Inhibition of lipid synthesis and glucose-6-phosphate dehydrogenase in rat skin by dehydroepiandrosterone

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ABSTRACT Lipid synthesis from acetate-1-^{14}C by rat skin was inhibited 44–56% by 2.5×10^{-4} M dehydroepiandrosterone (DHA) in vitro with or without addition of glucose in the incubation medium. This inhibition affected all the lipid fractions examined (hydrocarbons, sterols, sterol esters, tri-, di- and monoglycerides, fatty acids, and polar lipids) and could be reversed by NADPH. DHA also inhibited lipid synthesis from glucose-U-^{14}C and the formation of $^{14}CO_2$ from glucose-1-^{14}C, indicating interference with pentose cycle activity. Experiments with the 105,000 *g* supernatant fluid of rat skin homogenates demonstrated considerable activities of malic enzyme (ME) (12.6 nmoles of NADPH generated per min per mg of protein), of glucose-6-phosphate dehydrogenase (G6PD), and of 6-phosphogluconate dehydrogenase (6PGD) (17.5 nmoles of NADPH generated per min per mg of protein). G6PD was inhibited 98% by 2.5×10^{-4} M dehydroepiandrosterone, while 6PGD and ME were not affected. It can be estimated from these data that the pentose cycle may contribute 41–57% of the NADPH needed for lipid synthesis in rat skin; the remainder of the necessary NADPH is presumably supplied by malic enzyme.

SUPPLEMENTARY KEY WORDS 6-phospho-D-gluconate dehydrogenase · malic enzyme · polar lipids · neutral lipids · lipogenesis

A PREVIOUS report from this laboratory presented evidence that lipid synthesis in human skin is depressed during diabetic acidosis and after fasting (1). Subsequently, we demonstrated a similar depression of lipid synthesis in skin of alloxan-diabetic rats and starved rats; the data also indicated that the level of L-glycerol-3-phosphate may play a regulatory role in lipogenesis in this tissue (2, 3). These studies have demonstrated that lipid synthesis in the skin is subjected to physiological regulations, and have illustrated certain advantages in using skin as an experimental tissue. In continued studies, we have examined another pertinent factor concerning lipid synthesis in the skin, namely the supply of NADPH derived from the pentose cycle. Studies of Flatt and Ball (4) and of Wise and Ball (5) have estimated that the cycle contributes 50–60% of the NADPH necessary for lipogenesis in rat adipose tissue in the presence of insulin. These authors have emphasized the importance of ME in supplying the remainder of the NADPH necessary for lipogenesis. It would be interesting to know if similar situations exist in the skin.

Several laboratories have reported that DHA is an effective inhibitor of G6PD of a number of mammalian tissues (6–9), and a possible interrelation between the activity of G6PD and the level of DHA in obesity has also been suggested (10). These reports prompted us to examine the effects of DHA on lipid synthesis and on G6PD activity in rat skin. This paper describes our experiments which demonstrated that DHA effectively inhibited in vitro the formation of NADPH in the pentose cycle in rat skin with a concomitant partial inhibition of lipid synthesis. The data thus allowed us to examine the supply of NADPH provided by pentose cycle for lipid synthesis in this tissue.

Abbreviations: DHA, dehydroepiandrosterone (Δ^5-androsten-3β-ol-17-one); G6PD, glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase, EC 1.1.1.49); 6PGD, 6-phosphogluconate dehydrogenase (6-phospho-D-gluconate:NADP oxidoreductase [decarboxylating], EC 1.1.1.4); ME, malic enzyme (malate:NADP oxidoreductase [decarboxylating], EC 1.1.1.40).

MATERIALS AND METHODS

Sodium acetate-1-^{14}C (specific activity: 40 mCi/mmole or 50 mCi/mmole) was purchased from Nuclear-Chicago Corporation, Des Plaines, Ill. Glucose-U-^{14}C (specific activity, 14.7 mCi/mmole), glucose-1-^{14}C (specific activity, 5.6 mCi/mmole), and reference ^{14}C-labeled lipids, stearic acid-1-^{14}C, palmitic acid-1-^{14}C, cholesterol-4-^{14}C, and cholesteryl palmitate-1-^{14}C were obtained from New England Nuclear Corp., Boston, Mass. The sodium salts of glucose-6-phosphate and 6-phosphogluconate were products of Boeringher Mannheim Corp., New York. NADPH and NADP were purchased from Sigma Chemical Co., St. Louis, Mo. Dehydroepiandrosterone was a product of Mann Research Labs., Inc., New York, and was recrystallized from methanol before use. Sephadex (G-25 coarse) was purchased from Pharmacia Fine Chemicals Inc., Piscataway, N.J.; Unisil was from Clarkson Chemical Co., Inc., Williamsport, Pa.; Florisil was from Floridin Co., Pittsburgh, Pa.; nonradioactive lipids, squalene, tripalmitin, dipalmitin, diolein, monopalmitin, and monolein were from Pierce Chemicals Co., Rockford, Ill. Reagents were of analytical grade, and solvents were redistilled before use.

