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ADENYLATE ENERGY CHARGE OF RAT AND HUMAN CULTURED HEPATOCYTES

YOICHI MATSUI, HIROAKI KITADE, TOMOO KAMIYA, TOSHIKI KANEMAKI, YOSHIFUMI HIRAMATSU, TADAYOSHI OKUMURA, AND YASUO KAMIYAMA

First Department of Surgery, and Department of Medical Chemistry (T. O.), Kansai Medical University, 1 Fumizono, Moriguchi, Osaka 570, Japan

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SUMMARY

A simple and rapid method for the assay of adenine nucleotides (ATP, ADP, and AMP) was established to evaluate the adenylate energy charge (ATP+ADP/2)/(ATP+ADP+AMP) of cultured hepatocytes. The effects of inhibitors of glycolysis, fatty acid oxidation, or oxidative phosphorylation on the energy charge were examined. The energy charges of cultured hepatocytes in rats and human were almost identical and were maintained at a high level between 6 and 24 h after changing the media (rat: 0.908 ± 0.008 $n = 9$, human: 0.918 ± 0.014 $n = 6$, mean $\pm$ SD). Inhibition of glycolysis with sodium fluoride or oxidative phosphorylation with antimycin A irreversibly reduced both the adenine nucleotide contents and the energy charge. However, the inhibition of fatty acid oxidation with 2-tetradecylglycidic acid did not affect the nucleotide contents, and the energy charge only decreased transiently to recover within 8 h. When the inhibitor of oxidative phosphorylation was removed, the recovery in the energy charge preceded the recovery in the adenine nucleotide contents. These findings suggest that the adenylate energy charge is a more sensitive measure of the changes in energy metabolism than the adenine nucleotide contents. Furthermore, energy charge regulates adenine nucleotide contents in cultured hepatocytes. It is important to confirm that the high energy charge of the cultured hepatocytes is maintained when these cells are used for metabolic studies.

Key words: adenine nucleotides; adenylate energy charge; antimycin A; sodium fluoride; tetradecylglycidic acid; hepatocyte culture.

INTRODUCTION

Adenosine 5′-triphosphate (ATP) plays a critical role in the regulation and integration of cellular metabolism, and the cellular levels of this nucleotide are considered an index of cellular viability by several investigators (7,20). Various assays have been used to measure ATP in cultured hepatocytes, (e.g., enzymatic technique (19), luciferin-luciferase system (4,16,25) or high-performance liquid chromatography (HPLC) (3,5,8)), although they are all relatively insensitive, complicated, and time-consuming to perform.

Atkinson (1) proposed the concept of adenylate energy charge, designated as (ATP+ADP/2)/(ATP+ADP+AMP), as having an important role in the regulation of many enzymatic reactions and he suggested that the energy charge provides the cells with a sensitive intracellular control mechanism. Sequences, such as glycolysis and the citrate cycle, that lead to the regeneration of ATP and biosynthetic sequences that lead to the utilization of ATP are regulated by the energy charge. We have employed this parameter for many in vivo studies on liver tissue (13–15,21,22,24) because of its importance in metabolic regulation.

However, most studies that evaluated the energy state of hepatocytes in vitro have only measured the amount of ATP (3–5,8,16,25), and rarely has the concept of energy charge been applied (19). Thus the measurement of the adenylate energy charge of cultured hepatocytes to evaluate changes in energy metabolism has not yet been firmly established.

We have developed a rapid and simple assay for the selective analysis of ATP, ADP, and AMP in cultured hepatocytes. Using this method, the effects of inhibitors of the main energy production pathways on the energy charge were analyzed to determine whether this parameter is applicable to cultured hepatocytes as it is with in vivo liver tissues.

