EFFECTS OF CORTISOL ON CULTURED RAT HEART CELLS

Lipase Activity, Fatty Acid Oxidation, Glycogen Metabolism, and ATP Levels as Related to the Beating Phenomenon

J. V. ANASTASIA and R. L. McCARL

From the Department of Biochemistry, Paul M. Althouse Laboratory, The Pennsylvania State University, University Park, Pennsylvania 16802. Dr. Anastasia's present address is Procter and Gamble Co., Miami Valley Labs, Cincinnati, Ohio 45239.

ABSTRACT

This paper reports the determination of the ability of rat heart cells in culture to release [^{14}C]palmitate from its triglyceride and to oxidize this fatty acid and free [^{14}C]palmitate to $^{14}CO_2$ when the cells are actively beating and when they stop beating after aging in culture. In addition, the levels of glucose, glycogen, and ATP were determined to relate the concentration of these metabolites with beating and with cessation of beating. When young rat heart cells in culture are actively beating, they oxidize free fatty acids at a rate parallel with cellular ATP production. Both fatty acid oxidation and ATP production remain constant while the cells continue to beat. Furthermore, glucose is removed from the growth medium by the cells and stored as glycogen. When cultured cells stop beating, a decrease is seen in their ability to oxidize free fatty acids and to release them from their corresponding triglycerides. Concomitant with decreased fatty acid oxidation is a decrease in cellular levels of ATP until beating ceases. Midway between initiation of cultures and cessation of beating the cells begin to mobilize the stored glycogen. When the growth medium is supplemented with cortisol acetate and given to cultures which have ceased to beat, reinitiation of beating occurs. Furthermore, all decreases previously observed in ATP levels, fatty acid oxidation, and esterase activity are restored.

INTRODUCTION

In 1960 Harary and his associates (1) cultured cells from young rat hearts which retained their ability to "beat" in culture. The beating of these rat heart cells could be observed under a microscope, allowing one to study directly the effects of both chemical substances and physical conditions on a primary physiological event. The culturing of heart cells thus provides a system for the study of beating function as related to the environment and biochemical processes of the cell.

After a period of time, these cells in culture cease to beat. Changes in various biochemical reactions and processes are associated with this cessation of beating. Fujimoto and Harary (2) reported a shift from lipid to carbohydrate metabolism occurring as the cells age and stop beating. Also taking place during this time is a decrease in the enzymatic activities of malic and isocitrate dehydrogenase and an increase in glucose-6-phosphate dehydrogenase (3). Other experiments (4) have shown that heart cell cultures grown on lipid-deficient medium stop beat-

ing in a few days although this medium maintains growth as measured by an increase in protein content. In these experiments, beating could be restored by adding complete growth medium. When serum lipids or certain fatty acids were added to the lipid-deficient medium, there was also a restoration in beating although in the case of fatty acids the effect was transient. From the results of these experiments, it would seem that the actively beating cultures use various lipids to maintain their ability to beat and supplement energy requirements with carbohydrate metabolism. Cessation of beating with age would appear then to be correlated with loss or decrease in lipid metabolism.

It has been shown with isolated perfused heart techniques that fatty acids supply 80% of the energy utilized by the heart (5). No doubt the primary role of fatty acids is to serve as the specific ATP producer from their oxidation but no relationship has yet been made between ATP production, potential for fatty acid oxidation, and beating. The cultured rat heart system is amenable to this kind of study and it mimics the in vivo system. A knowledge of this relationship in in vitro cells would be basic to understand the energy requirements of beating in vivo. Furthermore, cultured heart cells stop beating with age and can be restimulated to beat with cortisol acetate (6). This allows one to study age-related changes in the cells and may give an insight into how these changes can be prevented or reversed. Therefore, the object of this present study was to determine the activity of the enzymes for fatty acid oxidation, ATP production, mitochondrial condition, and potential for ATP production in cultured rat heart cells. In addition, a further object of this work was to determine the effect of cortisol on lipid metabolism in cells which had been reinitiated to beat after they stopped beating with age.

