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STUDIES ON THE EFFECTS OF ESSENTIAL FATTY ACIDS ON GROWTH RATE, FATTY ACID COMPOSITION, OXIDATIVE PHOSPHORYLATION AND RESPIRATORY CONTROL OF HeLa CELLS IN CULTURE

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SUMMARY

1. Mammalian cells in culture provide a simple model to study the function of polyunsaturated fatty acids.

2. HeLa S_3 cells cultured in a lipid-deficient medium showed a pattern of essential fatty acid deficiency represented by an increase in 18:1 and a decrease in 20:4 and 18:2, a lack of growth and impaired mitochondrial function.

3. Addition of albumin-bound linoleic or arachidonic acid partially or totally prevented these changes.

INTRODUCTION

The metabolism of essential fatty acids and the physiological consequences of their deficiency have been extensively studied in intact animals[1,2] but the intimate details of their function have remained obscure. Several authors[3–8] have reported evidence of alterations in the function of mitochondria isolated from the livers of rats deficient in essential fatty acids, but the exact nature of the alterations was not revealed by these experiments. A simpler system for the study of the function of the polyunsaturated fatty acids was suggested by the report of HAM[9] that linoleic acid is a growth factor for the clonal growth of Chinese hamster cells.

The purpose of this investigation was to study the effect of essential fatty acids on the growth of HeLa cells and on their role in mitochondrial function using ADP:O ratios and respiratory control as indicators of physiological integrity. It was hoped that at this level, the factors responsible for the alterations in metabolism, seen in whole animal experiments, might be more readily apparent.

MATERIALS AND METHODS

Experimental medium

Stock cultures of HeLa S_3 cells were maintained in plastic flasks (Falcon Plastics) using a complete growth medium[10] *plus* 20% fetal bovine serum (Micro-

biological Associates) under a gas phase of 95 % air and 5 % CO_2. Fatty acids were prepared as already described[11].

Growth

Cells were seeded, after trypsinization (0.1 % trypsin), in plastic petri dishes in complete growth medium *plus* 20 % fetal bovine serum for 48 h, after which the medium was poured off, the cells rinsed gently with Hank's saline and the experimental medium added. Growth was measured through the increase in protein, using OYAMA's method and bovine albumin as a standard[12]. Each point in the experiments described in Figs. 1–4 is the mean of 3 determinations done in duplicate.

The concentrations of fatty acids used were chosen after a preliminary experiment, using a range of concentrations varying from 10^{-11} M to 10^{-5} M linoleic or arachidonic acid. The criteria used for choosing the optimal concentrations were based on qualitative evaluation of the formation of colonies and their formation of confluent sheets of cells.

Fatty acid analysis

The total lipids were extracted with 5 ml of chloroform–methanol (2.5:1, v/v) in a glass homogenizer. The homogenate was washed twice with 5-ml portions of solvent and all the washings combined, the extract filtered by gravity through a filter paper and the paper washed 3 times with the extracting mixture. The filtrate was evaporated to dryness at 45° under N_2. Later the residue was transmethylated with boron trifluoride–methanol reagent[13]. The methyl esters were weighed and analyzed by gas–liquid chromatography using a Barber-Colman Model 20 apparatus equipped with a 100-ft capillary column coated with Apiezon L. Fatty acids were identified by comparison with known standards and were quantitatively estimated by triangulation.

Oxidative phosphorylation and respiratory control

The cells were incubated as described above and, after 2 days in experimental medium, harvested gently with a rubber policeman after being rinsed once in Hank's saline. The cells, suspended in Hank's saline were centrifuged at 900 rev./min in a Model CL International centrifuge for 10 min at 5° and the supernatant was decanted and the cells suspended in a homogenization medium containing 75 mM sucrose, 225 mM mannitol and 0.1 mM EDTA (pH 7.1). The homogenization was carried out for 30 sec with a Teflon pestle in an ice bath. The oxygen consumption was measured using the system described by STRICKLAND, ZIEGLER AND ANTHONY[14] consisting of a closed system and an oxygen electrode (Yellow Springs Co.) connected to a recorder (Varian Co.). The volume of the vessel was 3.3 ml and the temperature at which the experiments were carried out was 25°. The duration of each experiment was 15–20 min.

