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Inverse Relationships between ATP and Fructose Diphosphate in Cultured Cells in Response to Change in External pH[1] (39789)

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Survival in biological systems depends on correct response to change in the external environment. If increase in external acidity results in net influx of acid, the response must be to arrange net efflux to sustain balance. Such an event must relate to alterations in the internal metabolism of the cell, and one can anticipate that certain of these could be identified and measured. Since the excretion of hydrogen ions requires energy, attention is drawn to reactions which utilize ATP. In a preliminary study, increased amounts of ATP were found in mitochondria when the hydrogen ion concentration of the surrounding fluids was raised from 10^{-8} to 10^{-7} *M* (1). In the present study, gain in ATP was observed in preparations of cultured cells when the hydrogen ion concentration was raised over the same physiological range. In these cells, changes in ATP are coupled closely to reciprocal variations in the content of fructose-1,6-diphosphate.

Methods. The human KB cells were made available through the generosity of Drs. T. Pinkerton and K. Berns. In their laboratory day-to-day maintenance was achieved using a minimal essential medium (Eagle, Microbiological Associates) supplemented with 5% horse serum. In our laboratory the cells were transferred to a second medium, RPMI-1640 (Associated Biomedics Systems). This second medium is supplemented with 10% bovine serum, and it also contains calcium and a larger assortment of amino acids. The cells were grown in closed, spinner flasks. Those to be used for the experiments were maintained for an extra day, 3 rather than 2 days. Under these conditions, the cell counts are high, 6–9 × 10^5/ml, and the pH is reduced to 6.6–6.9. Lymphocytes RPMI-1788, were obtained from Associated Biomedic Systems. Preparations of ascites tumor cells were made available by Dr. T. Spencer.

In the usual experiment with the human KB cells, aliquots (20 ml) of the original growth suspensions (0.2–0.4 mg of protein/ml) were incubated at 23 or at 37° for 5 min. The pH was adjusted with inclusions of NaOH or HCl. Incubations were terminated by centrifugation, and the pellets were immediately taken up in 6% perchloric acid. Perchlorate was precipitated on neutralization with KOH. When the ascites tumor cells were used, 0.5-ml aliquots of material from the mice were added to 2-ml incubation solutions to provide final concentrations of 2–4 mg of protein/ml. When these more concentrated suspensions were used, centrifugation was omitted, and the perchloric acid was added directly to terminate the experimental incubation periods.

Acid-soluble components were identified and measured using chromatographic and enzymatic techniques. Extracts were first placed on a 2-cm anion-exchange column (1-cm diameter, Dowex-1, 200–400 mesh, formate form). Six fractions were eluted using 5-ml volumes containing increasing concentrations of formic acid (0.2, 2.5, and 4.0 *M*) and eventually 4.0 formic acid with increasing concentrations of ammonium formate (0.3, 0.5, and 1.0 *M*). The procedure is a modification (2) of the method described by Siekevitz and Potter (3). Ammonium ion was removed with a cation-exchange column (Dowex-50, 200–400 mesh, hydrogen form) and samples were evaporated to dryness without exceeding 40° using the Buchler Evapo-Mix. Thin-layer chromatography of the fractions was carried out using plastic-coated sheets (polygram cell 330 PEI, Brinkman Instrument Co.) with acetic acid 1.0 *M*:LiCl 3.0 *M* (9:1) as the solvent. For the paper chromatography t-

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butyl alcohol:formic acid:water (76:5:14) was used as the solvent. Anions were located using bromphenol blue as the indicator. Phosphate components were identified using the Hanes–Isherwood spray (4). Certain of the preparations were labeled during incubations with ^{32}P for 24–48 hr prior to harvesting. Assay for the radioactivity of the staining components provided estimates of the sizes of the changes. The enzymatic assays involved measurements of uv absorption changes of NAD and NADP in accordance with the procedures described in "Methods of Enzymatic Analysis" which is edited by H. U. Bergmeyer (5). Alterations in ATP were measured with the use of two different enzyme systems; hexokinase together with glucose-6-phosphate dehydrogenase (W. Lamprecht and I. Trautschol, Vol. 4, p. 2101) or phosphoglyceric phosphokinase with glyceraldehyde phosphate dehydrogenase (D. Jaworek, W. Gruber, and H. U. Bergmeyer, Vol. 4, p. 2097). ADP and AMP were measured using lactic dehydrogenase and pyruvic kinase combined with adenylate kinase (D. Jaworek, W. Gruber, and H. U. Bergmeyer, Vol. 4, p. 2127). In the assay for fructose-1,6-diphosphate, α-glycerophosphate dehydrogenase and triosephosphate isomerase were combined with aldolase (D. Michal and H-O. Beutler, Vol. 3, p. 1314). Glucose-6-phosphate and fructose-6-phosphate were measured using phosphohexoseisomerase with glucose-6-phosphate dehydrogenase (G. Lang and G. Michal, Vol. 3, p. 1238). The enzymes were obtained from Sigma Chemical Co. There were no significant quantities of these organic phosphate components in the extracellular fluids. Protein content was determined in aliquots of the cell preparations after washing once with saline. A biuret method (6) was used.