MATERIALS AND METHODS

Culture of rat hepatocytes. Male Wister rats (6 to 8 wk old, 200 to 280 *g*) fed ad libitum were used. Hepatocytes were isolated by the method of Seglen (26) with minor modifications (17). Rats were anesthetized with sodium pentobarbital and the liver was initially perfused in situ with a 0.025% collagenase solution (Collagenase-Yakult; Streptomyces sp. C-51, 500 U/mg protein, Sanko Junyaku, Tokyo, Japan) for 5 to 10 min. Hepatocytes were then isolated from the digested liver as reported previously (23). The isolated hepatocytes, showing more than 80% viability by trypan blue exclusion, were suspended in culture medium at 5×10^5 viable cells/ml, seeded into plastic dishes (3.5 cm diameter; 9 cm^2, Falcon Plastic, Oxnard, CA) at a density of 1×10^6 viable cells/dish, and cultured in a CO_2 incubator. Williams' culture medium E supplemented with 10% newborn bovine serum, HEPES (5 m*M*), penicillin (100 U/ml), streptomycin (0.1 mg/ml), dexamethasone (10 n*M*), and insulin (10 n*M*) was used. To remove nonattached cells the medium was replaced by fresh serum-free medium

(1.5 ml/dish) 2 h after seeding, and the hepatocytes were cultured for 1 to 5 days with a change of medium every day. To determine the adenine nucleotide contents per cell, the number of cells attached to the dishes was determined for every experiment by counting the cell nuclei by direct microscopic observation with the aid of a ruled slide glass as described by Horiuti et al. (11). Adherent cell monolayers containing 0.8 to 1.1 $\times$ 10^6 attached cells/dish were used for all experiments.

Culture of human hepatocytes. Liver tissue (1.8 *g*) was obtained from a 74-yr-old male gastric cancer patient undergoing gastrectomy after informed consent was obtained. A normal part of the liver, which was resected along with metastatic cancerous tissue, was used. This tissue was histopathologically normal.

Hepatocyte isolation was performed within 1 h after resection according to the microperfusion technique (10) with the following modifications. The tissue was first perfused with a solution containing (in m*M*) 137 NaCl, 5.4 KCl, 0.5 NaH_2PO_4, 0.42 Na_2HPO_4, 10 HEPES, 5 glucose, 25 $NaHCO_3$, and 0.5 EGTA at pH 7.2 to 7.3. The second perfusion was performed with the same buffer containing 5 m*M* $CaCl_2$ and 0.05% of collagenase, but no EGTA. The isolated hepatocytes, showing 76% viability, were seeded onto collagen-coated plastic dishes (3.5 cm diameter; 9 cm^2, Falcon) at a density of 1 $\times$ 10^6 viable cells/dish, and cultured as described for the rat hepatocytes.

Assay of adenine nucleotides. Immediately upon removal of the cultured hepatocytes from the incubator, the dishes were placed on crushed ice and the culture medium was removed. A predetermined volume (0.2 to 2.0 ml) of ice-cold 7% perchloric acid (PCA) was added to each dish, and while still on ice the monolayer of cells was scraped off from one dish as one sample with a stainless steel scraper. Cells were scraped between 0 and 6 min after removal of culture media. In some studies cells were frozen by pipetting 1 ml of liquid nitrogen onto a cell monolayer immediately after removing the culture media. PCA (1 ml) was added just after the liquid nitrogen had completely evaporated and the cells were then scraped. Each sample was transferred with a pipette from a dish to a silicon tube. The samples were homogenized either by homogenizing for 15 s with a Polytron (Biotron Biotrona, Kuessnacht, Switzerland) or by sonicating for 30 s with a Sonifier (model 250 Branson, Danbury, CT). After adjusting the pH to 6.0 to 6.5 with 2 *M* tripotassium phosphate, the samples were centrifuged for 10 min at 10 000 $\times g$. The supernatant was filtered (0.45 μm type 13A Shimadzu Techno Research, Inc., Kyoto, Japan) and stored at $-80°$ C until the assays were performed. The final volume of each sample was measured to obtain the amounts of adenine nucleotides per dish. The final amounts were corrected for the number of cells per dish.

