)] followed by elution with acidified methanol. The radioactivity of a portion of the purified fatty acids was determined.

The neutral lipids were further fractionated by preparative TLC. Plates, 1 mm thick, were prepared with Merck Silica Gel G, the lipid was applied by multiple spotting, and the plate was run in benzene to a height of 10 cm. The separated bands of lipid were located by means of 2,7-dichlorofluorescein and scraped from the

plate. Sufficient lipid for radioactivity determination was obtained by the use of several such plates (up to 15); the amounts of silica scraped off with each fraction therefore amounted to several grams, and could not be added directly to the scintillation fluid. The lipids were first recovered by extraction with chloroform in a Soxhlet apparatus and their radioactivity was determined as described below.

Measurement of Radioactivity

Radioactivity was measured by means of a Nuclear-Chicago Model 701B Liquid Scintillation Spectrometer with 2,5-diphenyloxazole (PPO) and 1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) as scintillators. 15 ml of a solution containing 4 g of PPO and 50 mg of POPOP per liter of toluene was used for each determination. Lipid samples were dissolved directly in the scintillator solution; tissue samples were digested with either Hyamine hydroxide [*p*-(diisobutylcresoxy-ethoxyethyl) dimethyl benzyl ammonium hydroxide, 1 M in methanol; Nuclear-Chicago] or NCS™ (a quaternary ammonium hydroxide solubilizer available from Nuclear-Chicago), then dissolved in the scintillator solution. Results were corrected for quenching by means of an internal standard of hexadecane-^{14}C. Efficiency was determined daily by means of toluene-^{14}C standard; this averaged 71% under the conditions used. The size of the lipid sample was selected to give a counting rate of at least twice the background count, and the statistical error of counting was kept below 5%.

RESULTS

Incorporation of Perfused Acetate into Cutaneous Lipids

Dogs perfused with 5, 10, or 20 μc of acetate-^{14}C all showed incorporation into the skin lipids. The amount of activity incorporated ranged from 0.4 to 5.7% of the acetate perfused (Table 1) and incorporation into all isolated lipid fractions occurred (Table 2). In a preliminary report (8) this index, namely the percentage of perfused activity incorporated into the lipids isolated from the skin, was used as a measure of the extent of in-

TABLE 1 INCORPORATION OF PERFUSED ACETATE INTO CUTANEOUS LIPIDS

Dog No.	Amount Perfused	Lipid Incorporation: % of Total Perfused Activity	Lipid Incorporation: % of Total Tissue Activity	Specific Activity: Lipids	Specific Activity: Residual Tissue	Ratio of Specific Activities
	μc			μc/g		
1	20	1.79	21.4	0.175	0.140	1.25
2	20	2.07	31.4	0.104	0.130	0.80
3	20	0.43	20.6	0.114	0.060	1.90
4	10	2.89	21.4	0.305	0.278	1.10
5	10	5.67	16.6	0.107	0.222	0.48
6	10	3.10	25.8	0.762	0.186	4.10
7	5	1.80	12.3	0.014	0.048	0.40
Mean		2.54	21.4	0.226	0.152	1.43
SD		±1.64	±4.6	±0.256	±0.83	±1.28

TABLE 2 DISTRIBUTION OF INCORPORATED RADIOACTIVITY IN LIPIDS DERIVED FROM ACETATE

Fraction	Dog* 4	Dog* 5	Dog* 6	Average for Dogs 4–6	Dog 7*
	% total lipid activity			% total lipid activity	
Free fatty acids	5.6	9.4	9.7	8.2	23.5
Hydrocarbons	0.0	0.1	0.1	0.1	0.2
Sterol esters	0.9	0.8	3.5	1.7	0.6
Waxes	0.8	0.8	1.0	0.9	0.6
Methyl esters of fatty acids†	51.0	4.3	2.0	18.8	1.2
Triglycerides	13.2	47.5	71.6	44.1	25.0
Unidentified band‡	1.2	1.1	2.8	1.7	3.7
Free sterols	9.7	22.1	3.1	11.9	37.5
Polar lipids§	17.7	13.9	5.5	12.4	20.7

* Data not available for dogs 1, 2, and 3. Dogs 4–6 received 10 μc of acetate-^{14}C, Dog 7 received 5 μc.