Preparation and Incubation of Skin Specimens

Adult male Sprague-Dawley rats weighing 300–400 g were maintained ad lib. on Purina Laboratory Chow. They were caged singly for at least 48 hr before the experiments to assure that they were well fed. The rats were restrained, and the hair in the posterior dorsum was shaved with an electric clipper. Small skin specimens (60–70 mg) were removed with scissors,[1] and the underlying subcutaneous tissue was carefully scraped off with a scalpel. The specimen was immediately weighed and transferred into an incubation vial containing streptomycin (200 μg), gentamicin sulfate (200 μg), penicillin (200 units), and sodium acetate-1-^{14}C (4 μCi or 5μCi, 0.1 μmole) in 2 ml of Krebs-Ringer phosphate buffer (pH 7.4). DHA in various concentrations was added in 0.02 ml of propylene glycol, and 0.02 ml of propylene glycol without the steroid was added to the control vial.[2] Potassium acetate or glucose was added as indicated in the Results section. The mixture was incubated aerobically at 37°C in a Dubnoff shaking incubator for 6 hr.

In experiments with labeled glucose, the skin specimen was incubated in 2.0 ml of Krebs-Ringer bicarbonate solution which contained 5 mM glucose and the antibiotics, and was gassed with 95% O_2 and 5% CO_2. Incubations were with glucose-U-^{14}C (2.5 μCi per flask) or glucose-1-^{14}C (1.25 μCi per flask). DHA when used was in 2.5 $\times$ 10^{-4} M final concentration. For the collection of $^{14}CO_2$, the method of Snyder and Godfrey (11) was adapted. The incubation flask was fitted with a rubber cap (serum stopper), and a polyethylene center well (supplied by Kontes Glass Co., Vineland, N.J.) was attached to the cap. The mixture was incubated with shaking at 37°C for 2 hr. At the end of the incubation, 0.3 ml of 1M Hyamine hydroxide (Packard Instrument Co., Inc., Downers Grove, Ill.) was introduced by injection through the rubber cap into the center well, and 0.4 ml of 6 N H_2SO_4 was injected into the incubation mixture to stop the reaction and to liberate CO_2. The vessel was left at 37°C for 4 hr, after which the center well was carefully removed and placed directly into a counting vial for radioassay. In control experiments, the recovery of $^{14}CO_2$ from $NaH^{14}CO_3$ was greater than 90%. For the determination of ^{14}C in lipids, the skin specimen was rinsed gently with 0.9% saline, and the lipids were extracted according to procedure B as described below.

Preparation of Skin Homogenate

Rats were killed by a blow on the neck, and the skin was removed quickly from the shaved area of the posterior dorsum. After the removal of subcutaneous tissue, the skin specimens were minced and homogenized in 10 volumes of 0.25 M sucrose in an ice bath with a Polytron (Model PT 10) homogenizer. The nuclei and cellular debris were removed by centrifugation at 800 *g* for 12 min at 4°C. The supernatant fluid, referred to as skin homogenate, was used for incubation. The protein content of the skin homogenate, determined by the method of Lowry, Rosebrough, Farr, and Randall (12), was 2–2.5 mg/ml.

The skin homogenate (2–2.5 mg of protein) was added to 2 ml of 80 mM glycylglycine–KOH buffer (pH 7.5) containing 24 mM $MgCl_2$, 0.4 mM $MnCl_2$, 30 mM $KHCO_3$, 20 mM reduced glutathione, 16 mM ATP, 0.4 mM NAD, 0.4 mM NADP, 8 mM potassium citrate, 10 mM glucose-6-phosphate, 0.1 mM CoA, 200 μg of streptomycin, 200 μg of gentamicin sulfate, 200 units of penicillin, and sodium acetate-1-^{14}C (5 μCi, 0.13 μmole). Other additions are indicated in the Results section. Incubations were at 37° C for 2 hr.

Preparation of Tissue Extract

The skin homogenate was centrifuged at 105,000 *g* for 1 hr at 0°C in an International Equipment Co. ultracentrifuge (Model B-60). The supernatant was concentrated at 0°C to 1/4 its volume with Sephadex G-25. This concentrate was referred to as "the tissue extract," and was used for enzyme assays. The protein content of the tissue

[1] In some experiments, skin specimens were obtained under ether anesthesia. No notable difference was observed in results of lipid synthesis.

[2] Control experiments showed that the propylene glycol has no demonstrable effect on lipid synthesis in the skin.

extract, determined by the method of Lowry et al. (12) was 2.5–3.6 mg/ml.

Determination of ^{14}C in Lipids

The following three procedures were employed for the determination of ^{14}C incorporated into lipids in skin specimens:

(A) For estimation of the total amount of ^{14}C incorporated into lipids, the incubation was terminated by the addition of 2 g of potassium hydroxide and 15 ml of ethanol to each incubation vessel. The mixture was kept at 60°C overnight to allow hydrolysis of fatty acid esters and digestion of the tissue. The alkaline mixture was acidified with HCl to pH 1 and was then extracted four times with 100 ml of dichloromethane. Radioactive acetate in the extract was removed by Sephadex (G-25 coarse) column chromatography as described by Siakotos and Rouser (13). In control experiments, cholesterol-4-^{14}C (8.9×10^5 dpm) and palmitic acid-1-^{14}C (8.4×10^5 dpm) mixed with lipids extracted from 60 mg of rat skin, were recovered quantitatively from this column. Under similar conditions lipids from 60 mg of skin mixed with acetate-1-^{14}C (4.07×10^6 dpm) were recovered free of radioactivity. The ^{14}C in the column effluent was assayed with a Packard Tri-Carb Model 2002 liquid scintillation counter. The amounts of ^{14}C incorporated into the lipids per mg of skin (wet weight) were calculated.