The ATP, ADP, and AMP levels were determined by HPLC according to the method previously reported (9) with some modifications. A reverse-phase column (ODS-M Shimadzu Techno Research, Inc.) was used at a flow rate of 1 ml/min with a buffer of 0.1 *M* ammonium dihydrogen phosphate (pH 6.0) at a column temperature of 50° C. Absorbance was monitored at 254 nm. The injection volume was 40 μl for each sample. A solution containing 33 μ*M* each of ATP, ADP, and AMP (Oriental Yeast Co., Ltd., Tokyo, Japan), prepared with ultrapure water, was used as an external standard.

To examine the effects of homogenization or sonication on the adenine nucleotide contents of the standard solution, 1 ml of this solution was homogenized or sonicated in the same way as described for the sample solution.

To examine the effects of liquid nitrogen on the adenine nucleotide contents of the standard solution, 1 ml of liquid nitrogen was added to 1 ml of the standard solution. After evaporation of the liquid nitrogen, the standard solution was thawed and analyzed.

Metabolic inhibitors. Sodium fluoride, an inhibitor of glycolysis (2), was dissolved in the culture medium. Antimycin A, an inhibitor of mitochondrial oxidative phosphorylation (4,16), and 2-tetradecylglycidic acid, an inhibitor of fatty acid oxidation (12,18,27,28), were dissolved in dimethylsulfoxide. In preliminary experiments the minimum concentrations (*see* Fig. 4 legend) of sodium fluoride and antimycin A that caused an irreversible reduction in the adenine nucleotide content and the energy charge for at least 24 h were determined. The final concentration of 2-tetradecylglycidic acid was more than the minimum required for irreversible inhibition of fatty acid oxidation as previously reported (18,27,28). The 2-tetradecylglycidic acid was generously provided by McNeil Pharmaceutical (Spring House, PA). All other drugs were obtained from Sigma Chemical Co. (St. Louis, MO).

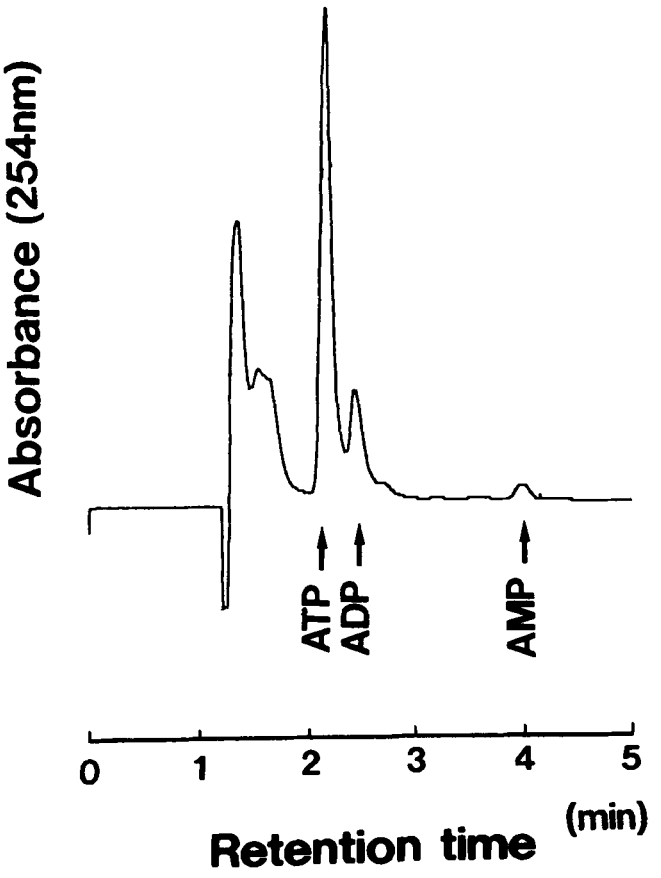

FIG. 1. A high performance liquid chromatogram of adenine nucleotides extracted from cultured rat hepatocytes with 1 ml of 7% PCA. This chromatogram yields the following values: ATP, 21.43; ADP, 5.15; and AMP, 0.82 (nmol/10^6 cells). Adenylate energy charge is 0.876.

Statistics. The significance of differences was analyzed by the unpaired Student's *t* test, and $P < 0.05$ was considered significant.