Formation of isotopically labeled lactate was measured to observe changes in rates of glycolysis. Chemical analysis was impractical due to high initial concentrations of lactate and due to the intolerance of the cells to washing. Glucose, labeled uniformly with ^{14}C was added to the original growth medium. After incubation, lactate was isolated using the column and paper chromatography and assayed for its radioactivity. Lactate is eluted by 2.5 *M* formic acid in the second of the column fractions and has an R_f value between 0.8 and 0.9 on the paper chromatogram. This area on the paper is free of both uv-absorbing and phosphate-staining materials.

Results. It was observed initially that large changes in ATP occurred when preparations of human KB cells, maintained in the original culture media, were exposed to different pH values. As shown in Fig. 1, a marked decrease in ATP occurred when the pH was increased over a narrow range between 7.4 and 7.8. Studies using column and thin-layer chromatography were initiated in order to identify other changes; and these served to demonstrate a large increase in a component having the characteristics of fructose-1,6-diphosphate. This component induced prompt positive staining for phosphate, but the color was noted to fade in 1–2 hr. Known additions of fructose diphosphate were eluted from the same column fraction, had the same R_f value on both paper and thin-layer sheets, and had identical staining properties. Enzymatic identification proved confirmatory, and changes in this and in other compounds were measured subsequently using enzymatic methods. Observed alterations in adenine nucleotides and in hexose phosphates are shown in Ta-

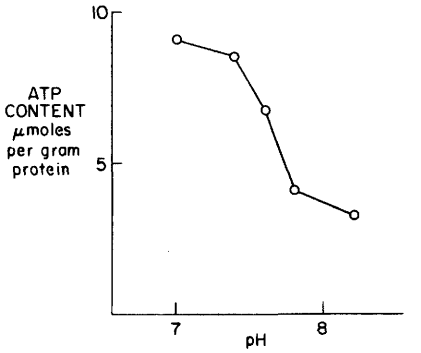

FIG. 1. Human KB cells were harvested during the plateau phase after growth. Aliquots (20 ml) of cells were suspended in the growth medium and incubated for 5 min at 23°. The initial pH was 6.9. The higher values were managed with micromolar additions of NaOH. Incubations were terminated with centrifugation and subsequent additions of 6% perchloric acid. Changes in ATP were measured by the first of the enzymatic methods described in the Methods section.

ble I. Emphasized by these data is the very large change in fructose diphosphate; and it can be seen that the phosphate available from the breakdown of ATP to ADP (3.4 μmole) and of ATP to AMP (8.8 μmole) is approximately correct to account for the gain of one of the phosphate groups of the FDP.

Techniques described by Coe (7) were utilized to measure participants in the second stage of glycolysis: triosephosphate, 2- and 3-phosphoglycerate, phosphoenolpyruvate, and pyruvate. Amounts of these components were found to be small (less than 0.5 μmole/g of protein), and significant changes were not observed.

The reciprocal relationship between ATP and ADP reverses when the pH is returned to normal. Events occur more rapidly when the direction of change is towards alkalinization. However, after reacidification 50–60% of theATP is regained in 10 min (Fig. 2).

The dramatic effect of change in pH upon the content of ATP and fructose diphosphate was observed consistently in preparations of KB cells which were grown in media containing fetal calf serum and which were harvested during a plateau phase after growth. The cultures were maintained in closed bottles for 1 extra day (3 rather than 2), and under these conditions accumulations of CO_2 and lactate resulted in reductions of the pH to 6.7–6.9. The lactate concentrations increased to 16–20 m*M* and the bicarbonate in the media was reduced to 5–10 m*M*. The phenomenon (Figs. 1 and 2) could not be demonstrated when the cells were harvested during the growth phase. Neither could it be reproduced in cultures of human lymphocytes. In these "inactive" preparations accumulations of lactate were much less, and the pH of the media never dropped below 7.0.