MATERIALS AND METHODS

Method of Heart Culture

Hearts from 1- to 3-day old rats were dissociated for culturing by trypsinization. The tissue was cultured in 60 × 15 mm plastic Petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) and grown at 37°C in a Hotpack CO_2 incubator (Hotpack Corp., Philadelphia, Pa.) under high humidity and 5% CO_2-95% air. The growth medium and culture procedure were as described by McCarl and Margossian (3), which are modifications of the procedures used by Harary and Farley (7). Supplies for complete growth medium (CGM) were obtained from Grand Island Biological Co., Grand Island, N. Y. Each plate in each experiment was inoculated with equal amounts of the cellular suspension of enzymatically dissociated heart tissue. Variation may occur in total amount of protein per plate in different experiments because of age and size of animals used.

Protein Determination

The measurement of protein was performed according to the procedure of Lowry et al. (8) as modified by Oyama and Eagle (9).

Preparation of Cultured Cells for the Measurement of Fatty Acid Oxidation and Esterase Activity

The cells were inoculated at a concentration of 1 heart per plate, i.e., if 50 animals were killed there would be 50 Petri dishes available for the experiment. The medium was changed every other day and assays were made 12 h after the last medium change. Plates were removed from the incubator and the cells on the plates were evaluated for beating. This was done by randomly looking at 20 areas on each plate. The beating is reported as a percentage of the 20 areas observed which contained beating cells or beating groups of cells. The medium from the plates was decanted and the cells on the plate were washed several times with a solution 250 mM in sucrose and 1 mM in EDTA. The cells were scraped off the plates with a rubber policemen and washed into centrifuge tubes with the above sucrose-EDTA solution. The cell suspension was then centrifuged at 5,000 *g* for 10 min in a Sorvall RC-2 centrifuge (Ivan Sorvall, Inc., Newtown, Conn.). The cell pellet was brought up to 3 ml with the above solution and homogenized in a Dounce homogenizer (Bellco Glass, Inc., Vineland, N. J.). A total of 5 strokes of the pestle were made to yield a uniform cell homogenate which could be pipetted reproducibly. Portions of this homogenate were used to assay for the activity of fatty acid oxidation. All the above procedures were performed at 1–4°C.

Assay for Palmitate Oxidation

The assay of palmitate oxidation was performed in 50-ml Erlenmeyer flasks fitted with rubber serum stoppers. Located in the bottom center of these flasks was a well containing 200 μl of 2 N KOH. The reaction was started with the injection of the homogenate. Subsequent oxidation of [1-^{14}C]palmitate was measured in 2.5 ml of medium containing 0.50–2.5 mg protein, 100 mM sucrose, 10 mM sodium phosphate, pH 7.4, 0.1 mM [1-^{14}C]palmitate, 0.02 mM fatty

acid-poor albumin, 2 mM ATP, 0.05 mM CoA, 1 mM nicotinamide adenine dinucleotide, oxidized form (NAD^+), 0.04 mM $MgCl_2$, 0.1 mM EDTA, and 80 mM KCl (10). Carnitine at a concentration of 1 mM was added as indicated. Duplicate incubations were made in a Dubnoff shaker (Precision Scientific Co., Chicago, Ill.) for 30 min at 37°C with mild agitation. The reaction was stopped by the addition of 1 ml of 4 N H_2SO_4 through the serum cap. The $^{14}CO_2$ was collected for 1 h at 37°C. 100 μl of the KOH from the center well was transferred to 10 ml of scintillation fluid containing dioxane, 1% 2,5-diphenyloxazole (PPO), 10% naphthlene, and 4% Cab-o-sil (Cabot Corporation, Boston, Mass.). Each scintillation vial was assayed for ^{14}C activity in a Beckman scintillation spectrometer LS-200B (Beckman Instruments, Inc., Fullerton, Calif.). A control was run for each experiment in which no homogenate was added.

Esterase Activity in Rat Heart Cells in Culture

Cells were prepared for the measurement of esterase activity in the same manner as they were for the measurement of fatty acid oxidation. The reaction mixture was identical with that used in fatty acid oxidation assays with the exception that 0.1 mM [1-^{14}C]tripalmitin was substituted for [1-^{14}C]palmitate.

