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# STUDIES *IN VITRO* ON SINGLE BEATING RAT-HEART CELLS

## X. THE EFFECT OF LINOLEIC AND PALMITIC ACIDS ON BEATING AND MITOCHONDRIAL PHOSPHORYLATION

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SUMMARY

1. Beating rat-heart cells cultured in a lipid-deficient medium showed: (a) lack of growth; (b) decrease and later a cessation of the beating; (c) impairment of oxidative phosphorylation and a decrease in the respiratory control; (d) a pattern of fatty acid deficiency as shown by the analysis using gas–liquid chromatography.

2. The addition of albumin-bound linoleic acid or arachidonic acids prevented partially or totally the effects of the lipid deficiency on the mitochondrial function and the fatty acid composition, but did not promote growth and did not improve the beating.

3. Medium supplemented with albumin-bound palmitic acid maintained the beating, but did not promote growth and did not affect the impairment of the mitochondrial function.

## INTRODUCTION

The finding that serum lipids or fatty acids can maintain or restore beating in cultured rat-heart cells[1] has focused our attention on a study of the mechanism of fatty acid action in these cells. Fatty acids have a unique role in mammalian cells. They may function equally in energy metabolism and in cellular structure. The evidence from studies in rat-liver mitochondria from whole animals[2,3] and mitochondria from cultured cells[4] is that unsaturated fatty acids are essential for mitochondrial function. This may reflect their role as a functional part of the membrane structure. It is also widely accepted that fatty acid oxidation is a primary source of energy for the mammalian heart[5].

The possibility that fatty acids serve as a specific energy source for contraction was made unlikely by the observation that ATP from glycolysis, alone, may support beating[6]. However beating rat-heart cells in culture lose their ability to oxidize fatty acids and their ability to beat at the same time[7]. Glycolysis does not change and one may surmise that glucose metabolism could serve as the main energy source. The

glycolytic capacity operating at its maximum level can maintain the ATP level[8] high enough for beating. It is not known, however, whether under the artificial culture conditions glycolysis can function at its maximum. It is possible that both fatty acid and glucose metabolism are necessary for maintenance of the correct ATP level and for long-term function.

Preliminary evidence obtained in our laboratory indicated that cells grown in medium without serum became depleted of essential fatty acids. It was therefore conjectured that the serum lipids and fatty acids were involved in maintaining the integrity of the cellular and mitochondrial membranes of the heart cells and in this capacity maintaining the ability of the cells to beat.

This report concerns observations of the relationship of the essential fatty acids, linoleic and arachidonic, and the non-essential acid, palmitic, to the beating of heart cells and to the respiratory control and oxidative phosphorylation of heart-cell mitochondria.

## MATERIALS AND METHODS

The general techniques for culturing rat-heart cells have been described elsewhere[9,10]. Basal medium *plus* 10 % human serum and 10 % fetal calf serum was used. This is the complete growth medium. The general procedure, unless otherwise indicated was as follows: the cells were placed in plastic petri dishes and cultured for 3 days in the complete growth medium described above. After this period of attachment and organization, the cells were rinsed once with Hank's saline and the experimental medium was added.

### *Experimental medium*

Linoleic, arachidonic and palmitic acids (Calbiochem) were stored under $N_2$ in sealed tubes. The fatty acid was added to sterile solution of NaOH, the mixture gassed with $N_2$ and the container closed tightly and heated in a water bath at 80°, with shaking for 5 min. An aliquot was transferred to basal medium containing human albumin (Pentex) free of lipids[11] in a stoichiometric relation of 2 moles of fatty acid to 1 mole of albumin, and 0.02 mM reduced glutathione to prevent peroxidation.

The fatty acids, albumin and glutathione were non-sterile, but the containers, solvents and growth medium were sterilized. No contamination was observed for the duration of this work. The controls contained albumin and glutathione in the same concentration used in the experimental groups, with any exception as indicated. The concentrations of fatty acids used were chosen after preliminary experiments in which different concentrations were tested.

### *Growth*

Protein determination was used as a measure of growth; cells plated in small plastic petri dishes (Falcon Plastics) were extracted and protein measured using a modification of Lowry's method[12].

### *Beating rate*

The beating rate was estimated by counting the number of beats in 2 different areas of each of 5 plates; each point represents an average of these determinations.