(B) For the determination of ^{14}C incorporated into various lipid fractions by skin, at the end of the incubation, the tissue was removed from the medium,[3] blotted lightly on filter paper and homogenized in a mixture of chloroform–methanol 2:1 with a Virtis 45 homogenizer. Tissue debris was removed by filtration on sintered glass funnels, and was washed with the solvent mixture until no radioactivity was detected in the wash. The filtrate was evaporated in a rotary evaporator. The lipid residue was dissolved in chloroform–methanol–water 19:1:0.1, and passed through a Sephadex (G-25 coarse) column as described above to remove radioactive acetate. After evaporation of the solvents, the recovered lipid mixture was dissolved in chloroform and percolated on to a column containing 5 g of Unisil (100–200 mesh) suspended in chloroform according to the procedure described by Borgström (14). Neutral lipids were eluted with chloroform (200 ml) and polar lipids with methyl alcohol (200 ml). Aliquots from each fraction were assayed for radioactivity.

The neutral lipids were dissolved in a small amount of hexane and chromatographed further on 5 g of Florisil hydrated with 7% water and suspended in hexane according to the procedure described by Carroll (15). Lipids were successively eluted from the column by the following solvents: (*a*) 15 ml of hexane; (*b*) 50 ml of 5% ether in hexane; (*c*) 75 ml of 15% ether in hexane; (*d*) 60 ml of 25% ether in hexane; (*e*) 40 ml of 40% ether in hexane; (*f*) 40 ml of 2% methanol in ether; and (*g*) 50 ml of 4% acetic acid in ether. The effluents were collected in 5-ml fractions, and aliquots were assayed for radioactivity. When reference compounds were chromatographed, squalene was eluted by (*a*), sterol esters by (*b*), tripalmitin by (*c*), cholesterol by (*d*), dipalmitin and diolein by (*e*), monopalmitin and monolein by (*f*), and palmitic acid and stearic acid by (*g*), respectively.

(C) For the determination of ^{14}C incorporated into lipids by the skin homogenate, the reaction was stopped by the addition of 10 ml of a mixture of chloroform–methanol 2:1. Total lipids were extracted according to the procedure of Folch, Lees, and Sloane Stanley (16). The extract was evaporated to dryness in a rotary evaporator, dissolved in chloroform–methanol–water mixture, and finally filtered through Sephadex (G-25 coarse) as described above to remove any contaminating radioactive acetate. The ^{14}C in the column effluent was assayed for radioactivity. The amounts of ^{14}C incorporated into lipids per mg of protein were calculated.

Enzyme Assays

To estimate the formation of NADPH in the pentose cycle, the method described by Löhr and Waller (17) for the assay of G6PD was adapted. A Cary Model 15 spectrophotometer was used for the determinations. Each sample cuvette contained, in a total volume of 3.0 ml of 1.3×10^{-3} M Tris buffer (pH 7.5), 1.5×10^{-3} M NADP, 0.5 ml of the tissue extract, and 2.0×10^{-4} M D-glucose-6-phosphate. The absorbency at 340 nm was recorded at 25° against a blank which was without the substrate, and the initial linear increase was used to calculate the rate of NADPH formation. In a separate assay for 6PGD, 2.0×10^{-4} M 6-phospho-D-gluconate was used as the substrate in place of the D-glucose 6-phosphate.

ME was assayed by the method of Ochoa (18) as modified by Wise and Ball (5). Each sample cuvette contained, in a total volume of 3.0 ml of 4.5×10^{-2} M Tris buffer (pH 7.4), 4.0×10^{-4} M NADP, 4.5×10^{-3} M $MgCl_2$, 5.6×10^{-4} M sodium malate, and 0.5 ml of the tissue extract. The increase in absorbency at 340 nm was recorded against a blank without the substrate.

The effect of DHA was determined by addition of the steroid in 0.02 ml of propylene glycol to each mixture which was assayed.

[3] The amount of ^{14}C-labeled lipids recovered from the medium was less than 4% of that recovered from the tissue.

RESULTS

Selection of Conditions for Incubation

The time course of the incorporation of ^{14}C from acetate-1-^{14}C into lipids by rat skin in vitro was previously reported (3). With skin specimens from normal rats, the reaction remained linear beyond 8 hr. 6 hr of incubation was chosen for the present study to give sufficient labeled lipids for analysis. The effect of pH on lipid synthesis from acetate-1-^{14}C is shown in Fig. 1. A broad peak of activity was observed from pH 7.4 to 7.8

Addition of glucose to the incubation medium greatly enhanced lipid synthesis by rat skin. Maximum enhancement was obtained at 5 mM glucose (Table 1).