The composition of the incubation medium was: 15 mM sucrose, 45 mM mannitol, 0.02 mM EDTA, 40 mM KCl, 20 mM $MgCl_2$ and 20 mM potassium phosphate buffer (pH 7.4) (ref. 15). The concentrations of the substrates* used were: 20 mM succinate, 15 mM β-hydroxybutyrate and 10 mM α-ketoglutarate; with the α-keto-

* Reagents: ADP sodium salt (P-L Biochemicals, Inc.), ATP disodium salt (Sigma), succinic acid disodium salt (Eastman), α-ketoglutaric acid (Calbiochem), DL-β-hydroxybutyric acid (ICN).

glutarate, 10 mM sodium malonate was used. The concentration of ATP used varied from 0.3 to 0.6 mM and of ADP from 0.1 to 0.2 mM.

At the completion of the experiment the protein of an aliquot of the homogenate was measured using LOWRY's method[16]. The protein varied from 1.5 to 2 mg for each experiment.

RESULTS

Growth

Albumin-bound linoleic acid promoted growth at both concentrations studied: $2.5 \cdot 10^{-8}$ M and $2.5 \cdot 10^{-7}$ M, while in the group incubated in medium supplemented with $1.25 \cdot 10^{-7}$ M albumin alone, the amount of protein per plate decreased throughout the 5 days of observation (Fig. 1). The lower concentrations of linoleic acid used, $2.5 \cdot 10^{-8}$ M, were slightly more efficient, showing 39% of the growth-promoting activity of serum.

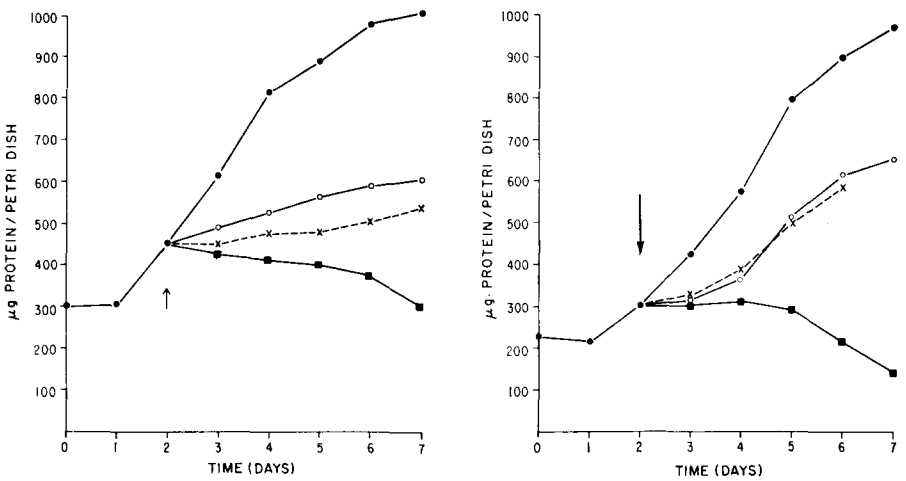


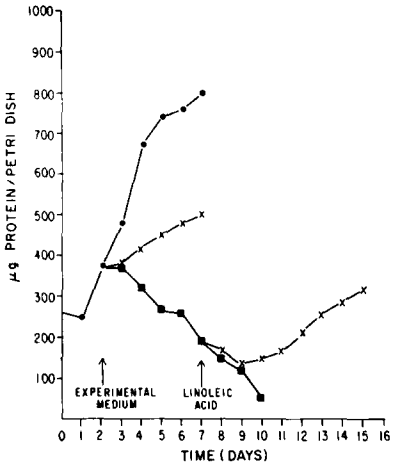
Fig. 1. Growth of HeLa S_3 cells in various media: ●—●, medium *plus* 20% fetal calf serum; ○—○, medium *minus* serum *plus* $2.5 \cdot 10^{-8}$ M linoleic acid; ×—×, medium *minus* serum *plus* $2.5 \cdot 10^{-7}$ M linoleic acid; ■—■, medium *minus* serum *plus* $1.25 \cdot 10^{-7}$ M albumin. Fatty acids were bound to albumin in a molar ratio of 2:1. At the arrow complete growth medium was substituted by the experimental medium or by fresh complete growth medium for the control.

