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LIPID METABOLISM OF PHOTOSYNTHETIC TISSUES

I. FATTY ACID SYNTHESIS BY EXTRACTS OF EUGLENA

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SUMMARY

A system for long-chain fatty acid synthesis from acetate or acetyl-CoA with soluble extracts of Euglena is described. When [1-^{14}C]acetyl-CoA is used as substrate, there is an absolute requirement for ATP and a divalent cation but no requirement for HCO_3^- nor is there inhibition by avidin. A marked specificity for NADH rather than NADPH is shown, but maximal rates are obtained only in the presence of both NADH and NADPH. These requirements can be shown either by radiochemical assay or by spectrophotometric assay. Apparent Michaelis–Menten constants are given for all known cofactors. From radiochemical and spectrophotometric assay, it is shown that 2 moles of reduced pyridine nucleotide are required for each acetyl unit incorporated into long-chain fatty acids. The major portion of radioactivity (67 %) is found in fatty acids with a chain length of 16–20 carbon atoms as determined by paper chromatography. No evidence for short-chain fatty acids as intermediates has been found.

INTRODUCTION

Many investigations have led to the point at which purified soluble-enzyme systems may be used to study the biosynthesis of fatty acids[1–4]. In all cases reported to date the formation of the naturally occurring fatty acids (C_{16} and C_{18} fatty acids) by net synthesis has been shown to proceed by the malonyl-CoA pathway. By the simple criterion of CO_2 requirement, this pathway has been shown to be universally distributed. In all of the fatty acid synthesizing systems which require CO_2 or a carboxyl-donor, the participation of protein-bound biotin in the carboxylation of acetyl-CoA has been either directly demonstrated[5–8] or implicated by the test of inhibition by avidin. In all syntheses by this pathway, a high specificity for NADPH compared to NADH is clear, and, in addition, a marked specificity for Mn^{2+} has become apparent.

Several workers have shown that the rate-limiting reaction in the malonyl-CoA pathway is the carboxylation of acetyl-CoA[9,10]. This reaction requires a relatively high concentration of HCO_3^- (about 0.01 M) to saturate the acetyl-CoA carboxylase (acetyl-CoA:CO_2 ligase (ADP), EC 6.4.1.2) and a non-physiologically high concentration (about 0.001 M) of a tricarboxylic acid[11,12] to activate the acetyl-CoA carboxylase. In some systems[13–18] evidence has been presented to indicate that the high HCO_3^-

requirement may be obviated by carboxyl-transfer via transcarboxylase activity.

The only other system of fatty acid synthesis known is by way of reversal of β-oxidation. Early experiments by STADTMAN AND BARKER[19-21] with soluble preparations from Clostridium demonstrated the conversion of acetate into butyrate and hexanoate; STANSLY AND BEINERT[25] showed the conversion of acetyl-CoA to butyryl-CoA with purified enzymes of the β-oxidation sequence in the presence of NADH and a reduced dye; and SEUBERT *et al.*[26], supplementing the enzymes of the β-oxidation sequence with the NADPH crotonyl reductase (NADPH:crotonyl-CoA oxidoreductase)[27], NADH, NADPH, acetyl-CoA, and hexanoyl-CoA, were able to demonstrate the synthesis primarily of octanoyl-CoA and decanoyl-CoA. However, in all these attempts to reverse the β-oxidation sequence, the longer-chain fatty acids (hexadecanoic and octadecanoic acids) were found only in insignificant amounts.

More recently, several workers[13-15,28-30] have re-investigated fatty acid synthesis from acetyl-CoA by liver mitochondria. The initial results[28] showed an absolute requirement for NADH and NADPH and also an absolute requirement for ATP. Omission of HCO_3^- from this system had no effect on the synthesis. Preliminary results suggested net synthesis of stearate[28], but later studies[29,30] with soluble enzymes derived from liver mitochondria showed that ATP could be replaced by an intermediate-length acyl-CoA acceptor and thus was very similar to the system studied by SEUBERT *et al.*[26]. It now appears that liver mitochondria possess three different pathways for fatty acid synthesis[13-18,29]: (1) the malonyl-CoA pathway; (2) a system for elongation of intermediate-length acyl-CoAs; and (3) an avidin-insensitive pathway for oleic acid synthesis, somewhat similar to a system first described using mitochondria from avocado mesocarp[31]. In this communication we wish to describe a soluble fatty acid synthesizing system from Euglena which shows an absolute requirement for ATP and a divalent cation, is independent of HCO_3^- and is avidin insensitive. Data to be published elsewhere will show that net synthesis of fatty acids occurs and that no elongation of added free fatty acids is involved.