The rate of uptake of acetate into lipids by rat skin was determined by incubation with acetate-1-^{14}C (5 μCi, 0.1 μmole) and various concentrations of potassium acetate (5×10^{-5} M to 1×10^{-3} M) in the presence of 5 mM glucose. From the total ^{14}C in the lipids and the specific activity of the acetate in the medium, the rate of uptake of acetate into lipids was calculated. The rate increased almost linearly with acetate concentration, and reached a plateau at 0.3 mM of acetate. Maximum rate was 18.2 nmoles/hr per g of skin (Fig. 2). Although increases in acetate uptake were achieved at higher acetate concentrations, the amounts of ^{14}C incorporated (dpm/mg of skin) under these conditions were depressed because of greater dilution of acetate-1-^{14}C with unlabeled acetate. To obtain sufficient incorporation of ^{14}C into lipids for assays, in subsequent experiments the acetate-1-^{14}C was not diluted with unlabeled K-acetate.

In experiments with skin homogenates, the various additions to the medium (ATP, CoA, NAD, NADP, $MgCl_2$, $MnCl_2$, GSH, $KHCO_3$, K-citrate, and glucose-6-phosphate), were tested at several concentrations. Optimal concentrations were those described under Materials and Methods. These were similar to systems described previously by other investigators (19, 20). The reaction rate was linear up to 2 hr, beyond which a decline was observed.

TABLE 1 THE EFFECTS OF GLUCOSE AND DHA ON INCORPORATION OF ACETATE-1-^{14}C INTO LIPIDS BY RAT SKIN

Glucose in Medium	^{14}C Incorporated		Inhibition by DHA
	No DHA	2.5×10^{-4} M DHA	
mM	*dpm/mg of skin*		*%*
0	928	430	53
2.5	2146	987	44
5.0	3514	1550	56
10.0	3100	—	—

Rat skin was incubated with a tracer amount of acetate-1-^{14}C (5 μCi, 0.1 μmole) and various amounts of glucose. The ^{14}C in lipids was determined by procedure A. Each value is the average of duplicate determinations.

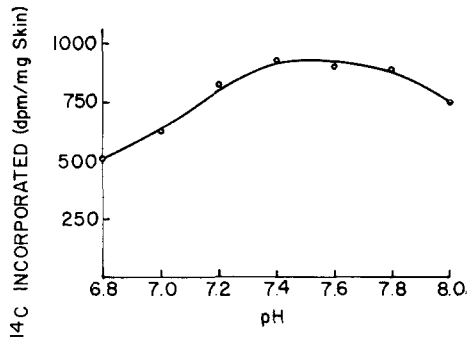

FIG. 1. Effect of pH on lipid synthesis by rat skin. Rat skin was incubated for 6 hr with acetate-1-^{14}C (4 μCi, 0.1 μmole) and antibiotics in 2 ml of Krebs-Ringer phosphate buffer adjusted to pH 6.8–8.0. The values are the averages of duplicate experiments.

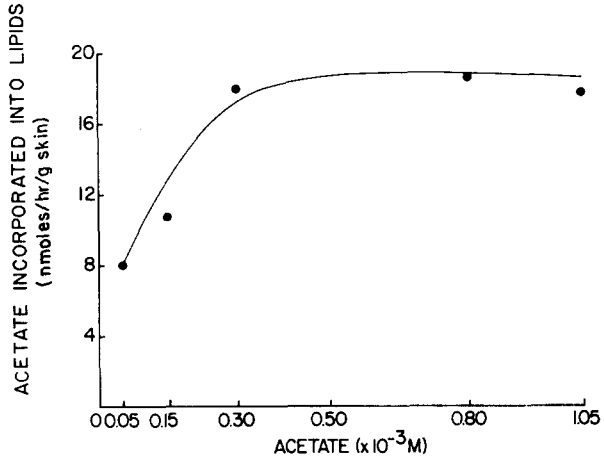

FIG. 2. Effects of acetate concentration on its incorporation into lipids by rat skin. Rat skin was incubated for 6 hr in 2 ml of Krebs-Ringer phosphate buffer pH 7.4 containing 5 mM glucose, antibiotics, acetate-1-^{14}C (5 μCi, 0.1 μmole), and various concentrations of K-acetate. The acetate incorporated into lipids (nmoles/hr per g of skin) was calculated from the specific activity of acetate-1-^{14}C in the medium and the total ^{14}C incorporated into lipids. The data are from the average of duplicate experiments.

Effect of DHA on Lipid Synthesis

The dose response of skin to DHA inhibition (in the absence of exogenous glucose) is shown in Fig. 3. An inhibitory effect of DHA was apparent at concentrations greater than 2.5×10^{-6} M; optimum inhibition (approximately 50%) was exhibited at 2.5×10^{-4} M. Further increase in the concentration of DHA did not appreciably increase the inhibition.

The effect of DHA on lipid synthesis from acetate-1-^{14}C by rat skin was examined in the absence and presence of 2.5 and 5.0 mM glucose. The results are shown in Table 1. The inhibitory effect of 2.5×10^{-4} M DHA was similar (44–56%) with or without the added glucose in the medium.

Effects of Preincubation with DHA

Skin specimens were preincubated with 2.5×10^{-4} M DHA for different time intervals up to 6 hr prior to the addition of acetate-1-^{14}C, and the amounts of ^{14}C incorporated from acetate-1-^{14}C into lipids in 6 hr were compared. The results indicated that the inhibitory effect of DHA on lipid synthesis of the skin was neither increased nor decreased by preincubation of the skin with DHA.