*Fatty acid analysis*

After different periods of incubation, the medium was removed, the cells rinsed once with Hank's saline, harvested gently with a rubber policeman and resuspended in Hank's saline, then centrifuged at 1500 rev./min in an International centrifuge, clinical model, for 10 min at 5°. The lipids were extracted from the sediment and transmethylated to yield the fatty acid methyl esters as described elsewhere[4].

*Oxidative phosphorylation and respiratory control*

Cells were incubated in the complete growth medium for 3 days at which time the growth medium was replaced with experimental medium. The cells were harvested on the 5th and 8th days as described above.

The materials and methods used for the homogenization and polarographic measurement of ADP:O ratios and respiratory control are described[4,13,14]. The homogenate fraction was added in a 0.20–0.35-ml volume containing 2.1–3.0 mg of protein obtained by pooling the cells from several petri dishes.

## RESULTS

*Linoleate and mitochondrial function and beating*

Cells were incubated in a basal medium *plus* serum (complete growth medium), albumin, linoleate and albumin, or linoleate, palmitate and albumin. The cells in the complete growth medium increased their beating rate to a maximum on the 4th day and thereafter maintained that rate (Fig. 1). The albumin and albumin–linoleate groups decreased their rate rapidly and stopped beating 2 days after the change of medium. On the other hand the albumin–linoleate–palmitate groups reached a maxi-

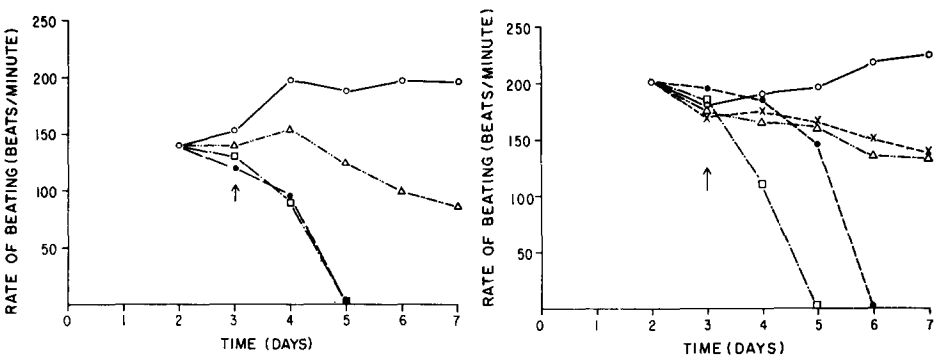


Fig. 1. Effect of linoleic acid and palmitic acid on beating: ○—○, complete growth medium; □—□, basal medium *plus* $10^{-6}$ M albumin; ●—●, basal medium *plus* $10^{-6}$ M linoleic acid; △···△, basal medium *plus* $10^{-6}$ M linoleic acid *plus* $10^{-7}$ M palmitic acid. At the arrow complete growth medium was substituted by the experimental medium or by fresh complete growth medium for the control.

Fig. 2. Effect of linoleic acid and palmitic acid on beating: ○—○, complete growth medium; □—□, basal medium *plus* $10^{-6}$ M albumin; ●—●, basal medium *plus* $10^{-6}$ M linoleic acid; ×—×, basal medium *plus* $10^{-7}$ M palmitic acid; △—△, basal medium *plus* $10^{-6}$ M linoleic acid *plus* $10^{-7}$ M palmitic acid. At the arrow complete growth medium was substituted by the experimental medium or by fresh complete growth medium for the control.

mum 1 day after the change of medium and this rate was maintained for an additional 3 days when the experiment was terminated.

The ADP:O ratio and the respiratory control showed no effects after 2 days of incubation in the experimental medium (5th day of total incubation) (Table I). After 5 days of incubation on the experimental medium, a loss in respiratory control developed in the albumin group but no change in the ADP:O ratio when succinate was used to measure these parameters. On the other hand, with α-ketoglutarate as the substrate both the ADP:O ratio and the respiratory control values decreased in the albumin group.