CULTURE OF EUGLENA AND PREPARATION OF ENZYME

Euglena gracilis Z strain* was cultured on the complex medium of HUTNER *et al.*[32], with aeration at room-light intensity (50 ft candles) in a 12-l boiling flask containing 4–6 l of medium. After 3–4 days, depending upon the size of the inoculum (usually equivalent to 6 g paste), the cells were harvested by centrifugation. Under these conditions, the yield of wet packed cells was 5–10 g of paste per liter of medium. All subsequent treatments were done at 0–4°. The paste was washed twice with cold distilled water and resuspended in 3 vol. of 0.05 M potassium phosphate buffer (pH 7.0), containing 0.002 M GSH. Several methods of rupturing the cells were tried. These included grinding in sand, alumina and sonication. Of these methods sonication for 2–3 min with a Raytheon 10-kC instrument was found to be the most convenient and yielded preparations of highest specific activity. Following sonication, extraction was continued for 20 min with occasional stirring, then whole cells were removed by centrifugation at 500 × *g* for 5 min.

Subsequent fractionation is shown in Table I. Following centrifugation at

* Obtained from DR. S. HUTNER, Haskins Laboratory, New York, N.Y.

100000 × *g* for 45 min, the clear yellow supernatant was filtered through a small pad of glass wool to remove floating lipids. The fatty acid-synthesizing enzymes were found in the supernatant fraction. Solid ammonium sulfate was added with stirring to give a solution which was 35 % saturated with respect to ammonium sulfate (based on its solubility at 25°). The mixture was stirred for 10–15 min before centrifugation at 20000 × *g* for 15 min. The resulting precipitate was discarded and the supernatant solution was brought to 60 % saturation with respect to ammonium sulfate, stirred for 10 min, and centrifuged at 20000 × *g* for 15 min. This precipitate was redissolved in 0.05 M potassium phosphate buffer containing 0.002 M GSH with a volume equivalent to one-third of the volume of the starting supernatant fraction, and stored at —15°. Before use the extract was routinely dialyzed for 8–10 h against 0.05 M potassium phosphate buffer (pH 7.0) containing 0.002 M cysteine. When stored and used under these conditions, the extract showed essentially no loss of activity over a period of 30 days.

Preliminary attempts at further purification of the extract with $Ca_3(PO_4)_2$ gel, ethanol and acetone fractionation were not successful. Treatment of the extract with ethanol or acetone resulted in complete loss of activity. Protein determinations were done by the method of LOWRY *et al.*[33].

ASSAYS

Radioisotope assays

The synthesis of fatty acids was measured by the extent to which [1-^{14}C]acetate or [1-^{14}C]acetyl-CoA was incorporated into fatty acids. The complete system contained potassium phosphate buffer (pH 7.0), 50 μmoles; NADH, 0.3 μmole; NADPH, 0.3 μmole; ATP, 2.5 μmoles; $MgCl_2$, 2.5 μmoles; [1-^{14}C]acetyl-CoA, 0.28–0.35 μmole (specific activity, either 1 μC/μmole or 10 μC/μmole); and enzyme, 0.5–1.5 mg protein. The final volume was adjusted to 0.5 ml with water. Where [1-^{14}C]acetate was used as substrate, the reaction mixture was supplemented with 0.5 unit of AS-1 fraction of acetyl-CoA synthetase (acetate:CoA ligase (AMP), EC 6.2.1.1) prepared from yeast as outlined by BERG[34].

After incubation at 30° for 30 min under aerobic conditions, 1 ml of 10 % methanolic KOH was added and the mixture deacylated at 80° under argon for 30 min (preliminary experiments showed no greater incorporation of [1-^{14}C]acetyl-CoA into fatty acids when argon was the gas phase during incubation). Extraction of the non-saponifiable fraction with hexane indicated no incorporation of radioactivity into this fraction, therefore in subsequent assays this extraction was omitted. Following deacylation an equal volume of water was added and the pH was adjusted to 2–3 with 2 N HCl using thymol blue as indicator. The fatty acids were extracted successively with four 2-ml portions of *n*-pentane. This procedure has been shown[35,36] to extract fatty acids of chain-length of 10 carbon atoms and longer. A small aliquot of the combined pentane extractions was plated to infinite thinness on stainless-steel planchets after first back-washing the pentane extractions with water. After 4–6 h at room temperature (23°), the radioactivity was measured in a gas-flow counter (Nuclear-Chicago Corporation) (28 % efficiency). The remainder of the pentane was removed from the sample under a stream of nitrogen. These samples of long-chain fatty acids were used subsequently for chromatographic analyses of the products.

After the pentane extraction of the long-chain fatty acids, diethyl ether extrac-

tions of the aqueous phase and water-wash of pentane extraction were done as described by WAKIL *et al.*[37] to recover possible short-chain fatty acids. The determination of radioactivity in this fraction and the preparation for chromatography have been described[35].