Reversal of the Inhibition by NADPH

The data in Table 2 indicate that the inhibitory effect of DHA on the incorporation of ^{14}C into lipids by whole skin specimens could be reversed by the addition of NADPH to the incubation medium. At concentrations less than 1.2×10^{-5} M, NADPH had no effect, but at 2.5×10^{-5} M or higher, a reversal of the inhibition by DHA was observed; and at 5.0×10^{-5} M of NADPH, the amount of ^{14}C incorporated into lipids was over 90% of the control.

This apparent reversal of DHA inhibition by NADPH was surprising, in view of the problem of permeability of the nucleotide across the cellular membrane. The experiment was repeated with two preparations of skin homogenate. The data in Table 2 show that lipid synthesis by the homogenate was also depressed by DHA, and the inhibitory effect could be reversed by NADPH. Addition of NADPH to the control had no apparent effect, indicating that adequate NADPH was generated for lipid synthesis.

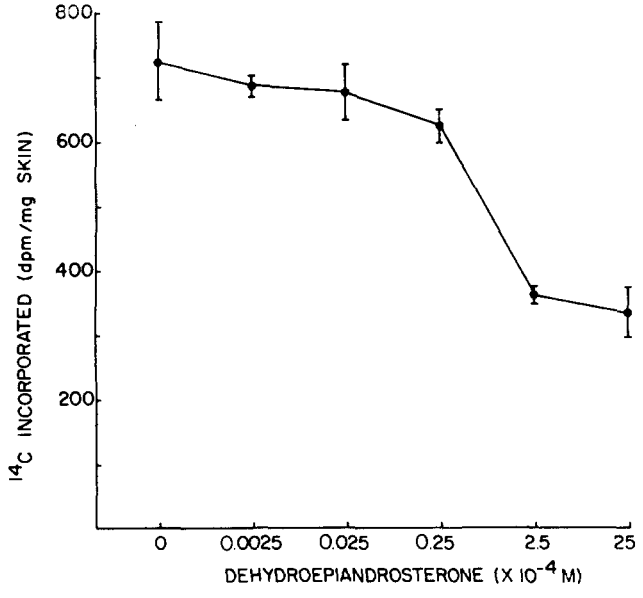

FIG. 3. Inhibition of lipid synthesis by DHA. DHA was added in 0.02 ml of propylene glycol to the incubation mixture consisting of Krebs-Ringer phosphate buffer pH 7.4, antibiotics, rat skin and acetate-1-^{14}C (4 μCi, 0.1 μmole). The amount of ^{14}C incorporated from acetate-1-^{14}C into lipids was determined by procedure A. The data were obtained from three determinations.

TABLE 2 THE EFFECTS OF DHA AND NADPH ON LIPID SYNTHESIS FROM ACETATE-1-^{14}C BY RAT SKIN AND SKIN HOMOGENATE

Additions	^{14}C Incorporated into Lipids		
	Whole Skin	Skin Homogenate I	Skin Homogenate II
		% of control	
None (Control)	100	100	100
NADPH (5.0×10^{-5} M)	94	100	97
DHA (2.5×10^{-4} M)	48	77	59
DHA (2.5×10^{-4} M) + NADPH (0.5×10^{-5} M)	43		
DHA (2.5×10^{-4} M) + NADPH (1.2×10^{-5} M)	45		
DHA (2.5×10^{-4} M) + NADPH (2.5×10^{-5} M)	78		
DHA (2.5×10^{-4} M) + NADPH (5.0×10^{-5} M)	91	91	102

The procedures for incubation are described in the text. Experiments with whole skin were in triplicate, and experiments with skin homogenates were in duplicate.

Effects of DHA and NADPH on Synthesis of Various Lipids

In an attempt to ascertain whether the observed inhibitory effect of DHA and its reversal by NADPH were on the entire lipid synthesizing system or on the synthesis of specific lipids, the ^{14}C-labeled lipids obtained after the incubations with skin specimens from four more rats were separated according to procedure B. It was found that the inhibition of ^{14}C incorporation by 2.5×10^{-4} M DHA in the neutral lipid fraction was more pronounced ($48 \pm 5\%$) than in the polar lipid fraction ($19 \pm 2\%$). This inhibition was reversed by NADPH, while NADPH alone had no stimulatory effect on the control.

The radioactive neutral lipids from one animal were further separated into seven fractions on the Florisil column, and the results are shown in Fig. 4. Except for the increase in ^{14}C in the hydrocarbon fraction which contained less than 5% of the total ^{14}C in neutral lipids, the addition of NADPH had no apparent effect on the ^{14}C incorporation in the other six lipid fractions. The addition of DHA to the incubation medium caused a decrease of ^{14}C incorporated into all of the following neutral lipid fractions: 38% in hydrocarbons, 46% in sterol and wax esters, 33% in triglycerides, 78% in sterols, 45% in diglycerides, 58% in monoglycerides, and 75% in fatty acids. Addition of NADPH effectively reversed the inhibition in each lipid fraction. This experiment was repeated with skin from two other rats, and the results were similar.