Concentration of ATP in Rat Heart Cells in Culture

Cells attached to culture plates were washed with a 250 mM sucrose—1 mM EDTA solution and the wash discarded. Cells were scraped from the plates with 6% $HClO_4$ and homogenized with a TenBroeck homogenizer for at least 20 strokes. The homogenate was then placed in an ice bath and the extraction of ATP was performed for 1 h. The $HClO_4$ was neutralized with 2 N KOH to pH 6.5. The homogenate and the neutralized $HClO_4$ solution was then placed in a boiling water bath for 3 min. Next, it was spun at 5,000 *g* for 10 min at 2°C and the supernatant solution was assayed for ATP by the luciferase method (11, 12).

Measurement of Endogenous Glucose and Glycogen

Cells were washed several times with distilled water and scraped from the plates with a rubber policemen and homogenized in a TenBroeck homogenizer in 2 ml of distilled water. The homogenate was then placed in a boiling water bath for 2 min. After this, it was centrifuged at 10,000 *g* for 10 min. The centrifuged pellet was washed, resuspended, and respun at 10,000 *g* for 10 min. The supernatant solutions were combined and assayed for their content of glucose and glycogen. The pellet was discarded.

For glycogen, 0.5 ml of the above supernatant was added to 0.5 ml of 0.1 M KH_2PO_4 (pH 7.4) in a small test tube. To this solution was added 50 μl of a 2 mg/ml solution of glucohydrolase obtained from Doctors Pazur and Knull, Biochemistry Department, Pennsylvania State University. The test tubes were allowed to remain at room temperature for 2 h. In this time period virtually all glycogen present was converted to glucose. A similar tube was carried through the same procedure, but the solution in this tube was injected with 50 μl of distilled water instead of glucohydrolase. In this way, the initial glucose concentration could be measured and the increase in glucose due to glycogen hydrolysis could be calculated. Glucose was then measured using the standard glucose oxidase:peroxidase:anisidine analysis (13, 14).

Measurement of ADP : O Ratio and Respiratory Control Rates

Measurements of oxidative phosphorylation and respiratory control rates were made according to the method of Ziegler et al. (15). Cells were prepared for assay in the same manner as they were for fatty acid oxidation experiments. The reaction medium consisted of 45 mM mannitol, 15 mM sucrose, 0.02 mM EDTA, 40 mM KCl, 20 mM $MgCl_2$, and 20 mM potassium phosphate buffer, pH 7.4 in 2.5 ml.

RESULTS

The ability of rat heart cells in culture to oxidize [1-^{14}C]palmitate to $^{14}CO_2$ and H_2O was determined on cells that had been in culture for various periods of time and which had also a variation in the number of areas observed to be beating. The ability of the cultured rat heart cell homogenates to oxidize [1-^{14}C]palmitate under optimal conditions (10) is shown in Fig. 1. The optimal conditions used are those described by Passeron et al. (10), using rat heart homogenates. Several of these conditions were evaluated to ascertain if they were also optimal conditions for the cultured system. It was found that all conditions employed in the heart homogenates were also optimal for the cultured system, with the exception of DL-carnitine: Passeron et al. reported a 30-fold increase in the rate of oxidation with added DL-carnitine, whereas the cultured system showed only a three- to fourfold increase.

As can be seen in Fig. 1, the ability of cultured rat heart cell homogenates to oxidize [1-^{14}C] palmitate to $^{14}CO_2$ decreased sharply during the first 48 h in culture. After this time, the cells