Spectrophotometric assays

For spectrophotometric assay of fatty acid synthesis, the complete assay system contained potassium phosphate buffer (pH 7.0), 50 μmoles; ATP, 5 μmoles; $MgCl_2$, 5 μmoles; [1-^{12}C]acetyl-CoA or [1-^{14}C]acetyl-CoA, 0.28–0.35 μmole; NADH, 0.1–0.15 μmole; and enzyme, 0.7–1.3 mg protein. The final volume was adjusted to 1.0 ml with water. The reaction for spectrophotometric assay was started usually with the addition of the enzyme and the rate was followed at room temperature (23°) by disappearance of the 340-mμ band either in a Beckman Model-DU or Cary Model-14 spectrophotometer. A slow oxidation of reduced pyridine nucleotide was observed in the absence of acetyl-CoA and ATP. This rate was subtracted unless otherwise stated from the rate of reaction that was dependent upon acetyl-CoA and ATP.

Resolution of fatty acids by chromatography

The long-chain fatty acids were resolved by the paper chromatographic system of KAUFMANN AND NITSCH[38] or on siliconized paper with 80% acetic acid[39]. After drying the papers in air at room temperature, vertical strips were cut from the paper, cut horizontally at 1-cm intervals and the distribution of radioactivity determined in a gas-flow counter. The distribution of radioactivity was compared with the location of known fatty acids chromatographed on the same sheet of paper. Standard fatty acids were visualized by reacting the mercury salts with *s*-diphenylcarbazide[40].

Fatty acids extracted by diethyl ether were chromatographed as their ammonium salts as described by REID AND LEDERER[41] and the distribution of any radioactivity, as determined above with long-chain fatty acids, was compared with location of known fatty acids.

MATERIALS

ATP, NADP, NADPH, and NADH were obtained from Sigma Chemical Company, CoA from Pabst Laboratories, avidin and biotin from Nutritional Biochemicals Corporation, and the [1-^{14}C]acetate and [1-^{14}C]acetic anhydride from New England Nuclear Corporation. The long-chain fatty acid standards were kindly donated by the Eldo Corporation. Gas chromatographic analyses of methyl esters of the acids showed them to be at least 99% pure. [1-^{14}C]Acetyl-CoA was synthesized by the method of SIMON AND SHEMIN[42]. Hydroxamate determinations were done by the procedure of LIPMANN AND TUTTLE[43].

RESULTS

When crude homogenates of Euglena were incubated with [1-^{14}C]acetate or [1-^{14}C]-acetyl-CoA in the presence of potassium phosphate buffer, ATP, $MgCl_2$, NADH, NADPH, or an NADPH-generating system, radioactivity was incorporated into long-chain fatty acids (Table I). Fractional centrifugation showed that the enzymic

activity was associated with the supernatant fraction resulting from the 100000 × *g* centrifugation for 45 min and thus the enzyme system can be considered soluble. However, no attempts have been made to isolate cell-particulate fractions under isotonic conditions. It is impossible therefore to make a statement on the intracellular distribution of the fatty acid-synthesizing enzymes.

TABLE I

FRACTIONATION OF THE FATTY ACID-SYNTHESIZING SYSTEM FROM EUGLENA

The assay procedure and the various components of the assay were as described in METHODS. The reaction was started with the addition of the various enzyme fractions. After a 30-min incubation and following deacylation, those fractions originally containing chlorophyll (and other pigments) were titrated to pH 2–3 using a pH meter with 2 N HCl before extraction of fatty acids. Otherwise the procedure was the same as outlined in METHODS. No extractable radioactive material was found in the non-saponifiable fraction of any enzyme fraction.

Fraction	*[1-^{14}C]Acetyl CoA converted into fatty acids (mμmoles)*	*Specific activity (mμmoles [1-^{14}C]acetyl-CoA /mg protein/30 min)*
Supernatant 500 × *g* for 5 min	18.8	6.2
Supernatant 1000 × *g* for 10 min	18.5	8.8
Supernatant 100000 × *g* for 45 min	17.5	15.9
Supernatant 0–35% $(NH_4)_2SO_4$	8.2	15.7
35–60% $(NH_4)_2SO_4$	51.6	37.0
Supernatant 60% $(NH_4)_2SO_4$	0.0	0.0

The supernatant fraction was fractionated with ammonium sulfate as described under METHODS. The data of Table I indicate that the highest enzyme specific activity was obtained in the fraction precipitable between 35 and 60% saturation with regard to ammonium sulfate. The fatty acids produced by this fraction were identical to those produced by the crude homogenate. Additions of other ammonium sulfate fractions to the 35–60% ammonium sulfate fraction gave no increase in incorporation of [1-^{14}C]acetyl-CoA. The increase in specific activity, beyond that of the crude homogenate, by fractional centrifugation and ammonium sulfate fractionation varied from about 6- to 10-fold.

