Extramitochondrial Fatty Acid Synthesis in Ehrlich Ascites Tumor Cells Propagated *in Vitro* and *in Vivo*[1] (36809)

B. N. PEDERSEN, A. GROMEK,[2] AND J. L. DAEHNFELDT
(Introduced by J. Kieler)
The Fibiger Laboratory,[3] Lundtoftevej 5, DK-2800 Lyngby, Denmark

The extramitochondrial, CO_2/HCO_3^- dependent fatty acid synthesis was first demonstrated by Lynen (1) and Wakil, Porter and Gibson (2). The need for cytoplasmic ATP and NADPH links this pathway tightly to glycolysis and pentose cycle. In this study we have tried to elucidate the role of the competition for nicotinamide-adenine cofactors.

Materials and Methods. Ehrlich near-diploid ascites tumor cells were propagated *in vivo* and *in vitro* under the designations ELDa and ELDc, respectively. STAD2FI hybrid mice were used for the *in vivo* propagation of the ELDa cells. The ELDc *in vitro* subline and methods of cultivation have previously been described (3).

Incorporation of ^{14}C-acetate into the lipid fraction of ELDc cells cultivated at different CO_2 tensions: For this study ELDc cells were cultivated in a modification of Eagle's MEM fortified with Tris (30 m*M*) and phosphate (2 m*M*) buffers and 20% dialyzed foetal bovine serum. The bicarbonate concentration of the medium was adjusted to the pCO_2 of the gas phase to give pH 7.4. The cells were cultivated in 60 ml culture vessels (Fibiger bottles) loosely stoppered with gauze and stacked in a pile in closed glass cylinders which were continuously aerated with a gas mixture containing 20% O_2 and 0–10% CO_2. Acetate and 1-^{14}C-acetate were added 24 hr after the start of the cultures. After incubation for 48 hr the cells were harvested and hydrolyzed in 15% metanolic KOH and extracted with hexane as described by Abrahams, Matthes and Chaikoff (4).

To eliminate the slight contamination of *n*-hexane with ^{14}C-acetate, the samples were acidified and dried to complete dryness in a desiccator gassed with N_2. By this procedure contaminating acetic acid was evaporated and the counts of blanks were reduced to the order of background.

Incorporation of ^{14}C-acetate and ^{14}C-acetyl-CoA into the lipid fraction of particle-free supernatants (PFS) of ELDa cells: ELDa cells were harvested on days 5 to 6 after ip inoculation. At this time the cells were in logarithmic growth phase. PFS was obtained from homogenates of cells washed 3 times with isotonic NaCl solution containing 1 m*M* EDTA. The cells were homogenized in 0.25 *M* sucrose in a Potter-Elvehjem glass–glass homogenizer cooled in crushed ice. Intact cells and debris were removed by centrifugation at 700*g* for 10 min. The supernatant was recentrifuged at 100,000*g* for 45 min. The supernatant obtained was considered particle free. All procedures were carried out at 4°.

Samples of PFS containing 5–7 mg protein were added to the basic medium specified in Table I in proportion 1:1. This medium was fortified as stated in the figure legends.

Determinations of acetate incorporation were started by the addition of 2 μCi of 1-^{14}C-acetate (sp act, 0.5 mCi/mmole) or 0.05 μCi of 1-^{14}C-acetyl-CoA (sp act 0.5 mCi/mmole) to 1 ml of the reaction mixture. Lipid extraction with *n*-hexane was carried out as stated for cultured cells.

The ^{14}C-activity of the fatty acids was determined by liquid scintillation counting. The scintillation mixture consisted of toluene and

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[2] On leave of absence from the Institute of Experimental Pathology, Warsaw, Poland.

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TABLE I. Basic Medium for the Study of Fatty Acid Synthesis in Particle-Free Supernatants (PFS).

Na-acetate	4	μmole	(Merck)
ATP (disodium salt)	4	μmole	(Sigma)
HS-CoA	0.05	μmole	(Boehringer)
Biotin	0.008	μmole	(Sigma)
Glutathione (reduced)	13	μmole	(Boehringer)
NADP (sodium salt)	2	μmole	(Boehringer)
$MgCl_2$	18	μmole	(Merck)
$MnCl_2$	0.25	μmole	(Merck)
Buffer:			
K-phosphate	100	μmole	(Merck)
$KHCO_3$	15	μmole	(Merck)
Total vol	1	ml, pH 7.4	

Triton-X-100 (2:1) with 0.5% PPO (5). Quench correction was performed by the external standard channels' ratio method. The counting efficiency was 85–90%, and samples were counted to give a counting error of 5% or less.

Protein determinations were performed as described by Lowry *et al.* (6).

The determinations of the NADPH generation in PFS were carried out in a mixture of basic medium and PFS, 1:1. Supplements were added to the different experimental groups as stated in the legend for Fig. 3. The NADPH concentration was determined spectrophotometrically at 340 nm in samples of 100 μl added to 900 μl ice-cold 0.1 *M* sodium bicarbonate.