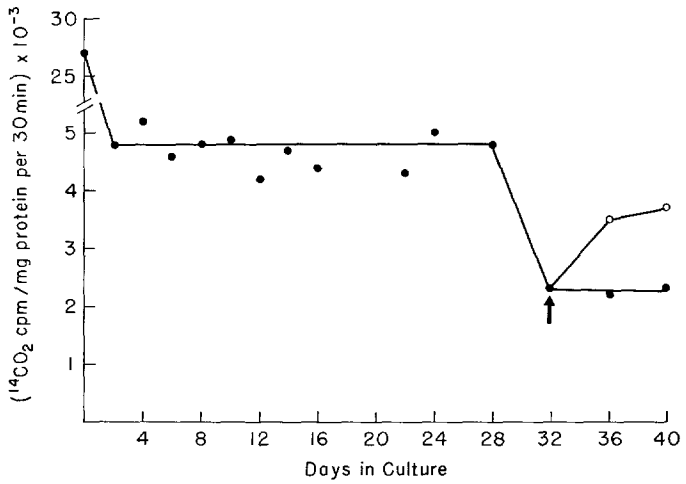

FIGURE 1 -●-●-, cells grown on CGM; -○-○-○-, cells treated with 10^{-6} M cortisol acetate. The effect of aging on the ability of rat heart cells in culture to oxidize [1-^{14}C]palmitate to $^{14}CO_2$. The conditions employed here are as described in the Materials and Methods section. Cortisol acetate was added (arrow) at least 24 h after the cells had stopped beating. Each point on this curve represents the average of duplicate incubations for one culture. No one determination differed by more than 7% from the average.

maintained a fairly constant rate of [1-^{14}C] palmitate oxidation until the 28th day in culture. This constant rate is equivalent to 4,800 cpm of $^{14}CO_2$ per mg of protein for 30 min or 0.29 nmol of palmitate oxidized per mg of protein for 30 min. This value of palmitate oxidation agrees with the values reported by Wittels and Bressler in work with whole hearts (16). When DL-carnitine (1 mM) was added to the incubation mixture, the amount of $^{14}CO_2$ produced from [1-^{14}C]palmitate increased three- to fourfold. This increased rate of oxidation is approximately 1 nmol of palmitate oxidized per mg of protein for 30 min.

When cells had aged in culture and beating had ceased completely on day 32, the rate of oxidation dropped from 4,800 cpm of $^{14}CO_2$ per mg of protein for 30 min to a value of 2,300 cpm of $^{14}CO_2$ per mg of protein for 30 min, a 52% decrease.

The decrease in fatty acid oxidation was reversed with the addition of cortisol acetate to the growth medium. Growth medium made 10^{-6} M in cortisol acetate was placed on cells which had stopped beating with age. Within 36 h these treated cells began to beat. When 100% of the areas observed were beating, the rate of fatty acid oxidation was again measured (day 32). The level of oxidation rose from 2,300 cpm to a level of 3,500 cpm of $^{14}CO_2$ per mg of protein for 30 min. The cells which had not received cortisol acetate treatment and hence were not beating maintained their low level of [1-^{14}C]palmitate oxidation.

The ability of cultured rat heart cell homogenates to hydrolyze [1-^{14}C]palmitate by general esterase activity and subsequently oxidize it was investigated also. Presented in Fig. 2 are data for the oxidation of palmitate from released [1-^{14}C]-tripalmitin by heart cells at various times after being cultured. These data show that the ability to release [1-^{14}C]palmitate from [1-^{14}C]tripalmitin decreased rapidly during the first 2 days in culture and then less rapidly but steadily until the cells stopped beating. When the cells ceased to beat, general esterase activity reached its lowest point at 80 cpm of $^{14}CO_2$ per mg of protein for 45 min. When cortisol acetate was added to the cells, the activity rose to 150 cpm of $^{14}CO_2$ per mg of protein for 45 min, an increase of 87.5%. At the same time, the cells in culture had once again started to beat rhythmically.

Fig. 3 represents data from a typical experiment analyzing for ATP in heart cells. Also represented in this figure is the percentage of beating in the heart cells at various times after being cultured. The cells maintained a steady concentration of ATP per milligram of protein while they were actively beating. Except for the fourth day in culture, the concentration of ATP remained around 1.2 nmol per mg of protein. When the cells lost their capacity to beat between days 28 and 32,