RESULTS

Chromatogram. A typical profile of the high-performance liquid chromatogram for ATP, ADP, and AMP for a hepatocyte sample is shown in Fig. 1. The three nucleotides were eluted within 5 min in all samples, with ATP eluting first at 2.19 min followed closely by ADP at 2.48 min and then by AMP at 4.05 min. ADP was quantified with respect to ATP by the method of vertical division of peak areas.

Effects of extraction volume. When the adenine nucleotides were extracted in 0.5 ml or less of PCA per dish, the recovery was lower than if 1.0 ml or more were used. One milliliter of PCA was most suitable both for optimal recovery of adenine nucleotides and for the accurate detection of low concentrations of AMP. All subsequent experiments used 1.0 ml of PCA per dish.

Effects of homogenization. Relative to untreated controls, homogenization of the samples significantly reduced the amounts of ATP and ADP recovered, and thus significantly reduced the energy charge (ATP: 21.6 $\pm$ 1.0 to 13.8 $\pm$ 1.3, ADP: 1.85 $\pm$ 0.05 to 1.16 $\pm$ 0.32, AMP: 0.44 $\pm$ 0.06 to 0.53 $\pm$ 0.08 nmol/10^6 cells, energy charge: 0.943 $\pm$ 0.005 to 0.929 $\pm$ 0.007, mean $\pm$ SD of

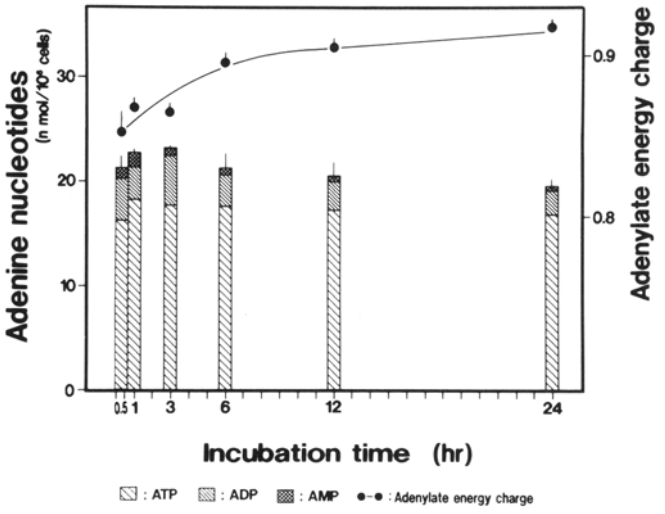

FIG. 2. Changes over time in the adenine nucleotide contents and the adenylate energy charge of cultured rat hepatocytes after a change of media. Data were collected on Day 2 of culture from 9 a.m. to 9 a.m. for hepatocytes plated at a density of 0.9×10^6 cells/dish. Values are expressed as means ± SD for three dishes at each time point.

four dishes for control and homogenized samples, respectively). In contrast, sonication of the sample solutions did not significantly affect the levels of adenine nucleotides or the energy charge relative to controls. Control samples were neither homogenized nor sonicated.

Whereas homogenization reduced the ATP, ADP, and AMP contents of the standard solution to the same degree as seen with the hepatocyte sample solutions, sonication had no effect. Inasmuch as homogenization and sonication were not necessary for optimal extraction of the nucleotides, all samples were extracted with PCA only.

Effects of liquid nitrogen. Liquid nitrogen significantly reduced the ATP content of cultured rat hepatocytes and significantly raised the ADP content. Consequently, the energy charge decreased significantly when compared to controls (ATP: 19.3 ± 1.1 to 17.1 ± 1.2, ADP: 3.24 ± 0.29 to 5.59 ± 0.24, AMP: 0.89 ± 0.08 to 0.91 ± 0.20 nmol/10^6 cells, energy charge: 0.893 ± 0.002 to 0.842 ± 0.015, mean ± SD of five dishes). The control values were obtained by extraction into ice-cold PCA only. The ATP, ADP, and AMP contents of the standard solution were not affected by liquid nitrogen. Consequently, we did not use liquid nitrogen in other extractions.