Studies were undertaken to observe effects upon rates of glycolysis in these preparations. Cell suspensions were incubated in the presence of uniformly labeled [^{14}C]glucose for 20 min. Lactate, as isolated from the medium using column and paper chromatography, was assayed for its radioactivity. The results shown in Table II were obtained using two different types of preparations of KB cells. The cells of the first were harvested during the growth phase. These were "inactive." The increase in pH produced no more than a 20% fall in ATP, and there was no detectable increase in fructose diphosphate. Alkalinization produced a marked stimulation in the rate of lactate formation. This result is in agreement with many previous observations of the effect of alkalinization upon glycolysis (8–10). On

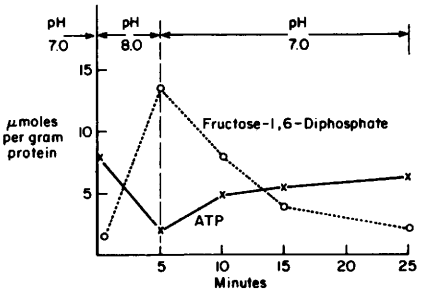

FIG. 2. Reciprocal effects of change in external pH upon the cellular content of ATP and fructose diphosphate. The experimental conditions are those described in Fig. 1. Reacidification was achieved with micromolar additions of HCl. Changes in ATP and in fructose diphosphate were measured enzymatically.

TABLE I. DIFFERENCES IN THE CONTENT OF VARIOUS PHOSPHATE COMPONENTS AFTER INCUBATION FOR 10 min AT 23°.[a]

phosphate components	pH 7.0 (micromoles per gram of protein)	pH 8.0 (micromoles per gram of protein)	Change
ATP	9.1	1.0	−8.1 (±1.8)
ADP	2.5	5.9	+3.4 (±1.5)
AMP	1.0	5.4	+4.4 (±1.2)
Fructose-1,6-diphosphate	1.5	16.1	+14.6 (±3.1)
Fructose-6-phosphate	0.3	0.1	−0.2 (±0.03)
Glucose-6-phosphate	1.0	0.2	−0.8 (±0.02)

[a] Experimental conditions are those described in Fig. 1. Components were identified with enzymatic methods. The values shown are averages from six experiments. Figures in the parentheses are standard deviations.

TABLE II. LACTATE PRODUCTION (NANOMOLES PER MINUTE).[a]

Phase	pH 7.0	pH 8.0
Growth	5.3	16.1
Plateau	4.5	1.9

[a] Suspensions of human KB cells were incubated for 20 min at 37° in the presence of ^{14}C-labeled glucose. After centrifugation, ^{14}C-labeled lactate was isolated from the medium by means of column and paper chromatography. The quantity of lactate was calculated on the basis of radioactivity recovered.

the other hand, the opposite result was obtained with the second preparation. In this case the cells were fully "active." When the pH was raised to 8.0, there was a large decrease in ATP and increase in FDP. In this preparation the rate of lactate formation, in place of being accelerated, was reduced by approximately 50%.

Effects of iodoacetate are shown in Fig. 3. Inhibition of glycolysis with this agent is due presumably to its effect upon the enzyme 3-phosphoglyceraldehyde dehydrogenase. Results obtained with an "active" preparation of the KB cells are shown on the left. Decrease in ATP and increase in FDP which occurred only at pH 8 in the absence of iodoacetate occurred also at pH 7 in its presence. Results obtained with a preparation of cultured human lymphocytes are shown on the right. These cells have not, in our hands, responded to alkalinization alone. However, in the presence of iodoacetate, reductions in ATP and increases in fructose diphosphate were observed. The response was greater at the higher pH.

The reaction in Ehrlich ascites tumor cells. The reciprocal relationships between ATP and fructose-1,6-diphosphate have also been observed in experiments with mouse Ehrlich ascites tumor cells. In the initial observations the RPMI-1640 growth medium was used, but the reaction can be demonstrated also when the cells are suspended in saline and alkalinized with bicarbonate when the only supplement is glucose. The requirement for glucose, as shown in Table III, becomes possible due to its virtual absence in the ascitic fluid at the start (11). Presumably glucose supplies the carbon source for formation of the fructose-1,6-diphosphate. Attempts to evoke the reaction *in vivo* in the peritoneal cavity were not successful, but it may be that sufficient alkalinity was not obtained due to the high tensions of CO_2. Although the bicarbonate of the ascitic fluid was increased above 50 meq/liter, the pH could not be raised above 7.5.