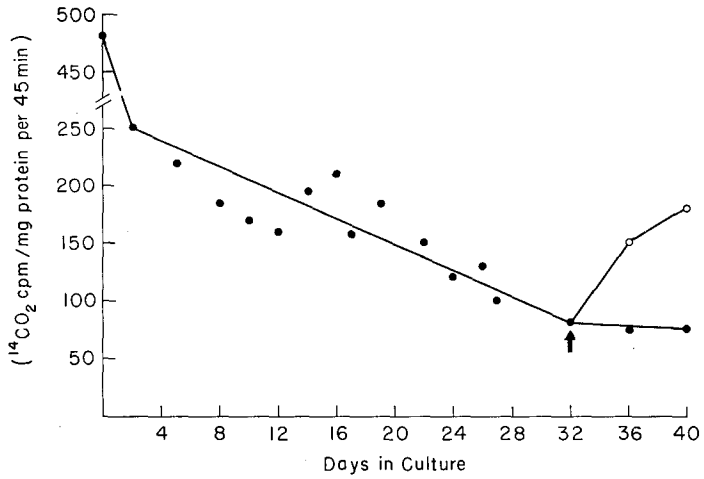

FIGURE 2 -●-●-●-, cells grown on CGM; -○-○-, cells treated with 10^{-6} M cortisol acetate. The effect of aging on the ability of rat heart cells in culture to produce $^{14}CO_2$ from [1-^{14}C]palmitate hydrolyzed from [1-^{14}C]tripalmitin. The conditions employed here are as described in the Materials and Methods section. Cortisol acetate was added (arrow) at least 24 h after the cells had stopped beating. Each point on this curve represents the average of duplicate incubations for one culture. No one determination differed by more than 7% from the average.

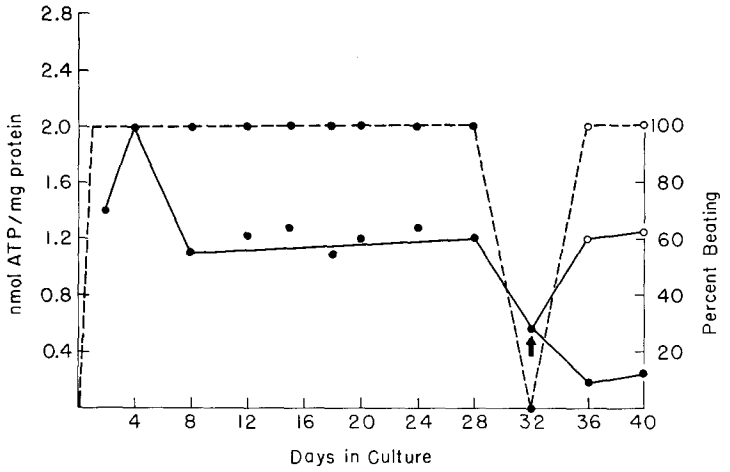

FIGURE 3 - - -, percentage of 20 areas observed to be beating; -●-●-, cells grown on CGM; -○-○-, cells treated with 10^{-6} M cortisol acetate. The effect of age of rat heart cells in culture on their concentration of ATP per milligram of protein. Cortisol acetate was added (arrow) at least 24 h after the cells had stopped beating. Each point on the curve represents the average of duplicate determinations. No one value differed by more than 10% from the average.

the concentration of ATP dropped from 1.2 nmol of ATP per mg of protein to 0.55 nmol of ATP per mg of protein. Furthermore, in cells which had received no cortisol acetate treatment the ATP concentration decreased to a low of 0.19 nmol of ATP per mg of protein by day 36. The remaining cells which did receive cortisol acetate treatment on the 32nd day in culture began to beat 36 h later, and by day 36, 100% of areas observed were beating. The concentration of ATP per milligram of protein in these cells rose to the same value as before they stopped beating, or 1.2 nmol of ATP per mg of protein.

Measurements of the levels of endogenous glu-

TABLE I

The Relationship between the Age of Cells in Culture and Their Endogenous Level of Glucose and Glycogen

Days in culture	Percent beating	μg glucose / mg protein	μg glycogen / mg protein
0	—	72	5
8	100	20	26
12	100	20	42
19	100	17	50
27	100	13	20
32	0	33	24
36	0	50	11

Each value represents two determinations on two different cultures.