† Provisional designation, on basis of R_f (TLC) only.

‡ Possibly diglycerides or esters of dihydroxy-sterols.

§ Includes phosphatides and other polar lipids.

TABLE 3 INCORPORATION OF PERFUSED AMINO ACIDS INTO CUTANEOUS LIPIDS

Amino Acid	No. of Perfusions	Lipid Incorporation as % Total Tissue Activity			Mean Efficiency of Lipogenesis*
					%
Glycine-1,2-^{14}C	2	0.30	0.95		2.9
Glycine-1-^{14}C	2	0.43	0.41		2.0
Glycine-1-^{14}C	2	0.78	0.75		3.6
Alanine-U-^{14}C	2	0.23	0.38		1.4
Isoleucine-U-^{14}C	3	1.10	4.80	2.20	12.6
Leucine-U-^{14}C	2	2.25	3.95		14.0
Valine-U-^{14}C	3	1.5	3.5	0.50	8.4
Phenylalanine-U-^{14}C	3	1.24	1.36	0.46	4.8

Amount perfused standardized at 10 μc.
* Expressed in terms of acetate incorporation (Table 1, column 4) as 100% efficiency.

corporation of the radioactive precursor. This index is inaccurate since both the size of the skin flap perfused and the lipid content of the skin showed very wide variations. In the seven dogs perfused with acetate, the dry weight of skin ranged from 2.4 to 9.0 g with an average of 6.2 g while the lipid content of the dry skin was 10.6–38.5% (average 22.3%).

To correct for these variations we considered two other indices: (*a*) the radioactivity of the isolated lipids expressed as a percentage of the radioactivity of the total perfused skin (lipid plus fat-free residual tissue), and (*b*) the ratio of the specific activity of the lipid to that of the residual fat-free tissue. Both indices have been used in Table 1. With the first index, the lipid incorporation ranged from 12.3 to 31.4%, mean 21.4 ± 4.6 (SD) %; coefficient of variation 21%. The second index gave a range of the ratio from 0.040 to 4.20, mean 1.43 ± 1.28 (SD); coefficient of variation 90%. Thus the results show wide variation, for which neither index gives adequate correction. In subsequent work the amount of precursor to be perfused was standardized at 10 μc and results were expressed in terms of the first index.

Of the incorporated radioactivity (Table 2), most appears in the triglyceride fraction (average 44.2% for the three dogs receiving 10 μc) though appreciable activity occurred in a band which just precedes the triglyceride band. This band has an R_f of 0.36, which is identical with that of the methyl esters of fatty acids. Further identification has not been attempted at this time and the fraction has been thus provisionally designated since such esters have been detected in other tissues (20). Unidentified traces of material are also found between the triglyceride band and the free sterol band, at an R_f of about 0.08; no attempt has been made to identify this material. Substances such as diglycerides and esters of dihydroxysterols have an R_f in this region.

Although there was again wide variation from dog to dog, the following general conclusion can be made. Acetate is incorporated to a significant extent into free fatty acids, free sterols, and the polar lipids, but not into hydrocarbons, sterol esters, or waxes. The polar lipid fraction consists of those compounds which remain at the origin in the TLC system we used. It would include phosphatides, monoglycerides, and "proteolipids" if present.

Incorporation of Perfused Amino Acids into Cutaneous Lipids

All the amino acids studied were incorporated into lipids (Table 3), and into all the major lipid fractions (Table 4). The percentage incorporated into lipid was lower than that of acetate; it varied from 0.3% with alanine to 3% with leucine, as calculated by the index of incorporation defined above. Much wider variations of results with the same precursor were observed than with acetate. Thus three perfusions with valine gave a mean incorporation of 1.8 ± 1.5 (SD) %; coefficient of variation 80%.