Assuming that the amount of ^{14}C from acetate-1-^{14}C is negligible in the glycerol moiety of glycerides and of

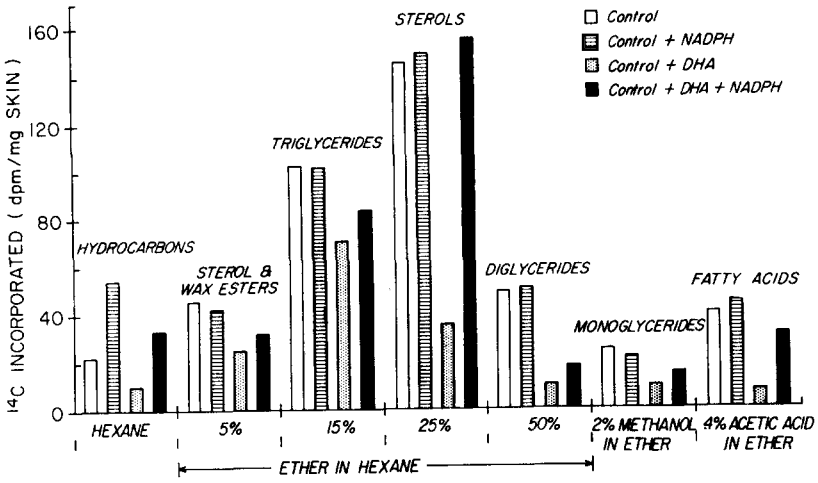

FIG. 4. The effects of DHA and NADPH on the synthesis of neutral lipids. The neutral lipids were separated on Florisil columns. Details of the method are described in the text. The height of the bars represents ^{14}C incorporated in each lipid fraction.

polar lipids (phospholipids), the total amount of ^{14}C in the glycerides, polar lipids, and fatty acids can be attributed to labeling of the fatty acyl chains. The decrease in the total amount of ^{14}C in these fractions caused by DHA is, therefore, caused by inhibition of fatty acid synthesis. Similarly, the decrease in the total amount of ^{14}C in the sterol and hydrocarbon (mainly squalene) fractions indicates the inhibition by DHA in the synthetic pathway of sterols. Calculations based on these considerations, using the data from three rats, showed that 2.5×10^{-4} M DHA inhibited fatty acid synthesis 54, 44, and 39% (average 46%), and sterol synthesis 85, 54, and 73% (average 71%). The small amount of ^{14}C in the sterol ester fraction, presumably distributed in both the sterol and the fatty acid moieties, was omitted from these calculations.

Effects of DHA and NADPH on Lipid Synthesis from Glucose-U-^{14}C

Skin specimens were incubated with glucose-U-^{14}C (2.5 μCi), with or without DHA (2.5×10^{-4} M) and NADPH (5.0×10^{-5} M) for 2 hr in Krebs-Ringer bicarbonate buffer, pH 7.4. The ^{14}C incorporated into lipids was determined, and the results are shown in Table 3. They indicate that DHA inhibited lipid synthesis from glucose-U-^{14}C approximately 40% and that there was a partial reversal of the inhibition by NADPH.

Effect of DHA on Formation of $^{14}CO_2$ from Glucose-1-^{14}C

The reversal by NADPH of the inhibitory effect of DHA in the above experiments with acetate-1-^{14}C and glucose-U-^{14}C suggested that the inhibition was probably caused by a curtailed supply of NADPH. Since a major source of NADPH for lipid synthesis is from the pentose cycle,

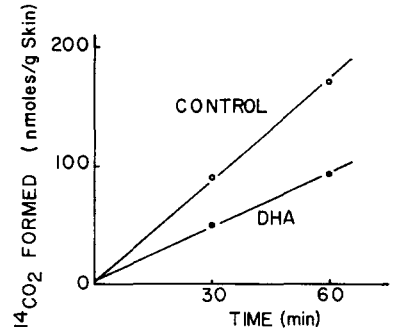

FIG. 5. The effect of DHA on the formation of $^{14}CO_2$ from glucose-1-^{14}C by rat skin in vitro. Rat skin was incubated with 1.2 μCi of glucose-1-^{14}C in 2 ml of Krebs-Ringer bicarbonate buffer pH 7.4 containing 5 mM glucose and antibiotics. DHA was added in 0.02 ml of propylene glycol to a final concentration of 2.5×10^{-4} M, and 0.02 ml of propylene glycol was added to the control flasks. The methods for collection and assay of $^{14}CO_2$ were similar to those described by Snyder and Godfrey (11). The data presented are the average of duplicate experiments.

the effect of DHA on $^{14}CO_2$ formation from glucose-1-^{14}C was studied. The data in Fig. 5 show that the formation of $^{14}CO_2$ from glucose-1-^{14}C was linear for at least 60 min and was inhibited 46% by 2.5×10^{-4} M DHA.

Effects of DHA on NADPH Generating Enzymes

To investigate further the mechanism of DHA inhibition, the effects of DHA on the three NADPH generating enzymes in the tissue extract were examined. Table 4 shows that there are considerable activities of the three enzymes in rat skin, and that DHA at all concentrations tested had no effect on 6PGD and ME, while it had a pronounced inhibitory effect on G6PD. At 2.5×10^{-4} M, DHA inhibited 98% of the NADPH produced in the pentose cycle.