Effects of on-ice condition. After removal of the culture media, the energy charge decreased gradually even when the samples were maintained on ice (0.954 ± 0.003 to 0.912 ± 0.008, mean ± SD of 3 dishes), although the cellular contents of adenine nucleotides remained constant (20.8 ± 1.1 nmol/10^6 cells, mean ± SD of 12 dishes) over the 6-min period. In contrast, in the presence of medium the adenine nucleotide contents and the energy charge were hardly affected by keeping the cells on ice for 10 min. Thus, our standard procedure for extraction required that PCA be added within 15 s after removal of the culture media.

Recovery percentages of the adenine nucleotides. The recovery percentages of ATP, ADP, and AMP through the extraction process were 89.2 ± 6.1%, 96.8 ± 5.7%, and 86.8 ± 3.7% (n = 6), respectively. These percentages were determined by adding standard solutions of ATP, ADP, and AMP together with PCA to a cell monolayer. Then the ideal extraction process just described was carried out.

Time course of changes in the adenylate energy charge. Figure 2 shows that the energy charge of cultured rat hepatocytes tended to increase in the first 6 h of incubation and then stabilized for the next 18 h. The total adenine nucleotide content tended to increase in the first 3 h and subsequently decreased.

For cultured human hepatocytes the changes in energy charge and total adenine nucleotide content over time were essentially the same as observed for the rat hepatocytes. However, the total adenine nucleotide content per cell was less than that seen with rat hepatocytes (Fig. 3).

The energy charge of the rat hepatocytes at 6 h after changing the media reached its highest level on Day 3 of culture (Day 1: 0.866 ± 0.008, Day 2: 0.891 ± 0.004, Day 3: 0.908 ± 0.0001, Day 4: 0.903 ± 0.005, Day 5: 0.897 ± 0.003, mean ± SD of 3 dishes), whereas the total adenine nucleotide content hardly changed during the 5 days of culture (22.5 ± 1.3 nmol/10^6 cells, mean ± SD of 15 dishes).

Effects of metabolic inhibitors. As shown in Figures 4 and 5, the inhibition of glycolysis or oxidative phosphorylation resulted in decreases in both the adenine nucleotide content and the energy charge level. These decreases were maintained for at least 24 h (results are only shown to 8 h). The inhibition of oxidative phosphorylation with antimycin A reduced the nucleotide content and energy charge more rapidly than did glycolysis inhibition with sodium fluoride. In contrast, the inhibition of fatty acid oxidation with 2-tetradecylglycidic acid produced only a transient reduction in the energy charge, which recovered within 8 h. This inhibitor did not affect total adenine nucleotide levels.

Figure 6 shows the changes in ATP, ADP, and AMP contents after antimycin A treatment. By 1.5 h the ATP levels had fallen dramatically, but ADP and AMP levels increased before beginning

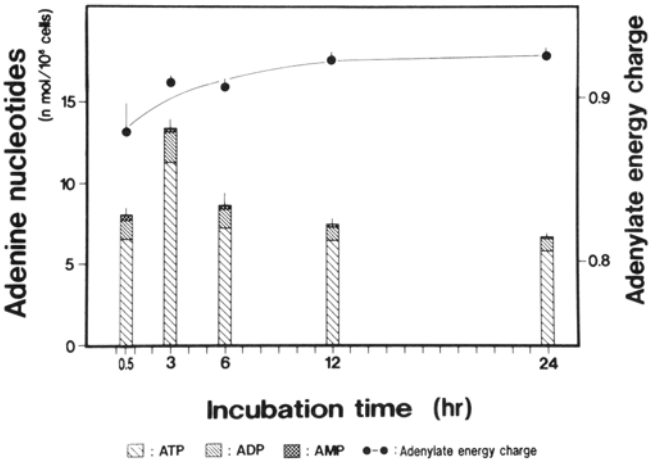

FIG. 3. Changes over time in the adenine nucleotide contents and the adenylate energy charge of cultured human hepatocytes after a change of media. Data were collected on Day 2 of culture from 9 a.m. to 9 a.m. for hepatocytes plated at a density of 0.8×10^6 cells/dish. Values are expressed as means ± SD for two dishes at each time point.