For incorporation of [1-^{14}C]acetyl-CoA into pentane-extractable fatty acids, this purified enzyme fraction showed an almost absolute requirement for ATP and magnesium ions (Table II, Fig. 1). Bicarbonate, which has been shown to be absolutely essential for fatty acid synthesis from acetyl-CoA via the malonyl-CoA pathway[44], did not enhance the incorporation of acetyl-CoA into fatty acids with Euglena extract. Addition of Mn^{2+} to the complete system with HCO_3^- also did not enhance incorporation. Both reduced pyridine nucleotides, NADH and NADPH, were required for optimum synthesis. Omission of NADPH resulted in only a 40–60% loss of incorporation, whereas the omission of NADH usually resulted in no synthesis at all. The same results were obtained under anaerobic and aerobic assay conditions. Under the specified conditions of assay the incorporation of acetyl-CoA into the fatty acids was proportional to the amount of extract added, as is shown in Fig. 2, and also to time of incubation (Fig. 3).

When the course of the reaction was followed spectrophotometrically by observing the disappearance of the 340-mμ band of reduced pyridine nucleotide, the

requirement for ATP also could be demonstrated (Fig. 4). Addition of acetyl-CoA to a reaction mixture complete except for ATP and acetyl-CoA did not increase the rate of oxidation of NADH and NADPH over that of the NADH oxidase (NAD:O_2 oxidoreductase) alone (Curve A, Fig. 4). With subsequent addition of ATP, where noted, an immediate sharp increase in rate of NADH and NADPH oxidation could be shown.

The order of addition of ATP and acetyl-CoA, did not appear to make a significant

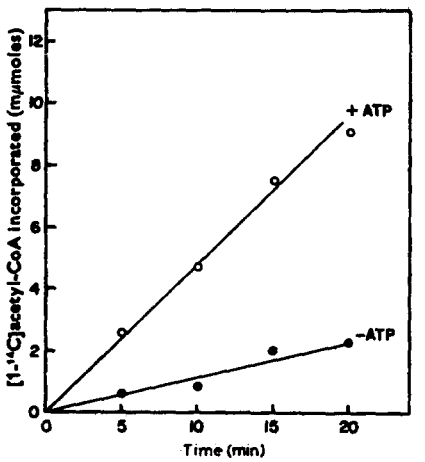

Fig. 1. Effect of ATP on incorporation of [1-^{14}C]acetyl-CoA into long-chain fatty acids. Except for omission of ATP from the complete system where noted, the concentration of components was exactly as described in METHODS. Incubation time was as noted.

TABLE II

COFACTOR REQUIREMENTS FOR ACETYL-CoA INCORPORATION INTO LONG-CHAIN FATTY ACIDS BY EUGLENA SOLUBLE SYSTEM

The complete system contained all components as described in METHODS. Omissions or additions (HCO_3^-) to the complete system were as indicated. Other details of assay were as described in METHODS.

	Acetyl-CoA incorporated into long-chain fatty acids (mμmoles)	
	Expt. I	*Expt. II*
Complete system	11.2	13.1
No ATP	1.1	0.9
No NADPH	6.4	5.6
No NADH	3.6	0.5
No NADPH; no NADH	0.0	0.1
No Mg^{2+}	1.2	1.0
Complete system		
+ 4 μM HCO_3^-	7.7	13.7
+ 10 μM HCO_3^-	8.7	11.2

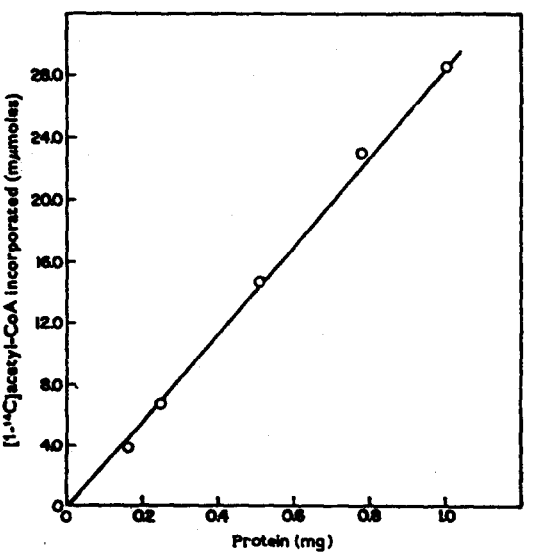

Fig. 2. Effect of increasing amount of protein on [1-^{14}C]acetyl-CoA incorporation into long-chain fatty acids. Conditions and components of system as described in METHODS.

difference in the initial rate of fatty acid synthesis, since, as shown in Fig. 4, addition of acetyl-CoA to a reaction mixture containing ATP also resulted in an immediate increase in rate of NADH and NADPH oxidation. A sustained rate was observed until the concentration of NADH and NADPH became rate-limiting. With addition of more NADH (Fig. 5) the rate of fatty acid synthesis increased immediately. This rate