TABLE 3 THE EFFECTS OF DHA AND NADPH ON LIPID SYNTHESIS FROM GLUCOSE-U-^{14}C BY RAT SKIN

Additions	Lipid Synthesized
	mμatoms of glucose carbon/hr per g of skin
None (Control)	624.3 ± 29.4
DHA (2.5×10^{-4} M)	373.8 ± 13.2
DHA (2.5×10^{-4} M) + NADPH (5.0×10^{-5} M)	531.0 ± 19.2

The procedures for incubation are described in the text. Each value represents the mean ± SEM of incubations from six rats.

TABLE 4 EFFECTS OF DHA ON NADPH GENERATING ENZYMES IN THE TISSUE EXTRACT

DHA Concentration	Pentose Cycle Activity	6-Phospho-D-gluconate Dehydrogenase	Malic Enzyme
	nmoles of NADPH generated per min per mg of protein		
None (Control)	17.5 ± 2.9	7.3 ± 1.1	12.6 ± 2.3
2.5×10^{-7} M	15.7 ± 2.2	—	12.9 ± 2.6
2.5×10^{-6} M	14.6 ± 1.8	—	12.5 ± 2.1
2.5×10^{-5} M	5.9 ± 0.9	—	13.6 ± 2.0
2.5×10^{-4} M	0.4 ± 0.1	8.4 ± 1.4	14.2 ± 1.9

Assay methods are described in the text. Each value represents the mean ± SEM of three determinations of a tissue extract prepared from six rats.

DISCUSSION

Table 1 shows that glucose enhances acetate uptake into lipids by rat skin. This is not surprising, since glucose utilization can supply the necessary reducing equivalents for fatty acid and sterol syntheses, the ATP necessary for the activation of acetate in the formation of acetyl CoA, and the glycerol-3-phosphate which accepts the acyl groups in the formation of phospholipids and glycerides. Glucose can also give rise to citric acid cycle intermediates which stimulate acetyl CoA carboxylase (21), and the CO_2 necessary for the formation of malonyl CoA. What is surprising is the fact that rat skin and human skin can synthesize lipids from acetate in vitro for many hours in the absence of added glucose or other cofactors in the supporting medium (3, 22). The content of glucose in rat skin is 16–24 mg/100 g of tissue, and in human skin it is 17–30 mg/100 g of tissue.[4] Apparently in experiments using acetate-1-^{14}C as a tracer, the endogenous glucose and its metabolites in the skin can support lipid synthesis without the addition of exogenous glucose to the incubation medium. Metabolism of endogenous glucose as well as glucose added to the incubation medium produces acetyl CoA, which is the substrate for lipid synthesis. The tracer acetate-1-^{14}C after its conversion to acetyl CoA, labels the acetyl CoA pool and thus the various lipid classes derived from acetyl CoA. It has been pointed out in a study by Duncombe (23) that to avoid disturbance of endogenous rate of fatty acid synthesis in adipose tissue, the labeled acetate should not exceed 10 mM. The concentrations of acetate-1-^{14}C in our experiments were well within this limit. Since the flux of acetyl CoA from glucose and other endogenous sources is not known, the rate of lipid synthesis cannot be calculated directly from the amount of ^{14}C incorporated into lipids and the specific activity of acetate-1-^{14}C. Such calculations from the data in Table 1 would give a value of 1.4 nmoles of acetate incorporated into lipids per hr per g of skin in the absence of exogenous glucose. This value is much below the true capacity of the tissue to synthesize lipids, because synthesis from endogenous acetyl CoA is not reflected. We have, therefore, expressed our results from experiments with tracer amounts of acetate-1-^{14}C in terms of dpm of ^{14}C rather than nmoles of acetate incorporated. The results presented in Fig. 3 were from experiments in which the acetate concentration was such that the reaction was approaching zero-order kinetics, and the maximum rate observed (at acetate concentration greater than 0.3 mM) was 18.2 nmoles of acetate-1-^{14}C per hr per g of skin. The true rate of lipid synthesis under the experimental conditions was actually higher than this, as the experiments were carried out in the presence of 5 mM glucose. Table 3 shows that the rate of lipid synthesis from 5 mM glucose was 624 mμatoms of glucose carbon per hr per g of skin. This is equivalent to 312 nmoles of acetate incorporated per hr per g of skin.

In spite of the handicap that the true rate of lipid synthesis could not be calculated from experiments with tracer amounts of acetate-1-^{14}C, they, nonetheless, yielded valid information with regard to the effect of DHA. Changes in the rate of ^{14}C incorporation into lipids from acetate-1-^{14}C must reflect proportional changes in the rate of lipid synthesis from acetyl CoA. The effects of DHA are shown in Table 1. Similar degrees of inhibition (44–56%) of lipid synthesis from acetate-1-^{14}C were observed in the absence or presence of the two levels of exogenous glucose. The experiments with glucose-U-^{14}C further indicated that 2.5×10^{-4} M DHA inhibited lipid synthesis 40% (Table 3).

