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RAPID LOSS OF ATP BY TUMOR CELLS DEPRIVED OF GLUCOSE: CONTRAST TO NORMAL CELLS

by

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SUMMARY: When starved of a carbon source, early passage normal cells (chick embryo fibroblasts, human skin fibroblasts and mouse splenic lymphocytes) are able to maintain their ATP content for 12 to 24 hours at levels essentially similar to those of cells fed glucose. In contrast, several malignant or transformed cell types (Py6, PyNil, Ehrlich acites tumor, P_{388}, CHO in suspension) under the same conditions of cultivation suffer a dramatic lowering in their ATP levels within the first hour of starvation. This sharply different response to glucose starvation and the loss of viability that accompanies loss of ATP are the principal findings reported here.

Among the several striking defferences known to distinguish malignant cells from their normal parent-cells or most normal cells in situ are differences involving glucose transport and utilization (1-9). Particular attention has been focused on the high rate of aerobic glycolysis (1) and glucose transport (5) observed in the malignant cells.

As a result of a series of studies on glucose transport (9-14) we have been led to consider the wastage of glucose and the effects of low glucose on ATP metabolism by eukaryotic cells in culture. In the process we have uncovered some major differences in the effects that low concentrations of glucose (including glucose starvation) have on a variety of cell types. According to their response to glucose starvation the cell types implicated in this study can be divided into three classes:

Class A: Cells able to maintain their ATP levels unchanged for 12-24 hours despite the absence of a carbon source. Early passage normal cells such as Chick Embryo Fibroblasts (CEF), Normal human skin Fibroblasts (NF3, NF6) and mixed mouse splenic lymphocytes have been found to belong to this class.

Class B: Cells undergoing a gradual decline in their ATP levels over a period of 6-12 hours when deprived of a carbon source. Non-transformed ("normal") cell lines such as Syrian Hamster Fibroblasts (Nil) and Chinese Hamster Ovary Fibroblasts growing as monolayers (CHO) have been found to exhibit such response to starvation.

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Class C: Cells suffering a precipitous loss of ATP within minutes after initiation of starvation for a carbon source. Transformed or spontaneously malignant cells have been found to demonstrate such behavior. Among the cell types implicated in this study were: mouse lymphosarcoma cells (P_{388}), Ehrlich Ascites Tumor Cells, polyoma virus transformed Nil cells (PyNil) and polyoma virus transformed BHK cells (Py6).

MATERIALS AND METHODS: Luciferin-luciferase (FLE-50), standard ATP (A-3127) and glycyl glycine were obtained from Sigma Chemical Co. (St. Louis, Mo.). Multiwell plates (3008) were obtained from Falcon Co. (Oxnard, Cal.); Kimble glass shell vials (15 x 45 mm) and plastic caps from Curtin-Matheson Co. (Woburn, Mass.). Regular and dialyzed or fetal calf sera were obtained from GIBCO (Grand Island, N.Y.) or Flow Laboratories (Rockville, Md.). Glucose- and pyruvate-free culture media were specially prepared by GIBCO.

Cell cultures: CEF primary and secondary cultures were prepared and maintained in BME or MEM supplemented with 4% fetal calf serum (FCS) or dialyzed fetal calf serum (DFCS) (10, 13, 14). NF3 and NF6 fibroblasts in early passage were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DME) (17). Nil and CHO cells were also grown as monolayer cultures in DME supplemented with 7-10% FCS or DFCS. Mouse spleen mixed lymphocyte cultures were prepared from spleens of adult Swiss mice (Charles River Laboratories, Wilmington, Mass.). Red blood cells were removed by lysis in hypotonic MEM. The mixed cultures of lymphocytes (85-90%) and macrophages (10-15%) were incubated at 2 x 10^6 cells/ml in DME or in RPMI 1640 (18) medium (Microbiological Associates, Bethesda, Md.) with DFCS (7 to 10%) with or without glucose. Ehrlich ascites tumor cells were grown in spinner cultures (Joklik's medium) (16) at approximately 5 x 10^5 to 10^6 cells per ml. P_{388} lymphosarcoma cells were grown as standing cultures in 15 x 100 mm screw-capped tubes in Fischer's medium (15). P_{388} cells were also obtained from advanced ascites tumors of CDF (C57BL/6xDBA/2F1) mice for direct use after washing with phosphate buffered saline (PBS). CHO cells were also grown as suspensions in spinner cultures (Joklik's medium). Py6 and PyNil cells were grown as monolayers in DME supplemented with 10% FCS or DFCS.