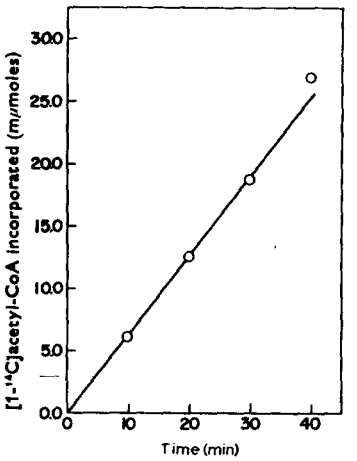

Fig. 3. Incorporation of [1-^{14}C]acetyl-CoA into long-chain fatty acids with 0.7 mg protein as a function of time of incubation. Conditions and components of system as described in METHODS.

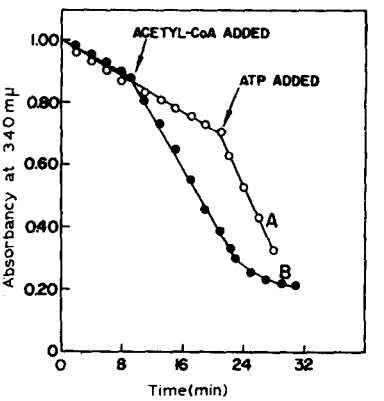

Fig. 4. Dependency on ATP and acetyl-CoA for oxidation of reduced pyridine nucleotide. Curve A (O—O): 0.28 μmoles acetyl-CoA added at 9 min after establishment of rate of reduced pyridine nucleotide oxidase in an assay (*cf.* METHODS) with ATP and acetyl-CoA omitted initially; 5.0 μmoles ATP then added at 22 min. Curve B (●—●): assay mixture (*cf.* METHODS) complete except for acetyl-CoA. At 9 min, where indicated, 0.28 μmole acetyl-CoA added. Absorbancy changes corrected for dilutions caused by additions.

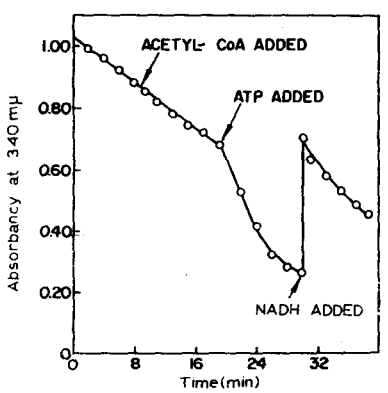

Fig. 5. Restoration of acetyl-CoA- and ATP-dependent oxidation of reduced pyridine nucleotide with further addition of NADH after rate had diminished. For details of assay mixture see METHODS. Absorbancy changes corrected for dilutions caused by additions.

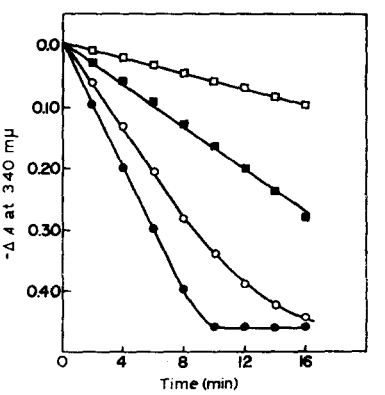

Fig. 6. Effect of increasing concentration of enzyme(s) on the ATP- and acetyl-CoA-dependent oxidation of reduced pyridine nucleotide. □—□, 0.26 mg protein; ■—■, 0.65 mg protein; O—O, 1.30 mg protein; ●—●, 1.95 mg protein. The rates given have been corrected for reduced pyridine nucleotide oxidase activity. For details of assay mixture see METHODS.

was less than that obtained initially with addition of ATP to the otherwise complete reaction, and probably can be explained by partial inactivation of the enzyme during the period of assay. In the complete reaction mixture the rate of fatty acid synthesis as measured spectrophotometrically was proportional to concentration of protein. The curves shown in Fig. 6 have been corrected for NADH and NADPH oxidation that was independent of acetyl-CoA or ATP (*cf.* Figs. 4 and 5). When the rate of oxidation of reduced pyridine nucleotide dependent upon acetyl-CoA was observed with measurement of radioactivity incorporated into long-chain fatty acids, curves were obtained as shown in Fig. 7. Curve A denotes the rate of NADH and NADPH oxidation in a reaction mixture without added [1-^{14}C]acetyl-CoA and curve B shows the rate in the complete reaction mixture. At the times indicated by the open circles (Curve C) aliquots of the reaction mixture were removed, and the quantity of radioactivity incorporated into fatty acids was determined as described in METHODS.