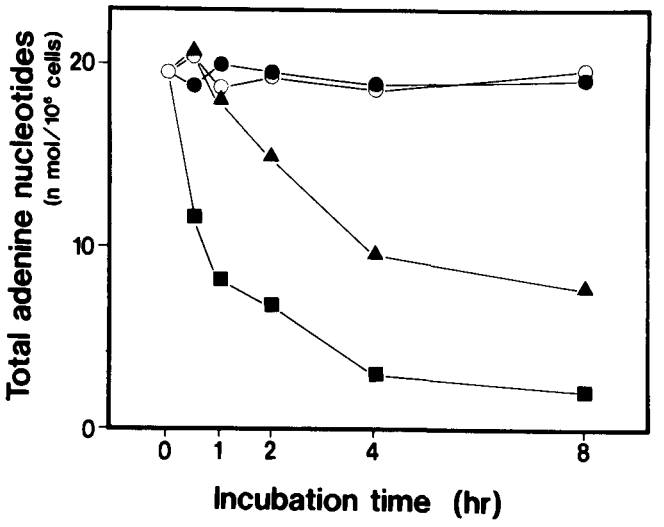

FIG. 4. Effects of metabolic inhibitors on the adenine nucleotide contents in cultured rat hepatocytes. Each inhibitor (30 μl) was added to the culture medium (1.5 ml) at the final concentration described below. Aliquots of vehicle (dimethylsulfoxide) alone were added to control cultures. Inhibitors were added 6 h after changing the media on Day 2 of culture for hepatocytes plated at a density of 1.1×10^6 cells/dish. Mean nucleotide levels in three separate culture dishes are presented. Standard deviations are within 10% of the means for all points. *Open circles* = controls, *solid circles* = 2-tetradecylglycidic acid (100 μ*M*), *solid triangles* = sodium fluoride (10 m*M*), *solid squares* = antimycin A (10 μ*M*).

to decrease slowly over the next few hours. Energy charge reached a minimum by 1.5 h and thereafter stabilized at a level of 0.4 to 0.6.

After inhibition of oxidative phosphorylation the decrease in energy charge preceded the decrease in total adenine nucleotide content (Fig. 7). Upon removing the inhibitor the energy charge recovered rapidly, although the total adenine nucleotide content continued to decrease for another 1.5 h. A slow recovery in total adenine nucleotide content began 2 h after removal of the inhibitor.

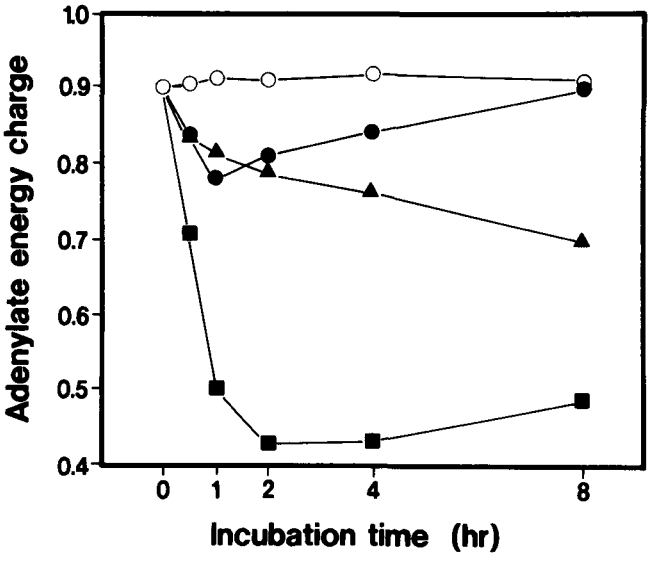

FIG. 5. Effects of metabolic inhibitors on the adenylate energy charge of cultured rat hepatocytes. Mean adenylate energy charge was calculated using the data presented in Fig. 4. Standard deviations are within 2% of the means for all points. *Open circles* = controls, *solid circles* = 2-tetradecylglycidic acid (100 μ*M*), *solid triangles* = sodium fluoride (10 m*M*), *solid squares* = antimycin A (10 μ*M*).