Results. Table II illustrates the effect of increasing CO_2 tensions on fatty acid synthesis in intact ELDc cells grown *in vitro*. At $pCO_2 = 0\%$ the incorporation of acetate is considered to be mainly mitochondrial and the table shows that the maximal rate at 5% CO_2 is 4 times the mitochondrial rate alone.

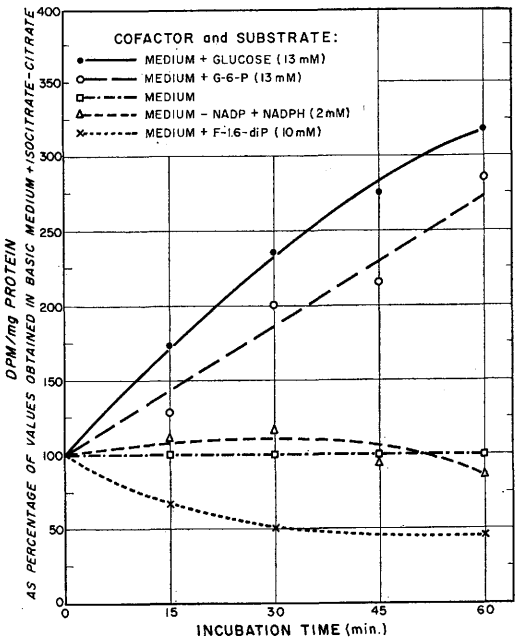

FIG. 1. Incorporation of 1-^{14}C-acetate into the lipid fraction of the particle-free supernatant of Ehrlich ascites tumor cells propagated *in vivo*. Cofactor and substrates were added as stated to basic medium with citrate-isocitrate 13 m*M*.

Figure 1 shows that NADPH may substitute for NADP in a system containing citrate–isocitrate. Glucose and glucose-6-phosphate (G-6-P) stimulate acetate incorporation above the level obtained in basic medium containing citrate–isocitrate while the addition of fructose-1,6-diphosphate (F-1,6-diP) has an inhibitory effect.

Using ^{14}C-acetyl-CoA as precursor, similar results were obtained (Fig. 2). With glucose and G-6-P a clear stimulatory effect, about 8 times the level of citrate–isocitrate + NADP, was found. The substitution of NADPH for NADP did not lead to an increased incorporation of acetyl-CoA. The addition of F-1, 6-diP to the basic medium containing isocitrate–citrate (13 m*M*) seemed only to have a

TABLE II. The Influence of CO_2 on the Incorporation of 1-^{14}C-Acetate into Fatty Acids of Ehrlich Hyperdiploid Ascites Tumor Cells Propagated *in Vitro*.[a]

CO_2 (%):	0	1	5	10
^{14}C-Acetate incorporation in % of 5% CO_2 value	25.4	30.2	100	93.9

[a] Calculations based on median values of 7–10 single determinations.

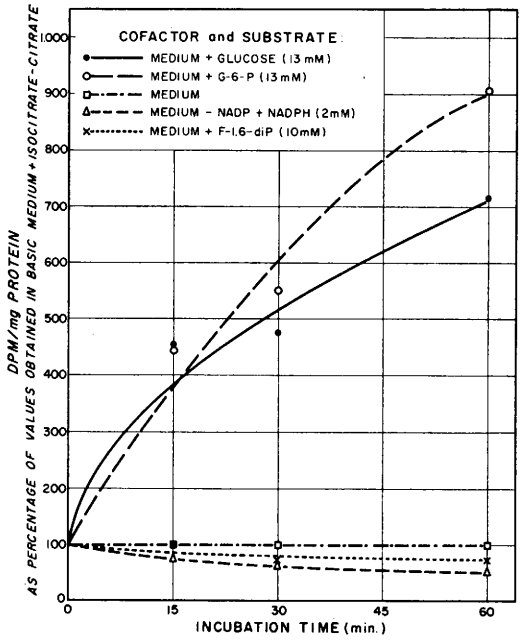

FIG. 2. Incorporation of ^{14}C-acetyl-CoA into the lipid fraction of the particle-free supernatant of Ehrlich ascites tumor cells propagated *in vivo*. Cofactor and substrates were added as stated to basic medium with citrate–isocitrate 13 m*M*.

slight or no effect on fatty acid synthesis.

It appears from Fig. 3, that the maintenance of NADPH concentration in a mixture of basic medium fortified with NADPH and PFS is dependent on the presence of suitable NADPH regeneration systems. The NADPH concentration can be stabilized apparently equally well by the addition of either glucose or isocitrate–citrate. The NADPH content is rapidly depleted if no regeneration system is added, or if F-1,6-diP or pyruvate is added. Even if isocitrate–citrate is added, the further addition of pyruvate and to some degree also the addition of F-1,6-diP will cause a rapid fall in the NADPH concentration.

Discussion. In the intact cell glucose serves as a direct precursor for fatty acid synthesis. However, in the particle-free supernatant practically all glucose consumption can be accounted for by the formation of lactate (90% or more) and consumption in the pentose cycle (2–4%). This means that the carbon chain of glucose does not contribute to the formation of fatty acids.