It is interesting that the inhibition of lipid synthesis by DHA could be reversed by the addition of NADPH (Tables 2 and 3), giving support to the interpretation that the inhibition was due to curtailed supply of NADPH, this being the case in experiments in which either acetate-1-^{14}C or glucose-U-^{14}C was used as substrate, and in experiments with the homogenates. Although at levels below 1.2×10^{-5} M, NADPH exhibited

[4] Glucose in rat skin and human skin was determined with glucose oxidase.

no effect, at 5.0×10^{-5} M, NADPH almost completely reversed the inhibitory effect of DHA. It is generally accepted that pyridine nucleotides are not permeable to cellular membranes. Our data indicate that at high concentrations of NADPH, perhaps some permeation can occur. The data also show that NADPH added to the control had no stimulatory effect, indicating that without DHA, NADPH was not a rate-limiting factor. It became rate limiting only when its supply was severely curtailed by DHA.

The inhibitory effect of DHA and the reversal by NADPH were found to affect all lipid fractions, and quantitatively the effect in each lipid fraction varied; greater effects were noticed in the neutral lipid fraction than on the polar lipid fraction. The polar lipid fraction is expected to contain phospholipids which are structural elements of cellular membranes and intermediates for the synthesis of glycerides. Our data show that this fraction contained approximately 20% of the total ^{14}C incorporated into lipids. It is interesting that the amount of ^{14}C in this fraction was held relatively steady (depressed only 19 ± 2% by DHA) in spite of severe impairment of lipid synthesis as reflected in other lipid fractions (33–78%, Fig. 4). In further analysis of the data, it was found that the inhibition of DHA in the sterol synthesis pathway was greater (71%) than in the fatty acid synthesis pathway (46%). These differences are worthy of note, and deserve further investigation.

The finding that DHA inhibits lipid synthesis agrees with previous findings on other tissues. For example, Gianelly and Terner (24) observed the inhibition of synthesis of cholesterol and triglycerides in rat mammary glands by DHA in vitro. Tepperman, De LaGarza, and Tepperman (25) reported the inhibitory effect of DHA on the incorporation of ^{14}C from acetate-1-^{14}C into fatty acids by rat liver slices. The inhibition of mammalian G6PD by DHA and other steroids has been demonstrated by Marks and Banks (6), McKerns and Keleita (7), Tsutsui, Marks, and Reich (8), and Levy (9). Our data in Fig. 5 indicate that DHA inhibits $^{14}CO_2$ formation from glucose-1-^{14}C. Since carbon 1 of glucose is decarboxylated to yield CO_2 by 6PGD, these data suggest that DHA may inhibit the activity of pentose cycle. But without further information on the extent of recycling of the carbon atoms in the glucose molecule, these data can provide little quantitative information on the pattern of glucose metabolism in the skin. The pitfalls in the evaluation of pathways of glucose metabolism have been discussed in detail by Katz and Wood (26). The notion that DHA affected pentose cycle activity was further substantiated by data in Table 4, which indicate that DHA inhibited G6PD in the 105,000 *g* supernatant of rat skin homogenates while both 6PGD and ME were not affected by DHA. The data further showed that the formation of NADPH from glucose-6-phosphate by the skin extract was almost completely (98%) inhibited by 2.5×10^{-4} M of DHA. Although DHA does not directly affect 6PGD, complete inhibition of G6PD would deprive 6PGD of its substrate, 6-phosphogluconate, and consequently preclude formation of NADPH by 6PGD.[5] Thus under these conditions, DHA blocked the formation of NADPH by both enzymes and the oxidation of carbon 1 of glucose to CO_2 in the pentose cycle. It was seen in the meantime, that lipid synthesis was inhibited optimally by 2.5×10^{-4} M of DHA (48 ± 7% in Fig. 3; 44–56% in Table 1, and 40% in Table 3). From these data, it could be calculated that the amount of NADPH contributed from the pentose cycle towards the over-all activity of lipid synthesis in rat skin was 41–57%. Table 4 shows that ME was not affected by DHA; it apparently could supply the remainder of the NADPH needed for lipid synthesis.

The above estimation of pentose cycle activity was based on the fact that DHA is a powerful inhibitor of G6PD, and on the assumption that DHA does not otherwise inhibit lipid synthesis. Table 2 shows that the reversal of DHA inhibition by 5×10^{-5} M NADPH was nearly complete. The data indicated that if DHA inhibited lipid synthesis by mechanisms other than curtailment of the supply of NADPH, their effect would have been quantitatively minor.

The plasma concentration of DHA is probably between 5×10^{-9} and 2×10^{-8} M (calculated from data of Saez and Bertrand [29]). This is two or three orders of magnitude below the concentrations to be effective in inhibiting lipid synthesis in vitro. It is, therefore, questionable if DHA functions as a physiological regulator of lipid synthesis in vivo. Nonetheless, the inhibition of G6PD by DHA has provided a tool to probe the activities of pentose cycle in the present study. Since DHA is one of the major steroids secreted by the adrenal cortex, its role in regulating lipid synthesis merits further attention.

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[5] Glucose oxidase [β-D-glucose:NAD(P) oxidoreductase, EC 1.1.1.47] and glucokinase (ATP:D-gluconate-6-phosphotransferase, EC 2.7.1.12) have been demonstrated in hepatic preparations. These enzymes can provide 6-phosphogluconate for 6PGD despite inhibition of G6PD. It has been suggested that these enzymes play minor roles in hepatic metabolism of glucose (27, 28). It is not known whether this pathway of glucose metabolism exists in the skin to furnish an additional source of reducing equivalents.

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