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Uptake and Utilization of 2-Phosphoenolpyruvate (PEP) by Malignant Cells. (32573)

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Although various carbohydrates are taken up by cells after a preliminary phosphorylation in the cell membrane(1,2), the latter is usually considered impermeable to phosphate esters(1,3), and it is generally thought that only the presence of phosphatases in the membranes(4) makes the uptake of phosphorylated compounds possible.

During our studies of the metabolism of cancer cells the question arose whether intact neoplastic cells are able to take up and metabolize 2-phosphoenolpyruvate (PEP) added to the medium. PEP is a direct precursor of pyruvate (PA) in the Embden-Meyerhof glycolytic pathway. It holds a key position in carbohydrate metabolism at a branching point, where glycolysis is coupled to the citric acid cycle(5), and it deserves, therefore, the attention of the experimental oncologist. The purpose of the present work was to elucidate the question whether intact malignant cells are able to take up and utilize 2-phosphoenolpyruvate.

Material and methods. The cells studied in the present investigation were the following:

1. A near-diploid line of the Ehrlich's ascites tumor (ELD), which was carried in inbred mice of the StA and the DBA 2 strain.

2. Yoshida's ascites tumor carried in inbred Wistar rats.

3. Earle's strain L-929 fibroblasts propagated *in vitro* in a medium (Fig. 1 a) composed of 20 per cent horse serum, and 80 per cent Tyrode's solution fortified with yeast extract to yield a final concentration of 50 mg%. The final glucose concentration was 5 mM. Penicillin and Streptomycin were added at concentrations of 250 i.u. and 25 micrograms per ml respectively. The medium was changed 3 times weekly, and each time the cultures were gassed with a mixture containing 5 per cent CO_2, 20 per cent O_2, and 75 per cent N_2. Cultures selected for biochemical studies received new medium 24 hours before the experiment. The cells were removed mechanically from the culture flasks without the use of trypsin.

Extensive studies to be reported elsewhere showed that although the L-929 fibroblasts do not produce any tumors in adult animals they cause progressive and metastasizing tumor growth in newborn C_3H mice. Thus, they

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may be classified as tumor cells of low degree malignancy.

Both *in vivo* and *in vitro* propagated cells were washed twice in Krebs-Ringer phosphate buffer before use. The experiments were carried out both with whole cells suspended in Krebs-Ringer phosphate or bicarbonate at pH 7.4 and with homogenates. The latter were prepared in a Potter-Elvehjem homogenizer. The cells were first frozen in Krebs-Ringer bi-carbonate buffer. Homogenization was then carried out during the subsequent process of thawing. This procedure was repeated twice.

Cells and homogenates in an amount corresponding to about 2.5 mg of protein were incubated in conical centrifuge tubes or Warburg vessels at 37°C in the presence of the trisodium salt of PEP, and the sodium salt of ADP both purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. In some experiments glucose (Merck, Darmstadt, Germany) was used as a substrate. After 30 or 60 minutes of incubation with air as the gas phase, the reactions were stopped by adding equal volumes of 0.6 M perchloric acid to the samples. The deproteinized centrifugates were used for further estimations.

PEP concentration was determined by the spectrophotometric measurements at 340 mμ of NADH oxidation(6,7) in the presence of pyruvate kinase (PK) and lactodehydrogenase (LDH). The reaction medium contained 0.1 ml of the deproteinized sample, 1.9 ml of 0.05 M triethanolamine buffer (pH 7.6), 0.02 ml 2 M KCl/0.5 M $MgSO_4$, 0.05 ml 14 mM NADH, 0.02 ml 0.1 M ADP, 0.05 ml lactodehydrogenase(0.75 mg LDH enzyme protein per ml), and 0.02 ml pyruvate kinase (1 mg enzyme protein per ml). For the spectrophotometric measurements 1 cm cuvettes were used. PEP utilization was calculated in terms of micromoles per mg of protein per hour.