For comparison purposes the extent of incorporation of acetate into lipids has been arbitrarily selected as representing 100% efficiency of lipogenesis. Efficiency of incorporation (Table 3) appears to be highest for the three

TABLE 4 DISTRIBUTION OF INCORPORATED RADIOACTIVITY IN LIPIDS DERIVED FROM AMINO ACIDS

	Glycine	Alanine	Isoleucine	Leucine	Valine	Phenylalanine
			% *total lipid activity*			
Free fatty acids	16.8	36.0	15.8	4.7	26.3	11.4
Hydrocarbons	0.2	0.3	0.7	0.2	0.3	0.2
Sterol esters	2.1	2.6	22.4	4.7	11.3	0.4
Waxes	1.9	1.7	4.9	4.5	3.5	1.0
Methyl esters of fatty acids*	10.9	4.7	15.3	29.8	25.6	1.2
Triglycerides	17.4	22.3	25.8	36.8	10.2	5.9
Unidentified band*	3.8	2.3	4.0	8.2	5.3	4.8
Free sterols	12.9	12.9	3.6	2.1	9.1	10.3
Polar lipids	33.1	17.1	7.5	9.2	8.5	64.9

* See footnote‡, Table 2.

branched-chain amino acids, valine, isoleucine, and leucine, and lowest for the straight-chain amino acids, alanine and glycine, with phenylalanine occupying a position between the two groups. The two carbons of glycine seem to be incorporated into lipids to about the same extent. The distribution of incorporated activity among the lipid classes was different for the different amino acids.

DISCUSSION

The principal reactant in lipogenesis is acetyl-CoA, which may be supplied by breakdown of carbohydrates, of fat, or of amino acids. Since acetate is incorporated into cutaneous lipids more actively than any other precursors studied, we have expressed the efficiency of lipogenesis from amino acids in terms of the extent of incorporation of acetate into lipid.

With a technique of this type, wide variations are to be expected. The area of skin perfused is different for different animals, and the lipid content of the skin also showed considerable variation. Attempts were made to correct for these variables by the use of different indices of the extent of incorporation. Wide variations were nevertheless observed in the results in spite of the correction for skin flap size; they may be attributed to many causes. Dogs available for experimental purposes are, for the most part, poor creatures; unwanted animals whose state of health and nutrition is questionable and which can rarely be adequately assessed. Hence, variation will be observed due to (*a*) previous malnutrition, which may cause changes in the enzyme levels in the tissues (21), and (*b*) changes in the permeability and physical state of the cutaneous blood vessels due to old age or ill-health. (In our experiments the dog surgery was performed from a few hours to 3 days after receipt of the animal from the suppliers. We experienced greater difficulty with the perfusion in older animals; skin circulation was often blocked by thrombosis.) Variations in the activity of skin enzymes due to other physiological causes might also be expected; in addition, the circulating blood contains both barbiturate and heparin, either of which could affect lipogenesis. These unavoidable factors are largely uncontrollable; they impose severe limitations on the procedure. For this reason care must be taken in the interpretation of results.

In addition to the limitations imposed by the uncontrolled variables of the perfusion technique, further limitations result from the low specific activity of the lipids obtained (sometimes as low as 0.004 μc/g). In addition to increasing the time required for accurate counting (and hence the length and expense of the investigation) this means that the lipid samples are too small for degradation or other detailed studies needed for elucidation of metabolic pathways. Attempts to obtain more active samples by recirculating the perfused blood have not been successful because of the blood vessel damage and blockage that result from prolonged perfusion. The perfusion of larger amounts of radioactive precursor (above 20 μc) has not been attempted. Indeed since the increase would, in some cases, need to be a thousandfold or more this becomes economically impracticable if ^{14}C is used as label. We consider, then, that the perfusion technique, while more valid from a biological standpoint and capable of providing valuable preliminary qualitative data, is not sufficiently reproducible to give reliable quantitative data, which will have to be obtained by indirect methods.