Fig. 2. Growth of HeLa S_3 cells in various media: ●—●, medium *plus* 20% fetal calf serum; ○—○, medium *minus* serum *plus* $1.4 \cdot 10^{-8}$ M arachidonic acid; ×—×, medium *minus* serum *plus* $2.8 \cdot 10^{-8}$ M arachidonic acid; ■—■, medium *minus* serum *plus* $1.4 \cdot 10^{-8}$ M albumin. Fatty acids were bound to albumin in a molar ratio of 2:1. At the arrow complete growth medium was substituted by the experimental medium or by fresh complete growth medium for the control.

With albumin-bound arachidonic acid, there was no difference between the two concentrations used: $1.4 \cdot 10^{-8}$ M and $2.8 \cdot 10^{-8}$ M (Fig. 2). As before, the group supplemented with $1.4 \cdot 10^{-8}$ M albumin showed a decrease in the amount of protein per plate. $1.4 \cdot 10^{-8}$ M arachdonic acid showed 56% of the growth-promiting efficiency of serum.

The experiments illustrated in Figs. 3 and 4 were designed to show the effect of linoleic and arachidonic acids in restoring growth to plates, which had shown a

decrease in the amount of protein prior to the addition of the unsaturated acids. After 5 days of culture in a medium supplemented only with $1.25 \cdot 10^{-8}$ M albumin, the medium was decanted from the plate and replaced with a medium containing albumin *plus* $2.5 \cdot 10^{-8}$ M linoleic acid (Fig. 3) or $1.4 \cdot 10^{-8}$ M arachidonic acid (Fig. 4). After a lag period of about 4 days the amount of protein per plate started to increase. Parallel experiments using a medium supplemented with serum albumin or the fatty acids from the start of the experiment were run and are shown as controls.



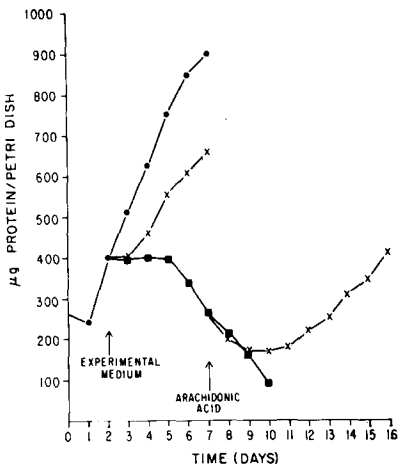


Fig. 3. Restoration of growth with linoleic acid: ●—●, medium *plus* 20% fetal calf serum; ×—×, medium *minus* serum *plus* $2.5 \cdot 10^{-8}$ M linoleic acid; ■—■, medium *minus* serum *plus* $1.25 \cdot 10^{-8}$ M albumin. Fatty acids were bound to albumin in a molar ratio of 2:1.

Fig. 4. Restoration of growth with arachidonic acid: ●—●, medium *plus* 20% fetal calf serum; ×—×, medium *minus* serum *plus* $1.4 \cdot 10^{-8}$ M arachidonic acid; ■—■, medium *minus* serum *plus* $1.25 \cdot 10^{-8}$ M albumin. Fatty acids were bound to albumin in a molar ratio of 2:1.