TABLE I

EFFECT OF LINOLEIC ACID AND PALMITIC ACID ON ADP:O RATIO AND RESPIRATORY CONTROL

For concentration of fatty acids, see Fig. 1 and text. The vol. of the vessel was 3.3 ml. The experiments were carried out at 25° and required 15–20 min for completion. The time represents days on experimental media. The ADP:O ratio was omitted when the respiratory control was too low to permit accurate measurement. The reaction medium consisted of 15 mM sucrose, 45 mM mannitol, 0.02 mM EDTA, 40 mM KCl, 20 mM potassium phosphate buffer (pH 7.4). The substrates were used in the following concentrations: 20 mM succinate, 15 mM β-hydroxybutyrate and 10 mM α-ketoglutarate and in the last case 10 mM sodium malonate was also added. The concentrations of ADP varied from 100 to 200 mM. R.C. stands for respiratory control.

| *Days in culture* | *Substrates for mitochondria* | *Expt. No.* | *Serum* | | *Albumin* | | *Linoleate* | | *Linoleate + palmitate* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | *ADP:O* | *R.C.* | *ADP:O* | *R.C.* | *ADP:O* | *R.C.* | *ADP:O* | *R.C.* |
| 2 | Succinate | 1 | 1.6 | 2.0 | 1.6 | 2.8 | 1.4 | 1.7 | 1.6 | 2.2 |
| | | | 1.4 | 1.9 | 1.5 | 1.9 | 1.6 | 2.1 | 1.5 | 1.9 |
| | | | 1.5 | 2.2 | 1.5 | 2.2 | 1.4 | 2.9 | 1.5 | 3.0 |
| | α-Ketoglutarate | 1 | 2.9 | 3.1 | 2.4 | 2.6 | 2.9 | 2.8 | 2.4 | 2.4 |
| | | | 2.7 | 2.8 | 2.9 | 2.4 | 3.1 | 2.7 | 2.8 | 2.6 |
| | | 2 | 3.0 | 2.6 | 2.7 | 3.0 | 2.9 | 2.0 | 2.5 | 2.0 |
| 5 | Succinate | 1 | 1.8 | 3.1 | 1.6 | 1.5 | 1.5 | 2.8 | 1.5 | 2.2 |
| | | 2 | 1.4 | 2.4 | 1.6 | 1.6 | 1.7 | 1.9 | 1.4 | 1.7 |
| | | 3 | 1.5 | 2.6 | — | 1.1 | 1.5 | 2.0 | 1.7 | 2.6 |
| | α-Ketoglutarate | 1 | 3.0 | 2.4 | 1.7 | 1.8 | 3.1 | 3.0 | 2.9 | 2.0 |
| | | 2 | 2.9 | 3.1 | — | 1.1 | 2.9 | 2.6 | 2.8 | 1.9 |
| | | 3 | 2.8 | 2.0 | — | 1.1 | 3.0 | 2.5 | 2.7 | 2.1 |

TABLE II

EFFECT OF LINOLEIC ACID AND PALMITIC ACID ON ADP:O RATIO AND RESPIRATORY CONTROL

See Fig. 2 and Table I for details. Cells 5 days on experimental media. R.C. stands for respiratory control.

| *Substrates for mitochondria* | *Serum* | | *Albumin* | | *Linoleate* | | *Palmitate* | | *Linoleate + palmitate* | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *ADP:O* | *R.C.* | *ADP:O* | *R.C.* | *ADP:O* | *R.C.* | *ADP:O* | *R.C.* | *ADP:O* | *R.C.* |
| Succinate | 1.7 | 2.9 | 1.6 | 1.5 | 1.7 | 2.4 | 1.7 | 1.8 | 1.6 | 2.2 |
| α-Ketoglutarate | 3.1 | 2.4 | — | 1.1 | 2.8 | 3.1 | 0.7 | 1.5 | 2.4 | 2.4 |
| β-Hydroxybutyrate | 2.4 | 2.4 | — | 1.1 | 2.5 | 3.0 | 0.5 | 1.2 | 2.1 | 2.6 |
| | 2.4 | 3.1 | 0.7 | 1.4 | 2.3 | 2.6 | — | 1.1 | 2.4 | 2.8 |

Linoleate appeared to be necessary for normal mitochondrial function although it could not support beating. The palmitate–linoleate medium supported beating, but it could not be concluded from these results that palmitate by itself would maintain beating.

*Linoleate and palmitate*

In another experiment, a similar series was used with the addition of an albumin–palmitate group. Here again the beating was best supported in the serum group (Fig. 2). It was also well maintained in the albumin–palmitate and albumin–palmitate–linoleate groups but decreased rapidly and stopped on the 5th and 6th days in the albumin and albumin–linoleate groups. Growth occurred only in complete growth medium (Fig. 3).