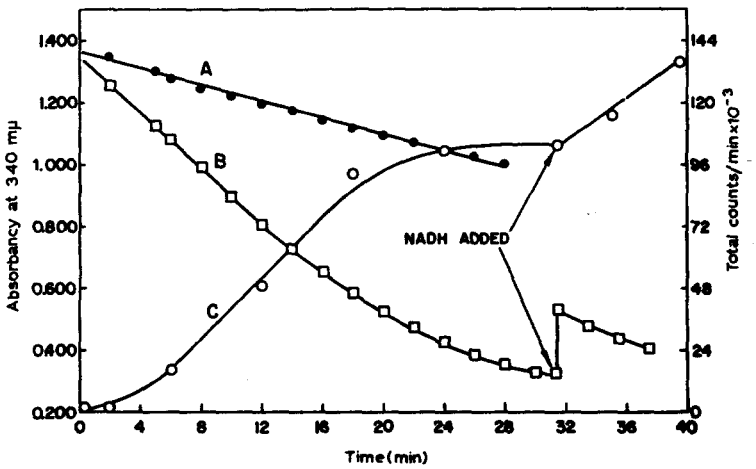

Fig. 7. The relation of rate of ATP- and acetyl-CoA-dependent oxidation of reduced pyridine nucleotide as determined spectrophotometrically to rate of incorporation of [1-^{14}C]acetyl-CoA into long-chain fatty acids. Curve A (●—●): rate of reduced pyridine nucleotide oxidation in absence of ATP (or [1-^{14}C]acetyl-CoA). Curve B (□—□): rate of reduced pyridine nucleotide oxidation in complete reaction mixture (see METHODS). Curve C (O—O): rate of incorporation of [1-^{14}C]-acetyl-CoA into long-chain fatty acids as determined by radioisotope assay (see METHODS). Additional NADH added to reaction mixture where indicated.

Except for some minor discrepancies, *e.g.*, the initial lag period in rate of fatty acid synthesis as determined by radioactivity (Curve C) compared to the lack of a lag period as determined spectrophotometrically (Curve B), a fairly close correlation between the two methods of assay can be obtained. Calculation of the quantity of NADH and NADPH oxidized at time 31.5 min after correction for NADH and NADPH oxidase gave a value of 98.6 mμmoles. A similar calculation based upon the radioactivity measurements gave a value of 48.2 mμmoles of acetyl-units incorporated. From these calculations it can be concluded that in the fatty acid-synthesizing system from Euglena, 2 moles of reduced pyridine nucleotide are oxidized per acetyl-unit incorporated into long-chain fatty acids. No attempt has been made thus far to determine the individual amounts of NADH and NADPH involved in this stoichiometry.

When the effect of concentration of NADH and NADPH on fatty acid synthesis was studied (Fig. 8), it could be shown that even in the presence of a saturating concentration of NADPH an additional amount of NADH was required. In the experiment (Fig. 8) saturating amounts of NADPH were employed while concentration of NADH was varied. When the effect of concentration of NADPH was studied, saturating amounts of NADH were used and the NADPH concentration varied.

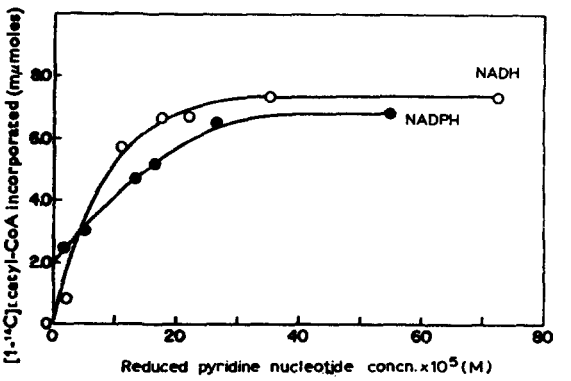

Fig. 8. Effect of increasing concentrations of reduced pyridine nucleotides on incorporation of [1-^{14}C]acetyl-CoA into long-chain fatty acids. Saturating amounts of NADPH were employed when concentration of NADH was varied. When effect of concentration of NADPH was studied, saturating amounts of NADH were used. For other details of the assay see METHODS.

From these curves (Fig. 8) it is apparent that the fatty acid-synthesizing system from Euglena shows a more striking dependence upon NADH than NADPH. Thus NADH can partially substitute in the requirement for NADPH but NADPH cannot satisfy the requirement for NADH. The apparent K_m for NADH was $6 \cdot 10^{-5}$ M.