TABLE II

The Relationship between the Age of Cells in Culture to Oxidative Phosphorylation and Respiratory Control Ratio

Days in culture	Beating	Respiratory control rate	ADP:O
3	100	2.15	1.6
9	100	2.3	1.79
10	100	2.0	1.92
12	100	2.0	—
16	100	2.1	1.76
17	100	2.7	1.8
20	100	2.2	1.73
24	100	1.5	2.1
36*	0	1.8	3.5
36*, ‡	100	2.0	1.65

Each number represents the average of three determinations unless otherwise indicated. The conditions employed are as described in the Materials and Methods section.

* Represents only one measurement in duplicate.

‡ Cells receiving cortisol acetate treatment.

cose and glycogen in cultured rat heart cells were made at various times after the cells were cultured. Table I presents these data. As rat heart cells aged in culture, they stored glycogen and maintained a fairly constant amount of free glucose until day 19 when the level of glycogen began to decrease. On days 32 and 36 when the cells had stopped beating, the level of glucose rose while the level of glycogen continued to fall.

The ADP:O ratio and the respiratory control rate are considered to be an indication of the integrity of the mitochondrial membrane to carry out oxidative phosphorylation (17, 18). For the analysis of these ratios, succinate was used as the substrate. Presented in Table II are the data for ADP:O ratios and respiratory control rates on rat heart cells at various times after being placed in culture. It can be seen that the ADP:O ratio for succinate oxidation is within the theoretical value throughout all ages of the cells in culture which were actively beating. When the cells ceased to beat, the ADP:O ratio for succinate increased to 3.5. However, upon reinitiation of beating with cortisol acetate, this value returned to 1.65.

DISCUSSION

This research was designed to answer several questions concerning the relationship between beating in rat heart cells in culture and lipid metabolism. Interest in lipid metabolism, specifically fatty acid oxidation in cultured rat heart cells, was stimulated by several experimental results. Previous experiments had shown that lipids played an important role in the beating phenomenon of cultured rat heart cells (3–5). Furthermore, it had been shown that as heart cells age in culture and eventually stop beating, they lose their ability to oxidize palmitate and their metabolism shifts from lipids to carbohydrates (2).

Fig. 1 represents the oxidation of [1-^{14}C]palmitate to $^{14}CO_2$ by the cultured rat heart cells under optimal conditions. These data indicate that culturing of heart tissue affects palmitate oxidation. The decrease in the ability of rat heart cells in culture to oxidize palmitate may be either a direct effect of culturing on the cells or selection in the population of cells in culture. After the cells attached and began to grow, they maintained a constant rate of palmitate oxidation. During the time when the rate of palmitate oxidation remained constant, there was also a fairly constant level of ATP in the cells. From day 2 until day 28, all areas observed were beating. Since it was previously shown that fatty acid oxidation was the primary source of energy for the heart (5), it would seem that this rate of oxidation was necessary to maintain the ATP at a concentration required for contraction.

The ability of cultured rat heart cells to perform oxidative phosphorylation is indicated in Table II. When cells were actively beating, the theoretical ADP:O ratio for succinate was maintained. Also, the cells showed a good respiratory control rate, indicating that the oxidative phosphorylation

system was stimulated by ADP to form ATP. When rat heart cells in culture were actively beating, they could oxidize fatty acids for the production of ATP via oxidative phosphorylation. An explanation for the increase in the ADP:O ratio from 2.1 to 3.5 when the cells stopped beating at day 36 will be given later.

As cells aged in culture and were actively beating, the level of glycogen increased. Evidently these cells have some control mechanism whereby they oxidize fatty acids and store glucose as glycogen, and the cessation of beating could result from the loss of this control and not from a loss of the contractile machinery. Furthermore, Cox observed a reinitiation of beating in cells that had stopped beating with age upon addition of ATP.[1] Free fatty acids and ATP selectively inhibit some of the key enzymes involved in the metabolism of glucose in liver tissue (19). Loss of control in these cultured cells could involve a combination of low ATP levels and low fatty acid concentration. In order to explore this possibility, experiments were designed to measure the ability of cultured cells to release fatty acids from triglycerides and to measure ATP levels in the cells. Data in Fig. 2 indicate that the ability of the heart cells in culture to release and oxidize palmitate from tripalmitin decreased as the cells aged in culture. Such a decrease in esterase activity could be harmful to cells if the free fatty acid concentration falls below a level necessary to help regulate enzymes of hydrolysis. Other work in our laboratory[2] has indicated that the free fatty acid pools in the cultured heart cells decreased with age. This supports our present finding of decreased esterase activity with age.