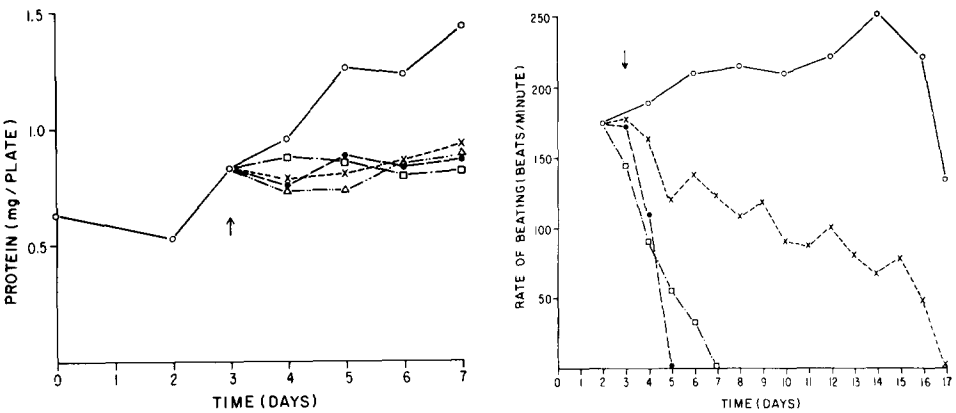


Fig. 3. Effect of linoleic acid and palmitic acid on growth. See Fig. 2 for details.

Fig. 4. Effect of linoleic acid and palmitic acid on long-term beating. See Fig. 2 for details.

The ADP:O ratio and the respiratory control responded in the same way as in the first experiment (Table II) with succinate as a substrate for the mitochondrial function. The respiratory control decreased in the albumin and the albumin–palmitate groups but the ADP:O ratio remained normal. Using α-ketoglutarate and β-hydroxybutyrate as substrates both the ADP:O ratio and respiratory control values decreased. Linoleate seems to be involved in maintaining a phosphorylation step unique in the oxidation of α-ketoglutarate and β-hydroxybutyrate but not in the oxidation of succinate. Palmitate supported beating despite its inability to maintain a high respiratory control and ADP:O ratio, while linoleate could not support beating despite its ability to maintain these parameters.

*Longer incubation period*

In the next experiment it was seen that these observations could be repeated for cells kept for a longer period of time. After 17 days, cells incubated on complete growth medium had not stopped beating (Fig. 4) while those in the palmitate group stopped on the 17th day. The cells in the groups lacking palmitate stopped beating between the 5th and 7th day.

The respiratory control and the ADP:O ratios were measured on the 5th and

TABLE III

EFFECT OF LINOLEIC ACID AND PALMITIC ACID ON ADP:O RATIO AND RESPIRATORY CONTROL IN A LONG-TERM INCUBATION

See Fig. 2 and Table I for details. Cells 5 and 14 days on experimental media. R.C. stands for respiratory control.

| *Days in culture* | *Substrates for mitochondria* | *Albumin* | | *Linoleate* | | *Palmitate* | |
|---|---|---|---|---|---|---|---|
| | | *ADP:O* | *R.C.* | *ADP:O* | *R.C.* | *ADP:O* | *R.C.* |
| 5 | Succinate | — | 1.1 | 1.6 | 2.2 | 1.7 | 1.3 |
| 14 | | — | 1.1 | 1.6 | 2.5 | 1.5 | 1.4 |
| 5 | α-Ketoglutarate | 1.2 | 1.5 | 3.1 | 2.8 | — | 1.1 |
| 14 | | — | 1.1 | 3.2 | 2.2 | 0.9 | 1.3 |
| 5 | β-Hydroxybutyrate | — | 1.1 | 2.5 | 3.0 | 1.0 | 1.6 |
| 14 | | — | 1.1 | 2.4 | 2.8 | — | 1.1 |

TABLE IV

EFFECT OF ARACHIDONIC ACID ON ADP:O RATIO AND RESPIRATORY CONTROL

See Fig. 5 and Table I for details. Cells 5 days on experimental media. R.C. stands for respiratory control.

| *Substrates for mitochondria* | *Serum* | | *Albumin* | | *Arachidonate* | |
|---|---|---|---|---|---|---|
| | *ADP:O* | *R.C.* | *ADP:O* | *R.C.* | *ADP:O* | *R.C.* |
| Succinate | 1.6 | 2.2 | 1.7 | 1.3 | 1.7 | 1.9 |
| | 1.7 | 2.0 | — | 1.1 | 1.6 | 2.1 |
| α-Ketoglutarate | 3.1 | 2.3 | 2.4 | 1.4 | 3.1 | 2.1 |
| | 2.8 | 1.9 | — | 1.1 | 3.0 | 2.2 |
| β-Hydroxybutyrate | 2.3 | 2.6 | 1.1 | 1.5 | 2.2 | 2.3 |
| | 2.2 | 2.2 | — | 1.1 | 2.2 | 1.9 |