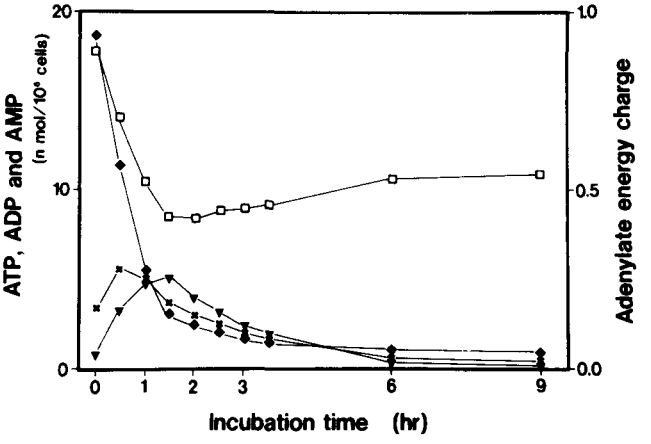

FIG. 6. Effects of an inhibitor (10 μ*M* antimycin A) of oxidative phosphorylation on ATP, ADP, and AMP contents, and on the adenylate energy charge in cultured rat hepatocytes. Antimycin A was added 6 h after changing the media on Day 2 of culture for hepatocytes plated at a density of 1.1×10^6 cells/dish. Average levels of the nucleotides for three separate culture dishes are presented. Standard deviations of the nucleotide contents and energy charge levels are within 7 and 2% of the means, respectively, for all points. ◆: ATP, ×: ADP, ▼: AMP, □: adenylate energy charge.

DISCUSSION

The ubiquitous involvement of adenine nucleotides in maintaining the functional and structural integrity of cells requires that they

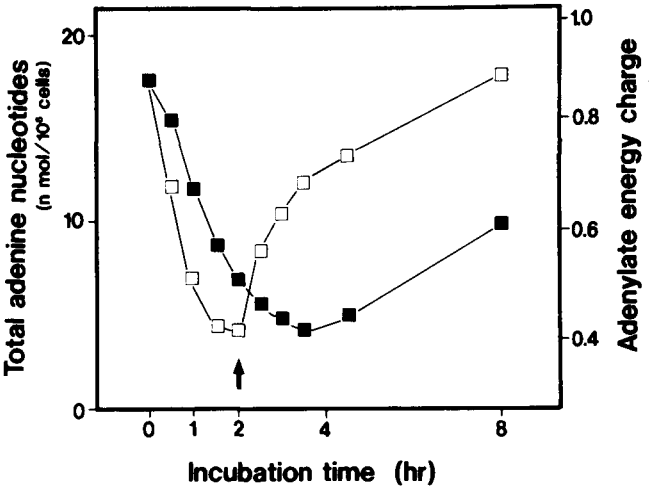

FIG. 7. Recovery of adenine nucleotide contents and the adenylate energy charge of cultured rat hepatocytes. The inhibitor of oxidative phosphorylation was washed out (*arrow*) by replacing with fresh media 2 h after the cell monolayers were exposed to 10 μ*M* antimycin A. The inhibitor was added 6 h after changing the media on Day 2 of culture for hepatocytes plated at a density of 1.0×10^6 cells/dish. Data represent the means of three separate culture dishes. Standard deviations of the nucleotide contents and energy charge levels are within 8 and 2% of the means, respectively, for all points. *Solid squares* = total adenine nucleotide content, *open squares* = adenylate energy charge.

be measured accurately in studies of cultured cells and tissues. With the procedure that we described, a dozen samples could be processed in 1.5 h. We found hepatocellular ATP levels that were nearly equal to those reported in other studies (4,8).