Measurement of ATP: ATP was measured by the method of Cole et al. (19) as modified by Wilson et al. (20). Cells in suspension were cooled to 0°C in an ice bath in 1 or 2 ml aliquots. The suspension was adjusted to 0.6 M in perchloric acid. After at least 20 minutes the acid extract was neutralized by a pretitrated volume of 3 M KOH and the samples were then processed for ATP determination by the Luciferin-luciferase method of Cole et al. (19) as modified by Wilson et al. (20). Monolayer cultures were washed with buffer, cooled and extracted with perchloric acid (0.6 N) for 20 minutes to 1 hour. The perchloric acid extract was transferred to a tube, neutralized by KOH and processed for ATP determination as above.

Protein determination: Aliquots from suspension cultures were centrifuged, washed and the cells were suspended in 2.5 to 5.0 ml of 0.1 N NaOH for use in the assay of Lowry et al. (21). After removal of the perchloric acid extract the cells of the monolayer were treated with 1 to 5 ml of 0.2 N NaOH. Aliquots of the dissolved cells were used in the Lowry et al. assay.

RESULTS

Effect of Starvation for Glucose on ATP Levels

Early passage normal cells: When starved for glucose chick fibroblasts and early passage human skin fibroblasts growing as monolayer cultures maintained their ATP concentrations close to those characteristic of cells provided with glucose-

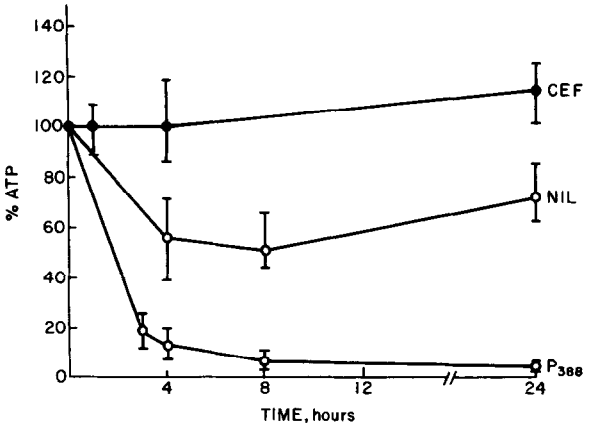


Figure 1

Effect of Starvation upon ATP Concentration of CEF, Nil, and P_{388} Cells

CEF and Nil cells were grown as described in Methods and Materials to a density representing one-half to near confluence. P_{388} cells were cultivated in Fischer's medium (10% horse serum) in suspension in standing tubes. Experiments were conducted with P_{388} cells at 10^6 cells per ml, 2 ml per tube. The cells were subjected to a change of medium containing glucose (5.5 mM) or no sugar. In the experiments reported above the fresh medium was serum-free. Essentially identical results were obtained with dialyzed calf or fetal calf serum (7 to 10%) as a supplement. ATP is expressed as % of ATP (nmoles per mg cell protein). ATP concentration of cells provided with glucose (5.5 mM) for the same time and under same conditions was considered 100%. The points on the curves represent the arithmetic mean of triplicate cell populations from 7 separate experiments, while the vertical lines indicate the range of deviation above and below the mean.

containing medium for prolonged periods, usually more than 24 hours. In fact, the concentrations of ATP in starved CEF were often considerably higher than those in CEF maintained on glucose (Figure 1). The conservation of ATP was observed in sparse as well as confluent CEF cultures attached either to glass or to plastic surfaces. NF6 cells also suffered little or no loss of ATP after 18-24 hours of starvation (Table 1). Glucose-free suspensions of mixed splenic lymphocytes in standing tubes (at 10^6 cells per ml) also maintained their ATP concentrations close to those of controls provided with glucose for the same period. In all cases the ATP levels in the control cultures provided with glucose varied not more than 15% over the 24-hour period.

Non-transformed cell lines: Nil hamster fibroblasts maintained their ATP concentrations less well than CEF or NF6 cells, undergoing a modest loss to 40 to 60% of ATP after 4 hours of incubation with or without dialyzed serum (Figure 1). CHO cells grown as monolayer cultures resemble closely Nil cells in their response to starvation (Table 1).

Transformed and spontaneous tumor cells: When P_{388} cells were subjected to

Table 1: Effect of Starvation upon ATP Concentration in Normal and Malignant Cells