ATP is required in substrate amounts as shown in Fig. 9. From this curve an apparent K_m for ATP of $4 \cdot 10^{-4}$ M was obtained. Certain other nucleotide tri-

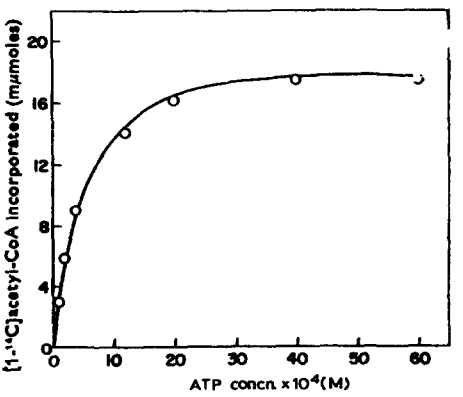

Fig. 9. Effect of increasing concentrations of ATP on incorporation of [1-^{14}C]acetyl-CoA into long-chain fatty acids. Details of the assay are given in METHODS.

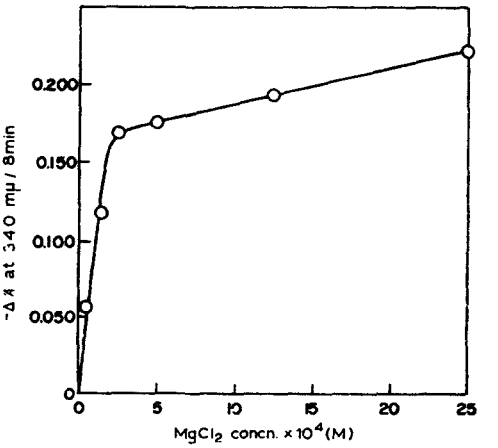

Fig. 10. The dependency of $MgCl_2$ concentration on the rate of acetyl-CoA- and ATP-dependent reduced pyridine nucleotide oxidation. For other details of the assay see METHODS.

phosphates will partially substitute for ATP as will be shown in a later publication.

The presence of either Mg^{2+} or Mn^{2+} was essential for fatty acid synthesis. The effect of increasing concentration of Mg^{2+} is shown in Fig. 10. Saturation of the system with Mg^{2+} was not obtained even at $2.5 \cdot 10^{-3}$ M, although at this concentration the system appeared to be approaching saturation asymptotically. Manganese ions were equally effective, but not more effective than Mg^{2+}, in satisfying the metal requirement for fatty acid synthesis. Other divalent cations have not been tried.

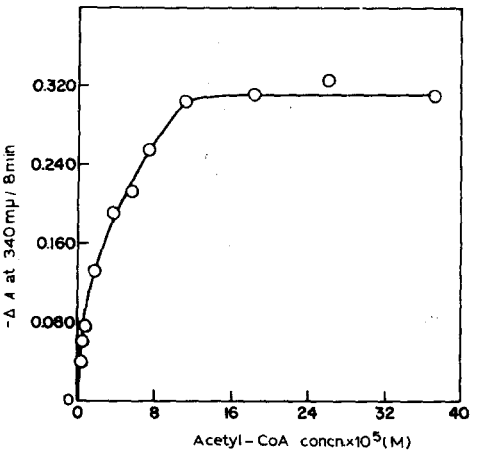

Fig. 11. Effect of increasing concentration of acetyl-CoA on the ATP-dependent oxidation of reduced pyridine nucleotide. Reaction mixtures were as described in METHODS except concentration of acetyl-CoA was varied as indicated.

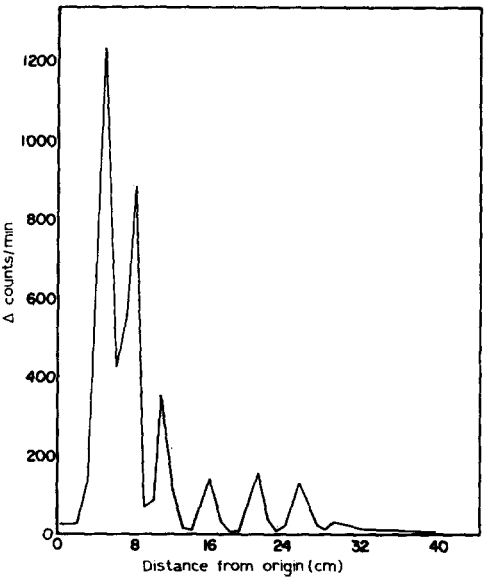

Fig. 12. Distribution of synthesized long-chain fatty acids in the paper chromatographic system of KAUFMANN AND NITSCH[38].

Saturation of the system with respect to acetyl-CoA, as determined by spectrophotometric assay, was attained at a concentration of $1.2 \cdot 10^{-4}$ M. The K_m value as estimated from the curve shown in Fig. 11 was $2.4 \cdot 10^{-5}$ M.

Addition of bicarbonate to the complete reaction had no effect on the amount of acetate (Table III) incorporated into fatty acids nor did pre-incubation of the extract with buffer and avidin at 4° for 10 min before addition of the other components and subsequent assay have any inhibitory effect on fatty acid synthesis (Table III). These results are dissimilar to those investigations in which fatty acid synthesis has been shown to involve the malonyl-CoA pathway.