The amount of PA produced was estimated by the spectrophotometrical determination of NADH oxidation at 340 mμ(8) in 1 cm cuvettes with a mixture containing 0.1 ml of the deproteinized sample, 1.9 ml 1.1 M K_2HPO_4, 0.05 ml LDH (0.75 mg protein per ml), and 0.05 ml 14 mM NADH. PA production was calculated in terms of micromoles per mg protein per hour.

Inorganic phosphate (P_i) was estimated according to the method described by Lowry-Lopez(9). The results were calculated in terms of micromoles per mg protein per hour.

Cell protein content was estimated with a Folin-Ciocalteau reagent as described by Oyama and Eagle(10).

Oxygen uptake was measured with conventional Warburg technique(11) in a Krebs-Ringer phosphate buffer at pH 7.4 with KOH in the central well. Each vessel contained cells corresponding to about 2.5 mg of protein. Substrates were added from the side arm to yield a final concentration of 5 mM. The manometric readings were made after 20 minutes of equilibration at 10 minute intervals for 1½ hour.

Lactodehydrogenase and pyruvate kinase were purchased from Boehringer und Soehne, Mannheim, Germany; the other reagents were Sigma products.

Results. The estimation of cellular PEP uptake was based on studies of the influence

TABLE I. Influence of Glucose and 2-Phosphoenolpyruvate (PEP) on Cellular Respiration.

		O_2 uptake (μmoles/mg protein/hr)			
Cell line	Substrate:	Endogenous	5 mM glucose	5 mM PEP	5 mM PEP + 5 mM ADP
Yoshida asc. tumor	Mean ± S.E.	0.531 ±0.033	0.413 ±0.017	0.488	0.596
	Per cent change	—	−22.5	−8.2	+11.2
	No. of experiments	6	6	3	3
ELD asc. tumor	Mean ± S.E.	0.384 ±0.019	0.257 ±0.015	0.342 ±0.016	0.423 ±0.019
	Per cent change	—	−33.2	−11.0	+11.0
	No. of experiments	12	11	11	4
Strain L-929 fibroblasts	Mean ± S.E.	0.618 ±0.026	0.499 ±0.032	0.566 ±0.027	—
	Per cent change	—	−19.4	−8.2	—
	No. of experiments	12	6	7	—

TABLE II. Utilization of 2-Phosphoenolpyruvate (PEP) by Intact ELD Ascites Tumor Cells.

Substrates and co-factors added to reaction medium (μmoles/2 ml)					
PEP, 10 μmoles	PEP, 10 μmoles ADP, 10 "	Glucose, 10 μmoles	Glucose, 10 μmoles PEP, 10 "	Glucose, 10 μmoles PEP, 10 " ADP, 10 "	
PEP utilization (μmoles/mg protein/hr)					
Mean values ± S.E.	0.440 ± 0.129	1.164 ± 0.162	0.162 ± 0.025	0.576 ± 0.103	1.374 ± 0.104
No. of experiments	13	10	5	6	6

of PEP on cell respiration, PEP utilization, and PA formation in intact cells.

The effect of PEP on cell respiration is shown in Fig. 1 and Table I, from which it

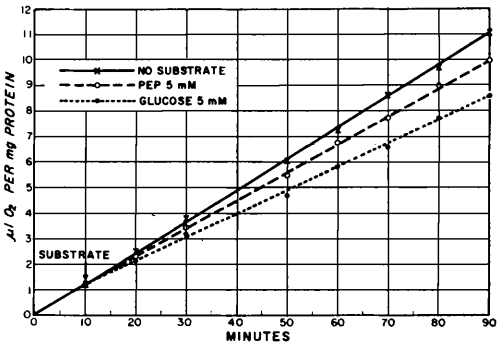

FIG. 1. Influence of phosphoenolpyruvate (PEP) and glucose on the respiration of Ehrlich's ascites tumor cells.

appears that PEP in all 3 cell lines exerted a depressing effect on cell respiration. However, this effect was only 33-42 per cent of that seen after the addition of glucose. Table I also shows that PEP in the presence of ADP not only was without any inhibitory effect on celluar respiration, but even showed a slight stimulation.