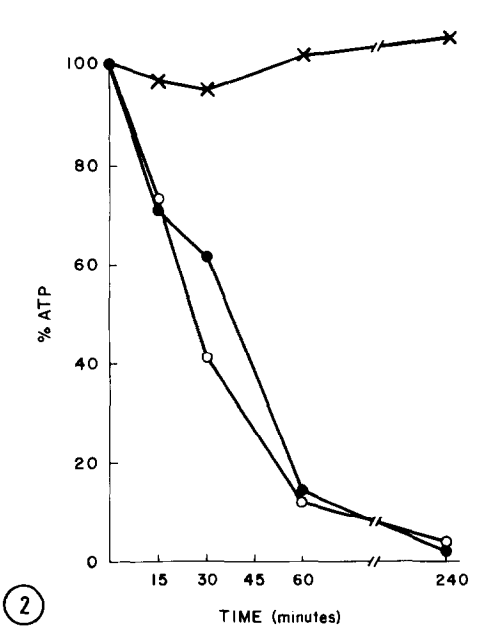
Cell type	ATP* (nmoles/mg cell protein)	% ATP Starved Cells** 4 hours	12-24 hours
1. CEF	(14.4 - 22.8)	107	128
2. NF	(5.3 - 6.4)	82	92
3. Mouse lymphocytes	(14.7 - 26.4)	83	80
4. CHO (monolayer)	(10.2 - 16.5)	90	N.D.
5. Nil	(8.3 - 16.3)	49	67
6. CHO (suspension)	(4.6 - 8.8)	28	N.D.
7. PyNil	(2.4 - 8.0)	13	1.3
8. Py6	(4.7 - 10.1)	13	N.D.
9. P_{388}	(3.7 - 9.5)	6	5.2
10. Ehrlich ascites	(31.3 - 36.1)	20	N.D.

Cells were grown for a period of 2 to 3 days under culture conditions specified in Methods and Materials. All growth media contained glucose as energy source. At the time of initiation of the ATP experiment the medium was changed to contain glucose (5.5 mM) or no energy source. Dialyzed calf or horse serum or serum-free medium was employed. The cultures were incubated for 4, 12 or 24 hours at 37°C with or without bicarbonate in the same type of medium and conditions (CO_2 incubator) in which the cells were grown.

* Range of ATP levels (nmoles per mg cell protein) for cells provided with glucose (5.5 mM) during 4, 12 or 24 hours. The ATP values were determined at the end of the 4, 12 or 24-hour incubation. The values presented are the range of values recorded in 4 to 5 independent experiments.

**The values given for starved cells are the per cent ATP compared to the cells incubated with glucose for the same time period in the same experiment. The percentages are an average of the percentages obtained in several experiments.

starvation for glucose their ATP content decreased rapidly and within four hours had fallen to 8 to 20% of the starting level, which in fact was the same as that of P_{388} cells provided with glucose (5.5 mM). The P_{388} cells were suspended in glucose free Fischer's medium (1 to 2 x 10^6 cells/ml) and incubated (37°C) in screw-capped tubes (5 ml/tube) standing in a 95% air and 5% CO_2 incubator. Addition of serum (dialyzed or undialyzed) to the medium did not alter significantly the fate of the ATP content. More careful observation of the time course revealed that the P_{388} cells began to lose ATP within minutes of incubation without a carbon source (Figure 2). Three additional tumor or transformed cells were equally sensitive to starvation: (1) Ehrlich ascites cells grown in suspension in gently agitated culture vessels; (2) Py6, a polyoma transformed BHK cell that requires attachment to a surface in order to grow; and (3) PyNil, a polyoma transformed Nil cell which



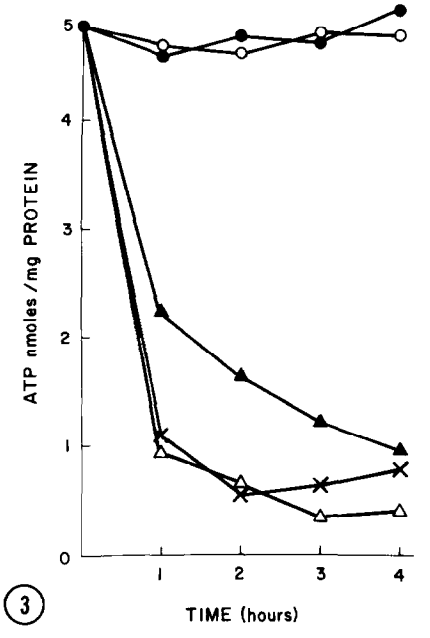


Figure 2

Effect of Starvation upon ATP Levels of P_{388} Cells

P_{388} cells cultivated in Fischer's medium (glucose 5.5 mM, 10% horse serum) were washed and resuspended at a concentration of 10^6 cells per ml in fresh Fischer's medium with (●) or without (O) dialyzed horse serum. ATP was measured on triplicate cultures at each of the time points indicated. ATP concentration expressed as % of zero time value for cells incubated in glucose (5.5 mM) containing medium. X = cells in medium supplemented with glucose (5.5 mM) and dialyzed horse serum.

Figure 3

Effect of Lowered Glucose Concentrations upon ATP Concentration of P_{388} Cells

Conditions same as for Figure 2. Medium contained 10% dialyzed horse serum. ● = glucose concentration 5.5 mM; O = glucose 2.75 mM; ▲ = glucose 1.65 mM; X = glucose 0.55 mM; Δ = no glucose.
ATP reported as ng per mg cell protein.

also requires attachment for growth. When starved, each of these representatives (Table 1) suffered an 80 to 90% reduction of its ATP concentration within four hours.