Fatty acid analysis

The proportions of fatty acids of the total lipids in cells incubated in complete growth medium *plus* 20% fetal bovine serum or $1.4 \cdot 10^{-8}$ M albumin are shown in Table I. Both groups showed a decrease in the relative amounts of 16:0 (palmitate) and 20:3 (eicosatrienoate) and an increase in 18:1 (oleate). The serum group showed no change in 14:0 (myristate) and 16:1 (palmitoleate), while in the albumin group both of these fatty acids increased. The 18:0 (stearate), 18:2 (linoleate) + 18:3 (linolenate), and 20:4 (arachidonate) increased in the 'serum group' and decreased in the 'albumin group'.

Two experiments are shown in Table II. In the first, the effect of the addition of $2.5 \cdot 10^{-8}$ M albumin-bound linoleic acid is compared to a control containing $1.25 \cdot 10^{-8}$ M albumin. In the second can be seen the effect of $2.8 \cdot 10^{-8}$ M arachidonic acid compared to a group containing $1.4 \cdot 10^{-8}$ M albumin, after 4 days of incubation in experimental medium. The main changes observed in the 'linoleate experiment' were: greater amounts of 12:0, 14:0, 18:2 + 18:3 and 20:3 and lower amounts of 16:1 and 18:1 in the group supplemented with linoleic acid compared with the 'deficient group'. The groups supplemented with arachidonic acid showed greater amounts of 14:0, 16:0, 16:1, and 18:0 and lesser amounts of a fatty acid with a retention time of 11.1 than the control.

TABLE I

EFFECT OF SERUM AND ALBUMIN ON FATTY ACID COMPOSITION

Cells are grown on normal medium and at zero time the experimental medium added. The time represents days on experimental media. The results are expressed as total lipid percentage of methyl esters in measurable peaks. See text for further details.

*Fatty acids**	*Fetal calf serum (20 %)*				*Albumin ($1.4 \cdot 10^{-8}$ M)*		
Days	*0*	*2*	*4*	*7*	*2*	*4*	*7*
12:0	1.1	0.9	0.7	0.7	0.0	0.0	0.8
14:0	2.9	2.7	2.7	2.4	2.8	3.4	6.1
Unidentified	2.3	1.5	2.4	1.1	2.3	1.1	1.9
16:0	46.0	38.0	26.0	28.0	25.0	23.0	24.0
16:1	3.8	3.6	3.5	3.7	5.7	4.5	11.0
Unidentified	1.0	1.4	2.6	2.7	0.0	0.0	0.3
18:0	10.0	14.0	18.0	18.0	8.5	5.5	8.8
18:1	8.0	12.0	15.0	16.0	11.0	11.0	19.0
18:2 + 18:3	1.5	2.7	6.4	6.8	1.5	2.4	0.6
20:3	14.0	13.0	5.7	6.5	7.8	8.4	4.9
20:4	2.5	3.7	9.5	14.0	5.8	0.0	1.1

* Carbon number:double bonds.

TABLE II

EFFECT OF LINOLEIC AND ARACHIDONIC ACIDS ON FATTY ACID COMPOSITION

Incubated 4 days on experimental medium. See Table I and text for details. Fatty acids were bound to albumin in a molar ratio of 2:1.

Fatty acids	*Expt. 1*		*Expt. 2*	
	Albumin ($1.25 \cdot 10^{-8}$ M)	*Linoleic acid ($2.5 \cdot 10^{-8}$ M)*	*Albumin ($1.4 \cdot 10^{-8}$ M)*	*Arachidonic acid ($2.8 \cdot 10^{-8}$ M)*
12:0	0	1.2	0	0
14:0	5.0	7.6	3.4	7.8
Unidentified	0.5	0.6	1.1	0.4
16:0	25.0	24.0	23.0	31.0
16:1	8.8	4.9	4.5	6.0
Unidentified	1.9	1.3	0	0.6
18:0	11.0	11.0	5.5	11.0
18:1	22.0	14.0	11.0	9.3
18:2 + 18:3	3.9	9.2	2.4	3.0
20:3	2.4	4.1	8.4	5.2
20:4	12.0	12.0	0	19.0

Oxidative phosphorylation and respiratory control

Tables III, IV and V record the effect of 2 days of incubation in a medium with albumin alone, compared with groups incubated in a medium supplemented with 20 % fetal bovine serum or albumin-bound linoleic or arachidonic acid.