Data from Fig. 3 show that on day 28 the level of ATP was 1.2 nmol of ATP per mg of protein. However, when the fatty acid oxidation multienzyme complex showed a 55% decrease in its ability to oxidize palmitate (Fig. 1), there was also a drastic drop in the concentration of ATP in the cells. On day 32, the concentration fell to 0.55 nmol of ATP per mg of protein and by day 36 fell to a low of 0.19 nmol. Changes in the activities of both esterase (Fig. 2) and the multienzyme complex (Fig. 1) caused the heart cells to stop beating. At this time the cells are apparently using carbohydrates as their major energy source. However, the energy produced from carbohydrate metabolism is not sufficient to maintain the high levels of ATP necessary for contraction and the other vital biochemical processes.

Data in Table II support the fact that cells shift their metabolism to carbohydrate catabolism when they stop beating. The ADP:O ratios were determined on cellular homogenates and not on isolated mitochondria. Therefore, the enzymes for glycolysis (i.e., substrate level phosphorylation) were present. When the cells stopped beating around day 28–36, the ADP:O ratio for succinate increased from 2.1 to 3.5. No substrate level phosphorylation occurs in fatty acid oxidation but does when glucose is metabolized via glycolysis. Substrate level phosphorylation occurs without the subsequent uptake of O_2 and although the added ADP would be phosphorylated to ATP there would be less O_2 used in the process. When the amount of ADP added is divided by the amount of oxygen used to phosphorylate it to ATP, the ratio would be high.

Thus, when the cells stopped beating the following occurred: (*a*) the ability to oxidize [1-^{14}C]palmitate decreased 52%. (*b*) The cellular concentrations of ATP dropped drastically. (*c*) Glycogen was mobilized and the overall content of glycogen decreased. (*d*) The ability of the cells to release palmitate from tripalmitin decreased fivefold compared with its initial activity. (*e*) ADP:O ratios indicated that there was a greater capacity for the cells to carry out substrate level phosphorylation (glycolysis). The summation of these events caused the cells to lose their ability to beat.

Cortisol acetate has the effect of reinitiating beating in rat heart cells in culture that had previously stopped beating (6). The mode of action of cortisol acetate on this process is not known although this compound has been shown to affect certain cellular enzymes (3). It is also known to affect the lipase activity in adipose tissue in vitro (20). The effect of cortisol acetate on the ability of rat heart cells in culture to oxidize [1-^{14}C]palmitate can be seen in Fig. 1. On the 32nd day in culture, the growth medium was supplemented with 10^{-6} M cortisol acetate. By day 36, all areas observed were beating. There was also an increase in the ability of these beating cells to oxidize palmitate. Cells which did not receive cortisol acetate treatment and hence were not beating maintained their low value of palmitate oxidation. This increased capacity to oxidize fatty acids resulted in an increased concentration of ATP. Energy was then available to the cell for rhythmic

[1] Cox, F. Personal communication.

[2] Swenson, P. Personal communication.

contraction. Table II shows that the ADP:O ratio approached the theoretical value of 2. Since the cells now have the capacity to oxidize fatty acids, they have reshifted their metabolism back to fatty acid catabolism. This could account for ATP production via oxidative phosphorylation as opposed to substrate level phosphorylation from carbohydrate catabolism. Cortisol acetate also has an effect on the activity of esterase. When cells were reinitiated to beat, the activity of esterase rose 88% over its low value when cells were not beating.

The data on the reinitiation by cortisol acetate indicate that cortisol acetate causes cells to switch their metabolism back to fatty acid oxidation. This oxidation now provides the necessary concentration of ATP for contraction. Furthermore, the increase in the ability of the cells to release free fatty acids from triglycerides may provide the necessary substrate levels not only for oxidation but also to regulate key enzymes in carbohydrate metabolism.