One characteristic feature of the cutaneous lipids of man and animals (21) is the high proportion of branched-chain fatty acids. If these are derived from branched-chain amino acids, as first proposed by Velick and English (22), we would expect preferential utilization of branched-chain amino acids for lipogenesis in skin, especially as compared to other tissues. The preliminary results reported here are in accordance with this expectation (Table 3) and contrast with the findings in adipose tissue [reviewed by Feller (23)], where the order of activity seems to be leucine > alanine > isoleucine > valine = glycine = phenylalanine.

The authors wish to express their appreciation for the collaboration of Dr. Cyril March in the dog surgery procedures. Skilled technical assistance was provided by Miss Evelyn Bornstein.

This work has been supported by Grants AM 06801-03 and AM 07137-05 of the Public Health Service and by the Dermatology Research Fund of this Department. Dr. Woo is in receipt of a Fellowship from the China Medical Board of New York.

Manuscript received 26 May 1966; accepted 1 November 1966.

REFERENCES

1. Schultze, E. J. 1872. *Prakt. Chem.* **7:** 163.
2. MacKenna, R. M. B., V. R. Wheatley, and A. Wormall. 1950. *J. Invest. Dermatol.* **15:** 33.
3. Srere, P. A., I. L. Chaikoff, S. S. Teitman, and L. S. Burnstein. 1950. *J. Biol. Chem.* **182:** 629.
4. Leider, M. 1949. *J. Invest. Dermatol.* **12:** 187.
5. Rosenfeld, G. 1906. *Zentr. f. inn. Med.* **40:** 986.
6. Nicolaides, N., and S. Rothman. 1955. *J. Invest. Dermatol.* **24:** 125.
7. Nicolaides, N., O. K. Reiss, and R. G. Langdon. 1955. *J. Am. Chem Soc.* **77:** 1535.
8. Wheatley, V. R., D. C. Chow, and F. Kennan. 1961. *J. Invest. Dermatol.* **36:** 237.
9. Ellis, R. A., and R. C. Henrikson. 1963. *In* Advances in Biology of Skin IV. The Sebaceous Glands. W. Montagna, R. A. Ellis, and A. F. Silver, editors. Pergamon Press, New York. 94–109.
10. Montagna, W. 1962. The Structure and Function of the Skin. Academic Press, New York. 2nd edition. 38–43 and 276–279.

11. Griesemer, R. D., and R. W. Thomas. 1963. *J. Invest. Dermatol.* **41:** 235.
12. Gaylor, J. L. 1963. *J. Biol. Chem.* **238:** 1643.
13. Freinkel, R. K. 1963. *In* Advances in Biology of Skin IV. The Sebaceous Glands. W. Montagna, R. A. Ellis, and A. F. Silver, editors. Pergamon Press, New York. 125–134.
14. Kandutsch, A. A., and A. E. Russell. 1960. *J. Biol. Chem.* **235:** 2256.
15. Brooks, S. C., V. C. Godefroi, and W. L. Simpson. 1966. *J. Lipid Res.* **7:** 95.
16. Kjaersgaard, A. R. 1954. *J. Invest. Dermatol.* **22:** 135.
17. Adachi, K., and D. C. Chow. 1962. *J. Invest. Dermatol.* **39:** 299.
18. Lipkin, G., V. R. Wheatley, and C. March. 1965. *J. Invest. Dermatol.* **45:** 356.
19. Hornstein, I., S. A. Alford, I. E. Elliott, and P. F. Crowe. 1960. *Anal. Chem.* **32:** 540.
20. Leikola, E., E. Nieminen, and E. Salomaa. 1965. *J. Lipid Res.* **6:** 490.
21. Wheatley, V. R., and A. T. James. 1957. *Biochem. J.* **65:** 36.
22. Velick, S. F., and J. English. 1945. *J. Biol. Chem.* **160:** 473.
23. Feller, D. D. 1965. *In* Handbook of Physiology: Section 5, Adipose Tissue. A. E. Renold and G. F. Cahill, Jr., editors. American Physiological Society, Washington, D.C. 363–373.