The oxidation of 3 substrates, succinate, α-ketoglutarate and β-hydroxybutyrate, was studied. In all cases the 'deficient group', incubated with albumin, showed a decrease in ADP:O ratios and respiratory control as compared with the 'serum group'. Therefore, uncoupling of the oxidative phosphorylation and a lo of

TABLE III

ADP:O AND RESPIRATORY CONTROL AS MEASURED WITH SUCCINATE

Cells were incubated for 48 h in lipid-free medium or in a medium supplemented with fatty acids. See text for details. Fatty acids were bound to albumin in a molar ratio of 2:1. R.C. stands for respiratory control.

Expt. No.	*Fetal calf serum (20%)*		*Albumin (10^{-8} M)*		*Linoleic acid ($2 \cdot 10^{-8}$ M)*		*Fetal calf serum (20%)*		*Albumin ($5 \cdot 10^{-9}$ M)*		*Arachidonic acid (10^{-8} M)*	
	ADP:O	*R.C.*	*ADP:O*	*R.C.*	*ADP:O*	*R.C.*	*ADP:O*	*R.C.*	*ADP:O*	*R.C.*	*ADP:O*	*R.C.*
1	1.9	2.8	—	1.1	1.2	1.8	1.8	3.0	1.0	1.4	1.6	1.9
2	1.8	2.9	0.9	1.3	1.3	2.3	1.9	2.8	0.9	1.7	1.4	2.2
3	1.7	3.5	1.1	2.2	1.6	2.8						

TABLE IV

ADP:O AND RESPIRATORY CONTROL AS MEASURED WITH α-KETOGLUTARATE

Conditions and concentrations as in Table III. See text for details. R.C. stands for respiratory control.

Expt. No.	*Fetal calf serum (20%)*		*Albumin (10^{-8} M)*		*Linoleic acid ($2 \cdot 10^{-8}$ M)*		*Fetal calf serum (20%)*		*Albumin ($5 \cdot 10^{-9}$ M)*		*Arachidonic acid (10^{-8} M)*	
	ADP:O	*R.C.*	*ADP:O*	*R.C.*	*ADP:O*	*R.C.*	*ADP:O*	*R.C.*	*ADP:O*	*R.C.*	*ADP:O*	*R.C.*
1	3.4	2.9	1.3	1.2	2.1	2.0	3.9	2.9	—	1.1	2.9	2.0
2	3.7	3.0	1.6	1.5	2.0	1.6	3.5	3.2	1.8	1.8	3.0	2.6
3	3.2	2.2	—	1.1	2.8	1.9						

TABLE V

ADP:O AND RESPIRATORY CONTROL AS MEASURED WITH β-HYDROXYBUTYRATE

Conditions and concentrations as indicated in Table III. See text for details. R.C. stands for respiratory control.

Expt. No.	*Fetal calf serum (20%)*		*Albumin (10^{-8} M)*		*Linoleic acid ($2 \cdot 10^{-8}$ M)*		*Arachidonic acid (10^{-8} M)*	
	ADP:O	*R.C.*	*ADP:O*	*R.C.*	*ADP:O*	*R.C.*	*ADP:O*	*R.C.*
1	2.8	2.2	—	1.1	2.4	1.3	2.8	2.0
2	2.7	1.9	0.8	1.3	2.6	1.8	2.5	1.9

spiratory control was observed in these cases. The addition of $2 \cdot 10^{-8}$ M linoleic or $^{1-8}$ M arachidonic acid partially or completely prevented these changes.