A typical spectrum of products obtained with incubation of [1-^{14}C]acetyl-CoA in the standard reaction mixture is shown in Fig. 12. About 67 % of the total radioactivity on the chromatogram (with 98 % recovery of the total radioactivity applied) was found in two peaks closest to the origin in the region where fatty acids of chain length of 16–20 carbons are found. A more detailed analysis of the products will be presented elsewhere, but it is sufficient to say here that the major products of the fatty acid-synthesizing fraction from Euglena are long-chain fatty acids and that this

same general distribution of products was observed from extracts pre-incubated with avidin (2 units/mg protein) or with standard reaction mixture that had been supplemented with 20 μmoles of HCO_3^-.

TABLE III

BIOTIN AND AVIDIN EFFECT ON [1-C^{14}]ACETATE INCORPORATION INTO PENTANE-EXTRACTABLE FATTY ACIDS BY EUGLENA FASF*

Lack of inhibition by avidin and lack of enhancement by HCO_3^- on incorporation of [1-^{14}C]acetate into long-chain fatty acids. Enzyme extract pre-incubated with avidin at 4° for 10 min before addition of other components of reaction mixture (see METHODS). Reaction started with addition of ATP. [1-^{14}C]Acetate concentration per assay was 2 μmoles (183000 counts/min per assay).

Additions	*Total counts/min incorporated*
None	22 600
0.2 unit avidin	19 800
0.5 unit avidin	22 600
0.7 unit avidin	21 800
0.2 unit avidin, 5 mμmoles biotin	21 200
0.5 unit avidin, 10 mμmoles biotin	19 700
0.7 unit avidin, 50 mμmoles biotin	20 610
10 μmoles HCO_3^-	19 600
10 μmoles HCO_3^-, 0.2 unit avidin	22 600
10 μmoles HCO_3^-, 0.5 unit avidin	20 000
10 μmoles HCO_3^-, 0.7 unit avidin	21 800
10 μmoles HCO_3^-, 0.7 unit avidin, 5 mμmoles biotin	21 800
10 μmoles HCO_3^-, 0.7 unit avidin, 10 mμmoles biotin	20 500
10 μmoles HCO_3^-, 0.7 unit avidin, 50 mμmoles biotin	20 100

* FASF, fatty acid synthesizing fraction.

DISCUSSION

The system for fatty acid synthesis as analyzed by us here shows many of the properties ascribed in the first publication of WAKIL *et al.*[28] to the fatty acid synthesis by intact rat-liver mitochondria. The requirement for both NADH and NADPH, as well as ATP and Mg^{2+}, the lack of a requirement for HCO_3^-, and the failure of avidin to inhibit the synthesis of fatty acids or change the distribution of products all suggest a pathway of fatty acid synthesis similar to the intact mitochondrial pathway but different from the malonyl-CoA pathway studied with soluble enzymes.

In the malonyl-CoA pathway a marked specificity for NADPH exists. In contrast the system described here has essentially no activity with NADPH alone. With omission of NADPH but with saturating amounts of NADH alone, the rate of fatty acid synthesis is 30–50 % of maximal rate when both NADH and NADPH are used at saturating amounts. These results may suggest that at least some of the enzymes of the β-oxidation sequence are functioning in this system.

As in the intact mitochondrial system and the malonyl-CoA pathway, there is an absolute requirement for ATP. Some possible explanations can be offered for the ATP requirement in this system: (1) deacylation of acetyl-CoA by an acetyl-CoA hydrolase (EC 3.1.2.1) with subsequent reactivation requiring ATP; and (2) activation of protein-bound free fatty acids and subsequent elongation with acetyl-CoA. Assays for acetyl-CoA hydrolase by spectrophotometric means[45,46] or by measurement of –SH release[47], however, have given no indication of hydrolase activity. Nor has any evidence

been obtained for activation of free fatty acids of 6–18 carbon atoms as measured by the hydroxamate method[48]. Moreover we have not been able to show elongation of ^{14}C-labeled free fatty acids when incubated with acetyl-CoA, ATP, Mg^{2+}, DPNH and TPNH as will be set forth in a following publication. Explanation for the ATP requirement for fatty acid synthesis with acetyl-CoA must await further details. The data presented here do not establish the identity of the fatty acids nor do they indicate whether they are formed by a mechanism resulting in net synthesis. Unpublished results of Schmidt degradations on the products, however, do indicate uniform labeling of the carbon atoms throughout the fatty acids and thus suggest that the fatty acid products are formed *de novo*. The avidin insensitivity and the cofactor requirements for incorporation of acetyl-CoA suggest a similarity to the system described by WAKIL *et al.*[28] and BARRON AND STUMPF[31] for oleic acid synthesis.

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