Discussion. The experiments in this report describe large and reciprocal changes in ATP and in fructose-1,6-diphosphate in response to relatively small changes in external acidity. The response was seen first in a line of cancer cells (human KB) which was cultured in a fully supplemented medium and allowed to grow an extra day in closed flasks. In the final preparation, the pH of the medium was reduced to 6.7 and the concentration of lactate increased to 15–20

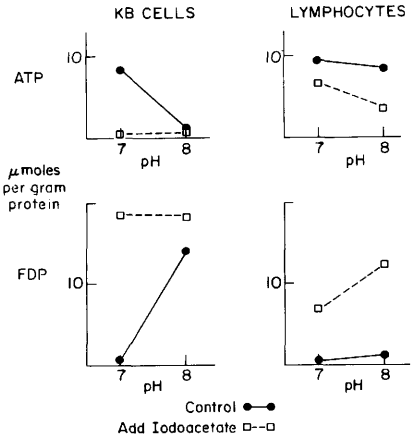

FIG. 3. Effects of iodoacetate. Results obtained with a preparation of KB cells are shown on the left, and those with a preparation of human lymphocytes are shown on the right. Experimental conditions are those described in Fig. 1. Control, ●——●; iodoacetate (10^{-4} *M*), □-----□.

TABLE III. REQUIREMENT FOR GLUCOSE IN ASCITES TUMOR CELLS (MICROMOLES PER GRAM OF PROTEIN).[a]

	pH 6.8		pH 8.2	
		+ Glucose		+ Glucose
ATP	16.1	14.2	15.1	3.0
FDP	0.6	3.1	1.1	25.4

[a] Freshly drawn mouse ascites tumor cells (0.5 ml) were incubated at 23° with 2 ml of saline (pH 6.8) or with 2 ml of saline containing 10 m*M* $NaHCO_3$ (pH 8.1) for 5 min prior to addition of perchloric acid. The concentration of glucose, when present, was 19 m*M*.

m*M*. The large changes in ATP and in the fructose diphosphate could not be demonstrated during earlier stages of growth, nor were they seen in cultures of lymphocytes which could not generate the higher concentration of lactate and of hydrogen ions. The reciprocal changes in the two components were demonstrated in preparations of mouse ascites tumor cells; and here, again, the pH was low, 6.7, and the concentration of lactate was high, 20 m*M*. Special interest attaches to two aspects of this reaction. First, the magnitude of the change in the fructose diphosphate is unusual. Reciprocal variations in the concentrations of ATP and fructose diphosphate have been described (12–14), but in these examples alterations in the fructose diphosphate have been small, amounting to no more than one-tenth to one-third of the change in ATP. We are unaware of other examples in which the size of the change in the fructose diphosphate actually exceeds that for ATP. The second point is the anomalous effect upon the rate of glycolysis. It has been observed repeatedly, in a great variety of experiments (8–10), that increase in pH is associated with acceleration of the rate of glycolysis; and yet in the presently described experiments, and in conjunction with perturbation to the concentrations of ATP and FDP, the rate of lactate formation is inhibited (Table II).

It is pointed out in Fig. 3 that the effect of increase in pH can be mimicked by adding iodoacetate which is known to block 3-phosphoglyceraldehyde dehydrogenase. It may be that the effect of the alkalinization (Figs. 1 and 2) is to inhibit one of the later enzymes of the glycolytic sequence. Such an action would decrease formation of ATP, and fructose diphosphate might accumulate due to decreased utilization. However, there are other possible explanations. An as yet undefined primary action of alkalinization might reduce the concentration of ATP. In this case the high levels of fructose diphosphate could be due to increase in its formation after release from an ATP-induced inhibition of phosphofructokinase (12, 15, 16).

The direction of change in ATP is appropriate in terms of a feedback mechanism for acid–base homeostasis. Accumulation of fructose diphosphate after alkalinization might be regarded as a temporary storage of high energy phosphate should there be need for a rapid resynthesis of ATP on return to an acid environment.

Summary. In a preparation of cultured cells (human KB) concentrations of ATP and of fructose-1,6-diphosphate are markedly sensitive to change in external pH. In a series of experiments when the pH was increased from 7.0 to 8.0, the average value for the content of ATP decreased from 9.1 to 1.0 μmole/g of protein while that for fructose-1,6-diphosphate increased from 1.5 to 16.1 μmole/g of protein. The changes reverse when the pH is returned to 7.0. The reaction is linked to inhibition rather than stimulation of the rate of glycolysis, and the effect can be simulated by adding iodoacetate without changing pH. The inverse relationship between ATP and FDP after changing pH has also been observed in Ehrlich ascites tumor cells.

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