DISCUSSION

e work of HAM[9] and DUBIN, CZERNOBILSKY AND HERBST[17] has shown that linoleic id is a growth factor for Chinese hamster cells and macrophages in culture, respectiv. SAVCHUCK, LOCKHARD AND LOY[18] were not able to demonstrate any growth eft t of a lysine salt of linoleic acid on Chang human-liver cells. In the latter

case the concentrations of the fatty acid used was 20–80 times higher than those used by the above mentioned authors[9,17]. This fact and possible different nutritional requirements for different cell types could explain the difference in the results. Working with beating heart cells in culture[19], we found that linoleic acid does not influence the growth or the beating rates but apparently is necessary for the normal functioning of the mitochondria[13]. It has been generally accepted that arachidonic is the essential fatty acid and that linoleic is necessary only through its conversion to the tetraenoic acid. The analysis of cells incubated with linoleic acid suggested the possibility of a lack of conversion of linoleic to arachidonic since addition of linoleic was not accompanied by an increase in the relative amount of arachidonic. Moreover, other experiments using radioactive linoleic acid, performed in this laboratory (HAGGERTY *et al.*[20]) have confirmed these preliminary indications.

The alteration in the relative amounts of fatty acids in the starved cells, shown in Table I, as compared to the serum controls, points to a state of fatty acid deficiency in these cells. The lack of an increase in the amount of 20:3, described as characteristic in the state of fatty acid deficiency in rats by MEAD[21] and others could be explained by the inability of these cells to convert 18:2 into 20:4. In HeLa cells, which appear to lack one of the enzymes for this conversion, one would not expect an increase in the level of 20:3. It is also possible that the 20:3 synthesized by these cells is not the usual 20:3 (refs. 5, 8, 11) found in essential fatty acid deficiency.

The addition of linoleic or arachidonic acids (Table II) increased their respective intracellular levels, but had no consistent effect on the other fatty acids. It is clear, however, that either acid promotes the growth of HeLa cells, although it appears that since they could not wholly replace serum, other factors are required.

These polyunsaturated fatty acids also have an effect on mitochondrial function. Cells incubated in lipid-free medium showed, consistently, an uncoupling of oxidative phosphorylation and a loss of respiratory control (Tables III–V). The addition of either linoleic or arachidonic acid partially prevented these effects. The relationship between essential fatty acids and energy metabolism has been stressed by many authors. Mitochondria from essential fatty acid-deficient rat liver showed an impaired phosphorylation coupled with oxidation of α-ketoglutarate, malate, glutamate and pyruvate, but no uncoupling with succinate as substrate[3,4]. Mitochondria from the same origin had a tendency to swell *in vitro*[5,6] and showed morphological changes related with changes in the respiratory activity[7]. ITO AND JOHNSON[8] suggested that essential fatty acid deficiency influences oxidative phosphorylation in rat-liver mitochondria indirectly, through changes in the structure of the lipoprotein membrane. In beating heart cells[19] essential fatty acid deficiency affected the phosphorylation coupled to α-ketoglutarate and β-hydroxybutyrate but not that coupled to succinate. Linoleic or arachidonic acid partially or completely prevented these changes[11]. These papers[3,4,11] presented evidence that essential fatty acid deficiency produced a defe in the electron-transport system at the level of NADH oxidation.

The low ADP:O ratios and loss of respiratory control suggests a disrupti of the electron-transport system in the cells incubated in essential fatty ac ree medium. The level of the ADP:O ratio and respiratory control is considered a s itive indicator of mitochondrial integrity[22–24]. The fact that the addition of both f y acids used prevented those changes may be interpreted in terms of a requirement r ether linoleate or arachidonate for maintaining the physiological integrity o he ito-

chondria. This effect may be a result of their function in the mitochondrial membrane, and it is possible that the role of these acids in promoting cell growth may also be a result of the maintenance of membrane integrity.

The fact that either linoleic or arachidonic acid fulfilled similar roles in growth and mitochondrial function in these cells, which are unable to convert linoleic acid to arachidonic[20], suggests the possibility of a functional interchange between these polyunsaturated fatty acids.

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