PEP utilization was measured as described. The results are presented in Table II, from which it is seen that intact ELD cells utilized PEP added to the medium. It is also seen that this utilization was greatly stimulated when the medium was fortified with ADP. These observations were made both in the presence and in the absence of glucose.

In order to rule out the possibility that the decrease in PEP concentration was due to hydrolysis the changes in P_i concentration were followed. As shown in Table III only minute amounts of P_i accumulated in the ELD cells and the L-929 fibroblasts in the absence of ADP. In the presence of ADP a considerable increase in P_i accumulation was seen indicating the formation of ATP and its dephosphorylation by ATP-ase.

PEP utilization was accompanied by the accumulation of PA as shown in Table IV. As it is seen only small amounts of PA accumulated in the ELD cells and in Earle's strain L-929 fibroblasts in the absence of exogenous ADP. However, when ADP was

TABLE III. Accumulation of Inorganic Phosphate in Tumor Cell Suspensions in the Presence of PEP, Glucose, and ADP.

Substrates added in amounts of 10 μmoles/2 ml	Change in inorganic phosphate conc. (μmoles P_i/mg protein/hr) ELD-asc. tumor cells No. of experiments	Mean ± S.E.	Strain L-929 fibroblasts No. of experiments	Mean ± S.E.
PEP	13	+0.062 ± 0.025	4	+0.016 ± 0.055
PEP + ADP	11	+2.650 ± 0.211	4	+0.744 ± 0.069
Glucose	6	−0.238 ± 0.020	—	—
PEP + glucose	6	+0.394 ± 0.033	—	—
PEP + glucose + ADP	5	+2.880 ± 0.086	—	—

TABLE IV. Metabolism of 2-Phosphoenolpyruvate—Pyruvate Formation in Intact Tumor Cells.

Substrates added in amounts of 10 μmoles/2 ml	Pyruvate accumulation (μmoles/mg protein/hr) ELD-asc. tumor cells No. of experiments	Mean ± S.E.	Strain L-929 fibroblasts No. of experiments	Mean ± S.E.
PEP	7	0.033 ± 0.005	4	0.024 ± 0.009
PEP + ADP	12	1.289 ± 0.122	4	0.692 ± 0.163
Glucose	6	0.114 ± 0.044	—	—
PEP + glucose	6	0.044 ± 0.011	—	—
PEP + glucose + ADP	6	1.732 ± 0.345	—	—

added to the medium, a considerable increase in PA accumulation was observed both in the presence and in the absence of glucose.

Finally a comparison was made between PEP utilization and PA formation in intact cells and in their homogenates. Fig. 2 a and b show that initially the rate of PEP utilization and PA formation in intact and homogenized ELD cells in the presence of ADP was the same. However, after a few minutes of incubation the rate of PEP utilization and PA formation in the intact cells decreased.

Discussion. The results of the present investigations indicate that PEP and ADP as well may be taken up by intact tumor cells. The mechanism of this uptake is unknown. Hydrolysis was ruled out as an explanation of PEP utilization, but it cannot *a priori* be excluded that the reaction of PEP and ADP takes place on the surface of the cells, or that the permeability of the membrane depends on cell damage. If this was the case PEP utilization and PA formation would be expected to follow a linear curve as in the homogenized material. Only the rate of the reactions might be lower. However, PEP utilization and PA formation followed a hyperbolic curve, indicating the presence of a limiting factor which is removed by homogenization. It seems reasonable to assume that this factor is associated with the intact cell membranes, and since clear signs of pinocytosis were frequently observed in cultures of Ehrlich's ascites tumor cells and of strain L-929 fibroblasts in this laboratory other explanations for PEP uptake in the present cells may not be needed. If this is so PEP should only pass the membrane in one direction, and the factors which control pinocytosis(12) would also control PEP and ADP uptake. Comparative studies of PEP utilization and PA formation in intact cells and in their homogenates only revealed quantitative differences regarding the velocity of the reaction, as one would expect if the above mentioned explanation for PEP and ADP uptake in intact cells is correct.