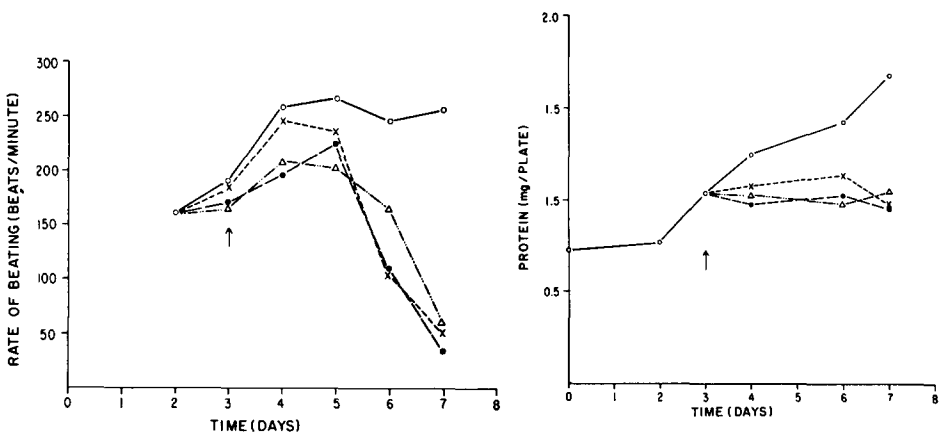


Fig. 5. Effect of arachidonic acid on beating: O—O, complete growth medium; ●—●, basal medium *plus* $5 \cdot 10^{-7}$ M albumin; ×—×, basal medium *plus* $10^{-7}$ M arachidonic acid; △—△, basal medium *plus* $10^{-6}$ M arachidonic acid. At the arrow complete growth medium was substituted by the experimental medium or by fresh complete growth medium for the control.

Fig. 6. Effect of arachidonic acid on growth. See Fig. 5 for details.

on the 17th day in culture (Table III). Here again the ADP:O ratio decreased in the albumin and the albumin–palmitate groups but only when α-ketoglutarate and β-hydroxybutyrate were used as substrates for respiration. The respiratory control decreased in these groups independent of which substrate was used. It was thereby clearly shown that the beating was related to the presence of palmitate, itself, and not to linoleate or the preservation of mitochondrial phosphorylation.

*Arachidonate*

Although it has been shown that linoleate can be converted to arachidonate in these heart cells[15], it was of interest to determine whether arachidonate could maintain mitochondrial function and beating. Arachidonate at $10^{-6}$ M and $10^{-7}$ M was indistinguishable from the albumin controls in so far as the beating was concerned. The rate decreased from 250 to 50 beats/min. Cells on complete growth medium maintained the rate (Fig. 5). There was no growth except in the complete growth medium group (Fig. 6). The results with arachidonate were similar to those with linoleate (Table IV) in that it maintained both the respiratory control and the ADP:O ratio but not the beating.

TABLE V

EFFECT OF THE DEFICIENCY OF ESSENTIAL FATTY ACIDS ON THE FATTY ACID COMPOSITION

The concentrations used were: Albumin $5 \cdot 10^{-7}$ M and linoleic acid $10^{-6}$ M. The results are expressed as total lipids percentage of methyl ester in measurable peaks. See text for details.

| *Fatty acids** | *2 days incubation* | | | *4 days incubation* | | |
|---|---|---|---|---|---|---|
| | *Serum* | *Albumin* | *Linoleate* | *Serum* | *Albumin* | *Linoleate* |
| 16:0 | 17.0 | 26.0 | 32.0 | 20.0 | 44.0 | 28.0 |
| 16:1 | 3.5 | — | 0.2 | 0.5 | 1.1 | 0.3 |
| 18:0 | 13.0 | 21.0 | 20.0 | 18.0 | 32.0 | 19.0 |
| 18:1 | 12.0 | 13.0 | 12.0 | 15.0 | 11.0 | 12.0 |
| 18:2 | 7.3 | 8.5 | 6.9 | 9.1 | 0.5 | 8.3 |
| 18:3 | 0.5 | — | 0.2 | 0.4 | 0.4 | 0.5 |
| 20:3 | 0.8 | 0.8 | 0.1 | 1.5 | — | 1.2 |
| 20:4 | 12.0 | 19.0 | 15.0 | 18.0 | 2.2 | 9.7 |

* Carbon number: double bond.