Nevertheless glucose has a marked stimulatory effect on 1-^{14}C-acetate as well as 1-^{14}C-acetyl-CoA incorporation into fatty acids in PFS.

From Figs. 1 and 2 it appears that the same stimulation may be obtained with G-6-P, while F-1,6-diP has a slight inhibitory effect. These findings indicate that metabolites later than G-6-P/F-6-P do not stimulate acetate incorporation. The finding that G-6-P can replace glucose is strongly suggestive of involvement of the oxydative part of the pentose cycle in the stimulatory effect. This interpretation is supported by the results in Fig. 3. The stabilizing effect of glucose on the NADPH concentration is explained by a net production of NADPH in oxydation of G-6-P and 6-phosphogluconate. As it can be seen from the effect of F-1,6-diP on NADPH

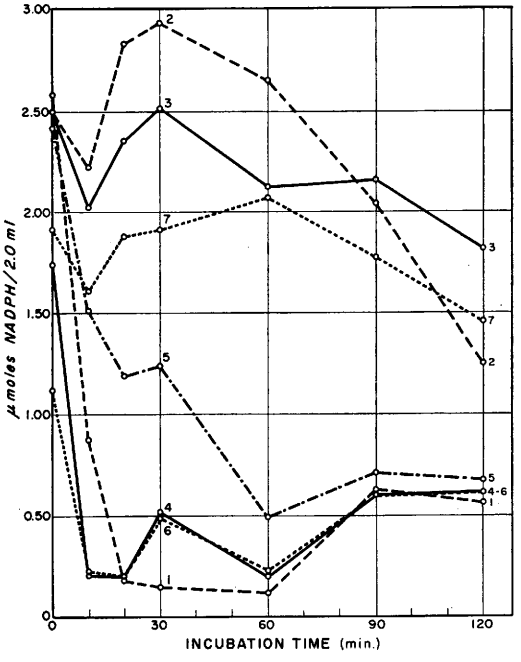

FIG. 3. The content of NADPH in basic medium with NADPH (1.7 m*M*) mixed with particle-free supernatant of Ehrlich ascites tumor cells in proportion 1:1. Substrates were added as follows: (1) no substrate; (2) glucose, 13 m*M*; (3) citrate–isocitrate, 13 m*M*; (4) pyruvate, 13 m*M*; (5) fructose-1,6-diphosphate, 13 m*M*; (6) citrate–isocitrate, 13 m*M* + pyruvate, 13 m*M*; (7) citrate–isocitrate, 13 m*M* + fructose-1, 6-diphosphate, 13 m*M*.

level, this metabolite will tend to depress the concentration of NADPH, probably by consumption of NADPH in pyruvate reduction (7). Because of the very low activity of fructose-1,6-diphosphate phosphatase in the Ehrlich ascites cells (8), F-1,6-diP cannot contribute to NADPH formation via F-6-P/G-6-P. That the NADPH content is not exhausted by F-1,6-diP in the presence of citrate–isocitrate, means that the production of NADPH from other regenerating systems, here citrate–isocitrate, can only partially keep pace with the consumption. Apparently in this system citrate–isocitrate acts as more than simply activator of the fatty acid synthesizing system. The production of NADPH in isocitrate oxydation also is essential. In addition to the production of NADPH from glucose, it should not be forgotten that glycolysis will also give rise to ATP formation necessary in fatty acid synthesis.

As shown by the depressing effect of F-1, 6-diP on the NADPH concentration in Fig. 3, the NADPH generating capacity from citrate–isocitrate is limited. This probably means that the stimulating effect of glucose and G-6-P on acetate incorporation, even in the presence of citrate–isocitrate, can be accounted for by the net production of NADPH and the ATP formation mentioned.

Summary. The presence of extramitochondrial CO_2/HCO_3^- dependent fatty acid synthesis in Ehrlich hyperdiploid ascites tumor cells cultivated *in vitro* was demonstrated by the stimulatory effect of increasing CO_2 tensions (0–5% atm) on the ^{14}C-acetate incorporation into hexane extracts of KOH hydrolysates of intact cells.

The incorporation of ^{14}C-acetate into fatty acids of particle-free supernatants of Ehrlich ascites tumor cells propagated *in vivo* was shown to be dependent on NADPH regenerating systems, such as citrate–isocitrate, glucose or glucose-6-phosphate. The last two compounds, however, stimulated the ^{14}C-acetate as well as the ^{14}C-acetyl-CoA incorporation into fatty acids even in the presence of citrate–isocitrate + NADP. Under the same conditions fructose-1,6-diphosphate produced an inhibition of the acetate incorporation.

Determinations of the NADPH concentration of particle-free supernatants fortified with different NADPH generating systems showed that only glucose and citrate–isocitrate could stabilize the NADPH level, while fructose-1,6-diphosphate and pyruvate would lower or completely exhaust the NADPH content.

The mechanism of the stimulatory effect of glucose on acetate incorporation in particle-free supernatants is discussed.

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