Our observations also show a high rate of pyruvate kinase activity in the ELD and the L-929 cells. Under the condition of the present experiments ADP was found to be a rate-limiting factor, which points to the competition for a phosphate acceptor as the explanation of the depressing effect exerted by PEP on cell respiration. This would be in agreement with the explanation usually given (13) for the Crabtree effect(14), and it explains why the effect of PEP is smaller than the effect shown by glucose, which requires

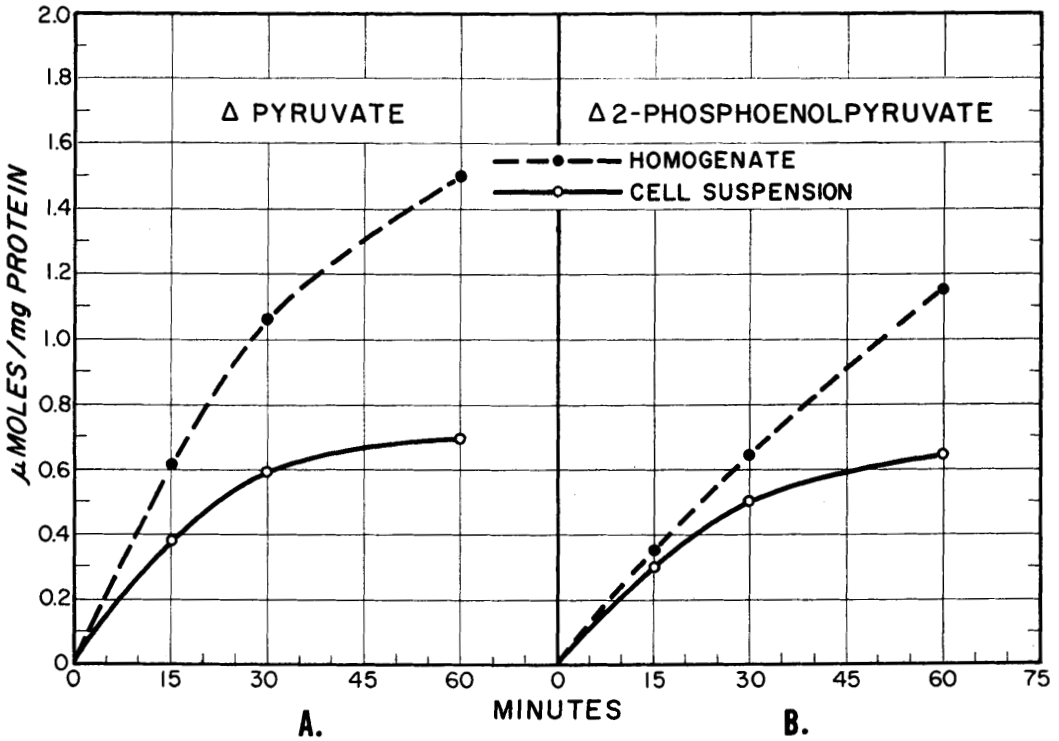

FIG. 2. Pyruvate accumulation (a) and phosphoenolpyruvate utilization (b) in homogenized and intact Ehrlich's ascites tumor cells.

two molecules of ADP for each molecule required by PEP for dephosphorylation to PA.

Summary. Exogenous PEP was found to be taken up and metabolized to PA by intact tumor cells. Pinocytosis was considered to be the most likely mechanism of uptake and at the same time to be a limiting factor. ADP was shown to be a limiting factor in PEP metabolism.

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