In fact, when followed more closely the ATP loss was in all cases significant within 15 minutes of the withdrawal of glucose and approached the 4-hour level within the first hour (Figure 2).

Minimal Glucose Required to Sustain ATP in P_{388} Cells

Whereas glucose concentrations from 5.5 mM (1 mg/ml) to virtual absence had no significant effect on ATP levels in early passage cells, P_{388} lymphosarcoma cells required a minimal glucose concentration of 2.75 mM (500 μg/ml) to maintain their

ATP concentration. Concentrations of 1.65 mM (300 μg/ml) or below were not sufficient to prevent a significant loss of ATP within 1 hour (Figure 3). A glucose concentration of 1.65 mM slowed the rate of loss, while one-third that concentration (0.55 mM) was indistinguishable from starvation. PyNil and Ehrlich ascites tumor cells demonstrated a similar glucose requirement to maintain their ATP levels (data not shown).

Cell Viability

As a second criterion of the cellular response to starvation for glucose, the integrity of the cells was followed by counting the cells remaining attached to the surface and determining what proportion of those excluded trypan blue (monolayer cultures) or by estimating the viable cells in suspension cultures as that fraction excluding trypan blue (Table 2).

DISCUSSION:

Of major interest in this report is the striking and consistent difference in response to starvation that characterizes normal cells on the one hand and malignant cells on the other. The ability of cells recently established in culture from normal animal tissues to maintain their ATP concentration when starved for sugar contrasts sharply with the inability of malignant or transformed cells to do the same. Thus, CEF, NF and mouse splenic lymphocyte cultures have as early passage cells or direct isolates behaved uniformly in retaining their ATP levels in the face of glucose starvation. In contrast, Ehrlich ascites, P_{388}, PyNil and Py6 cells responded as malignant cells to starvation by rapidly losing ATP. The so-called normal or non-transformed cell lines are intermediate but closer to early passage cells than to malignant or transformed cells.

The ATP levels as estimated by us for CEF cells correspond to reported values (26, 27). Fodge and Rubin (26) found that neither the pH at which the cells were grown (pH 6.7 to 7.6) nor the serum concentration (0 to 10%) altered significantly the ATP pool. Colby and Edlin (27) found little change in chick fibroblast ATP concentration as cells grew through exponential to the stationary phase.

Although glucose starvation does not result in loss of ATP by normal cells the addition of β-adrenergic stimulants such as L-epinephrine and DL-isoproterenol to the medium of starving cultures has been shown to induce a precipitous reduction in the ATP levels (32).

Sporadic references to glucose starvation of various ascites tumor cells (23, 24, 29) and of SVT2 cells (25) have been reported, but the investigators did not enlarge upon the simple citation of the loss of ATP observed.

Although the phenomenon of the conservation of ATP in the face of starvation is fully documented for bacteria and yeast (19, 22, 30, 31), no satisfactory explanation of the mechanism has been proposed. Whatever the control mechanism re-

Table 2: Viability of Cells Starved for Glucose

Cell type	Time (hours)	Glucose	% Attached*	% Viable**
CEF	24	+	100	96
		-	102	88
NF6	24	+	100	97
		-	94	89
Nil	24	+	100	91
		-	90	78
PyNil	24	+	100	89
		-	29	12
P_{388}	3	+	NA	93
		-	NA	28

NA = not applicable

Cells were grown to near confluence (NF3, CEF, Nil, Py6, PyNil) or to 2 x 10^5 cells per ml in suspension culture (P_{388}). The monolayer cultures were washed, drained, and the medium replaced by fresh medium with glc (5.5 mM) or no energy source.

* The percentage of cells attached was computed from the protein values by accepting the glucose-fed cultures as 100%.

** Cells were released from the surface by trypsinization and exposed to trypan blue for 3-5 minutes. Unstained cells are considered as viable. Each value is the average of triplicate determinations from each of two experiments. In no case was the variation greater than 10%.

The P_{388} cells were harvested by centrifugation for 10 min. at 1000 x g, washed and returned to Fischer's medium (17) with sugars as indicated. Viability was estimated by direct addition of trypan blue to the suspended cells. The % viable cells is of residual intact cells.

sulting in conservation or maintenance of ATP by normal cells, it is apparently lost in malignant cells. In neither case is glycogen utilized (D. Sens, unpublished data) nor are amino acids or other medium constituents responsible for the differences shown here, for normal cells are capable of maintaining their ATP levels even when cultivated in inorganic salt buffers (33).

Any attempt to explain the difference between the response of normal and malignant cells to sugar deprivation would be primarily speculative. Hopefully studies currently in progress will lead to some understanding of the molecular signals involved in the loss of regulatory function by malignant cells.

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