Homogenization was found to degrade the adenine nucleotides in both the sample solution and the standard solution. High energy phosphate bonds are thought to be dissociated by homogenization. Although many investigators have employed mechanical homogenization, sonication of tissues provides more accurate determinations of adenine nucleotides. Liquid nitrogen, which is commonly used to freeze tissues before extracting the adenine nucleotides, was found to affect the energy charge. Because liquid nitrogen did not affect the ATP, ADP, and AMP contents of the standard solution, this treatment preferentially promotes enzymatic hydrolysis of ATP and thus should not be used when adenine nucleotides are to be measured in cultured cells.

In view of the decrease in energy charge even when the cells are kept on ice, PCA should be added as quickly as possible, preferably within 1 min after removing the media. ATP-consuming reactions continue to occur after the media is removed even if the cells are kept on ice.

From the time-course studies it seems that cells have not recovered from the trauma of isolation after 1 day of culture, although from an energy charge point of view rat hepatocytes have recovered their energy metabolism after 2 days of culture. Because both the adenine nucleotide levels and the energy charge fluctuate in the first 6 h after a change of media for both rat and human hepatocytes, the energy metabolism of cultured hepatocytes should be examined at least 2 days after cell isolation and 6 h after media changes.

Even though the adenine nucleotide content in human hepatocytes was about half that of rat hepatocytes, the energy charge in these cells for the two species was the same. This maintenance in energy charge, despite a reduced content in total adenine nucleotides, indicates that a high level of energy charge (more than 0.9) is necessary for hepatocytes despite a difference in species.

Inasmuch as the inhibition of fatty acid oxidation caused a transient fall in the energy charge, the maintenance of adenylate energy charge partly depends on β-oxidation of fatty acids. However, the recovery of the energy charge, even in the presence of the inhibitor, indicates that the glycolysis is activated to supply the oxidative substrates to mitochondria in place of fatty acids when β-oxidation is inhibited (28).

On the other hand, the decreases in both the energy charge and the nucleotide content were less severe after the inhibition of glycolysis than after the inhibition of oxidative phosphorylation. This result probably indicates that fatty acid oxidation supports energy production to some extent when glycolysis is blocked, as described in in vivo study (22). However, the irreversible decrease in the energy charge after the inhibition of glycolysis shows that fatty acid oxidation alone is not sufficient to maintain the energy metabolism of cultured hepatocytes.

Because rapid decreases in both the nucleotide content and the energy charge were observed after inhibition of oxidative phosphorylation, anaerobic glycolysis alone cannot maintain the energy metabolism of the hepatocytes. These cultured cells seem to maintain a high energy balance aerobically by using a combination of glucose and fatty acids.

Upon the inhibition of oxidative phosphorylation, the rapid and drastic decrease in ATP was accompanied by substantial but transient increases in ADP and AMP levels. Both of these changes drive down energy charge, but as ADP and AMP begin to decline, the energy charge recovers slightly and stabilizes at a value between 0.4 and 0.6. This maintenance of energy charge in cultured hepatocytes, albeit at a lower level, probably reflects an increased activity of AMP deaminase as was observed in vivo by Chapman and Atkinson (6) using the purified enzyme. AMP deaminase probably prevents further decrease in energy charge at the expense of the amount of adenine nucleotides in such inhibitory state. The concept that energy charge can regulate adenine nucleotide levels is supported by our observations that changes in energy charge precede the changes in adenine nucleotide levels when oxidative phosphorylation was inhibited and when the inhibitor was removed. Moreover, high energy charge is necessary for the recovery of total adenine nucleotide amount, via de novo or salvage pathways or both, that has been lost.

In this study, we have confirmed that the high adenylate energy charge of the hepatocytes is maintained by oxidative phosphorylation whereas the oxidative substrates are supplied mainly by glycolysis and partly by β-oxidation. Furthermore, our results indicate that the continual exchanges between ATP, ADP, and AMP occur at a rapid rate. Inasmuch as the adenylate energy charge is quite sensitive to anoxia and to the recovery from anoxia, assay of the ATP levels only is not sufficient to analyze energy metabolism in cultured hepatocytes. It is essential to confirm that the high energy charge of the hepatocytes is maintained when these cells are used for metabolic studies.

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