This work was supported in part by United States Public Health Service grant no. HL-10018 and Pennsylvania State University Agricultural Experiment Station grant no. 1858.

Authorized for publication as paper no. 4280 in the journal series of the Agricultural Experiment Station.

Received for publication 26 July 1972, and in revised form 11 December 1972.

REFERENCES

1. HARARY, I., and B. FARLEY. 1960. *In vitro* studies of single isolated beating heart cells. *Science (Wash. D. C.).* **131**:1674.
2. FUJIMOTO, A., and I. HARARY. 1964. Studies *in vitro* on single beating rat heart cells. IV. The shift from fat to carbohydrate metabolism in culture. *Biochim. Biophys. Acta.* **86**:74.
3. MCCARL, R. L., and S. S. MARGOSSIAN. 1969. Restoration of beating and enzymatic response of cultured rat heart cells to cortisol acetate. *Arch. Biochem. Biophys.* **130**:321.
4. HARARY, I., R. L. MCCARL, and B. FARLEY. 1966. Studies *in vitro* of single beating rat heart cells. IX. The restoration of beating by serum lipids and fatty acids. *Biochim. Biophys. Acta.* **115**:15.
5. BING, R. J., A. SIEGEL, I. UNGAR, and M. GILBERT. 1954. Metabolism of the human heart. II. Studies on fat, ketone and amino acid metabolism. *Am. J. Med.* **16**:504.
6. MCCARL, R. L., B. F. SZUHAJ, and R. T. HOULIHAN. 1965. Steroid stimulation of beating of cultured rat heart cells. *Science (Wash. D. C.).* **150**:1611.
7. HARARY, I., and B. FARLEY. 1963. *In vitro* studies on single beating rat heart cells. I. Growth and organization. *Exp. Cell Res.* **29**:451.
8. LOWRY, O. H., N. J. ROSENBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* **193**:265.
9. OYAMA, V. I., and H. EAGLE. 1956. Measurement of cell growth in tissue culture with phenol reagent (folin-ciocalteau). *Proc. Soc. Exp. Biol. Med.* **91**:305.
10. PASSERON, S., M. A. SAVAGEAU, and I. HARARY. 1968. Optimal conditions for palmitate oxidation by rat heart homogenates. *Arch. Biochem. Biophys.* **128**:124.
11. STANLEY, P. E., and S. G. WILLIAMS. 1969. Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. *Anal. Biochem.* **29**:381.
12. ROBERTON, A. M., and R. S. WOLFE. 1970. Adenosine triphosphate pools in *Methanobacterium. J. Bacteriol.* **102**:43.
13. KESTON, A. S. 1956. Specific colorimetric enzymatic analytical reagents for glucose. Abstracts of Papers of the 129th Meeting of the American Chemical Society. 31C.
14. TELLER, J. D. 1956. Direct quantitative colorimetric determination of serum or plasma glucose. Abstracts of Papers of the 130th Meeting of the American Chemical Society. 69C.
15. ZIEGLER, F. D., E. H. STRICKLAND, and A. ANTHONY. 1963. Polarographic studies of energy metabolism in rat liver homogenates. *Am. J. Physiol.* **205**:241.
16. WITTELS, B., and R. BRESSLER. 1965. Lipid metabolism in the newborn heart. *J. Clin. Invest.* **44**:1639.
17. CHANCE, B. 1959. Quantitative aspects of the control of oxygen utilization. *Regul. Cell Metab. Ciba Found. Symp.* 91.
18. KLINGENBERG, M., and T. BUCHER. 1960. Biological oxidations—"classical" dehydrogenases. *Annu. Rev. Biochem.* **29**:669.
19. WEBER, G., M. A. LEA, and N. B. STAMM. 1968. Sequential feedback inhibition and regulation of liver carbohydrate metabolism through control of enzyme activity. *Adv. Enzyme Regul.* **6**:101.
20. STAFFORD, W. L. 1967. Effects of cortisol and of actinomycin D *in vitro* on the hormone-sensitive lipase of adipose tissue. *Biochem. J.* **103**:75P.