*Fatty acid analysis*

Three groups of cells were analyzed for their fatty acid content; one group grown on complete growth medium, the second on albumin and third on albumin and linoleate. At the end of 2 days the albumin and albumin–linoleate groups showed similar changes as compared with the complete growth medium group. There was an increase in 16:0, 18:0 and 20:4 and a decrease in the relative amount of 16:1. These changes were apparently not related to the presence or absence of linoleate.

On the 4th day, however, changes occurred which differed from those on the 2nd day. The albumin–linoleate and complete growth medium groups showed similar patterns except for a difference in 20:4. The albumin group differed markedly from both the albumin–linoleate and serum groups. There was an increase in 16:0, 16:1, 18:0 and a decrease of 18:2, 20:3 and 20:4. Arachidonate (20:4) and linoleate (18:2) exhibited the most marked decreases in the albumin medium which, of course, was lipid deficient.

## DISCUSSION

Respiratory control is thought to be associated with an intact and functional mitochondrial membrane[16–18]. The decrease in this function, brought about by a lack of essential fatty acids, may be a reflection of an impaired membrane. No direct evidence for this hypothesis has been presented. It depends upon the assumption that the main role of the unsaturated fatty acids in mitochondria is for membrane integrity, and the observations that conditions that affect membrane integrity also affect respiratory control. However, whatever other effect it may have, essential fatty acid deficiency does not affect long-term beating.

The observation that mitochondrial function may be impaired without seriously affecting the beating and also that maintenance of mitochondrial function is not sufficient for the maintenance of beating, experimentally separates these two functions. That reduced mitochondrial formation of ATP does not affect the maintenance of beating supports the previous conclusion that heart cells can beat with ATP formed from glycolysis as well. An impairment of mitochondrial function of itself would not affect the beating, if sufficient energy from other sources were available.

If we postulate, on the other hand, that palmitate is important as an energy source, we must consider that the loss of fatty acid oxidation could affect the cell only from a quantitative viewpoint. The lack of palmitate should, according to this hypothesis, lead to a depletion of energy reserves and an ATP level too low for beating. This could be the case if the complete lack of fatty acid as an energy source not only eliminated it as a source of ATP but led to a depletion of ATP from the other sources such as from glycolysis. If we consider that palmitate is important as an energy source, the question arises as to why it is that linoleate added to cells with intact mitochondria is not effective in maintaining beating.

Studies carried out in several laboratories indicate that linoleate is oxidized as efficiently as palmitate and to the same products[19,20]. Differences in their effects should therefore be sought in some other function. A possibility exists that despite measures to protect against peroxidation, some linoleate peroxide could be formed before or during incubation to exhibit its well-known toxic effect. It has been shown that in HeLa cells, the range of concentration of arachidonic and linoleic acids needed for growth is very narrow[4] and that above this range growth is severely inhibited. Previous observations in this laboratory[1] have indicated that linoleate may partially restore beating in quiescent cells for a short period of time, but cannot maintain the beating for longer periods.

Another possible explanation may lie in the removal of linoleate as an energy source for preservation as an essential membrane or other organelle constituent. Although, as documented above, this is unlikely for linoleate itself, its conversion to arachidonate, a pathway shown to occur in these cells[15], might remove it from the site of oxidation. It has been shown by COLLINS[21] and others that arachidonic acid is preserved from catabolism in its function in certain phospholipids and, indeed, that the turnover rate of those phospholipids in which it is present, is lower.

Palmitate may, however, be involved in supporting beating in another capacity. Accompanying the loss of beating, in a lipid-deficient medium, is a change in the synthesis of certain enzymes. Malic and isocitric dehydrogenases, creatine kinase, calcium-activated adenosine triphosphatase decrease and glucose-6-phosphate de-

hydrogenase increases[22]. These changes are partially prevented or reversed by inclusion of serum lipids in the medium[23]. One may speculate that palmitate somehow affects protein synthesis. Perhaps it serves as a precursor for compounds that act as regulators of the kind of enzymes synthesized in the specialized cell.

Whatever the mechanism, fatty acids seem somehow to be involved in heart cell maintenance although the evidence presented here would indicate that their involvement is not through their function in mitochondrial membranes.

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