A DIRECT STUDY OF INTRACELLULAR GLYCOLYSIS IN EHRLICH's ASCITES TUMOR

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SUMMARY

The use of high specific activity tracers combined with 2-dimensional paper chromatography and rapid sampling techniques has permitted a direct study of the kinetics of glycolysis within the ascites tumor cell. It has been shown that the glycolytic intermediates undergo characteristic transient concn. changes directly following the feeding of glucose, and that these transient changes give evidence of the manner in which the cell ultimately achieves steady state. The evidence indicates that hexokinase activity is governed by product inhibition, and phosphohexokinase activity by an inhibition which is in accordance with the concn. of ATP.

INTRODUCTION

For historical reasons our view of glycolysis has been compartmentalized and relatively little work has been concerned with the direct study of glycolysis inside the intact cell. One exception has been the work of CHANCE whose spectral studies have led him to measure the oxidation state of cofactors and cytochromes inside the cell during glycolysis, even during the short period of transition that the cell undergoes when it is abruptly given glucose substrate. CHANCE AND HESS studied this transition in some detail with ascites tumor cells, where they observed transient states in cytochrome *b* and DPN oxidation, and in glucose and oxygen uptake during the first minute[1]. They presented several theories to explain the way in which the cell ultimately regulates the rate of sugar utilization.

Our expts. on Ehrlich's mouse ascites tumor cells have borrowed some of the techniques used by CALVIN and WILSON and some of their associates for the study of photosynthesis in algal cell suspensions[2]. With the aid of tracers, rapid killing, and 2-dimensional paper chromatography, we have tried to look into the glycolytic system while it undergoes the transition between no net activity and active glycolysis. In order to be able to make chromatographic separation of our extracts on paper, samples had to be kept below 30 μl in size, as larger volumes of physiological salt solution contain amounts of salts that cannot be accommodated without serious streaking. Conventional methods of desalting were not adequate for use in these studies as organic phosphates had to be retained. To get sufficient radioactivity into such small samples, our expts. employed high specific activity [^{14}C]glucose or high activities of [^{32}P]phosphate.

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METHODS

Tumor

Our substrain of the Ehrlich mouse ascites tumor was carried in adult C57 black mice by passage every 8 days of 0.1 ml inocula I.P. Material for reinnoculation was removed through the exposed but intact peritoneum with a sterile syringe and used directly. Grossly hemorrhagic tumor was discarded. Tumor for metabolic expts. was removed to 5 vols. of cold buffer and washed twice by centrifugation in a table top International centrifuge (power on for 40 sec). This effectively removed any contaminating red cells and replaced the ascites serum with buffer of known composition[3]. The tumor was resuspended to approximately 10 % by vol., and used as such. All cell volumes were measured by centrifuging samples for 15 min in hematocrit tubes.

Buffer and substrates

All solutions were made up to physiological tonicity and pH. The [^{14}C]glucose expts. all employed sodium-phosphate LOCKE's solution[4], as suspending medium. The [^{32}P]phosphate expts. employed a suspending medium containing a bicarbonate buffer but similar cation concentrations as in LOCKE's solution, excepting Mg^{++} and Ca^{++}, whose concns. were increased in keeping with their increased solubilities in the presence of lowered amounts of PO_4^{-3}. The buffer was gassed with 5 % CO_2 in air before and during use. The composition in mmoles/l is as follows: $NaHCO_3$-12, Na_2HPO_4-1, NaCl-132, KCl-5.5, $CaCl_2$-1, $MgCl_2$-0.6.

Radioactive substrates

220–240 μC/mg glucose was obtained in part from Dr. E. W. PUTMAN and more was synthesized by a modified method of PUTMAN AND HASSID[5]. This synthesis involves the exposure of an excised canna lily leaf to CO_2 of 30 % ^{14}C content for 18 h with illumination. Following this, the photosynthesized sucrose is isolated chromatographically on paper in 2 separations with different solvents (phenol–water and butanol–propionic acid–water). The sucrose is then inverted and the resulting mixture of fructose and glucose is separated again by 2-dimensional chromatography. The glucose may be stored frozen in water with about 2 % impurities being formed by decomposition in nine months. Radioactive phosphate was obtained from Donner Laboratory Clinic in neutral isotonic solution and contained about 0.02 μg carrier phosphorus/μC ^{32}P. Over 100 μC were used to label 1 ml of cell suspension.

Automatic rapid sampler

Rapid sampling of the cell suspension was made possible with the aid of a device built around a modified 3-way pyrex stopcock (see Fig. 2). The spring loaded inner plug (a) may be rotated by hand or engaged to a motor driven gear train and rotated at 1/3 rev./sec or slower. The hole in the plug (h) is of 25 μl volume. It is first sucked empty (groove(s) leads to air) and then partially evacuated by a vacuum line (d). As it comes into contact with the incubation chamber (e) it fills with cell suspension, and it is then flushed of contents with distilled water under about 6 ft. pressure (c) ejecting the sample (through (b)) into a tube of hot ethanol. The time of ejection is electrically recorded as a conductivity increase between two electrodes (l) with the

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aid of an Esterline Angus recorder. The vols. employed have been such that the final suspension of killed cells is approximately 80 % ethanol; convection rapidly mixes the suspension. The entire apparatus is enclosed in a temperature jacket and is also

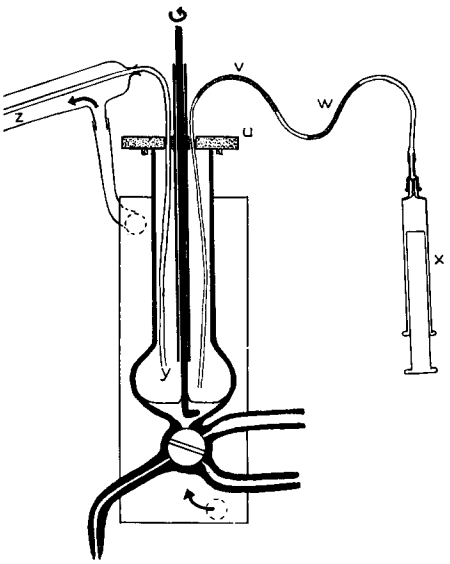

Fig. 1. Diagramatic cross-section of the Automatic Rapid Sampler (see text).

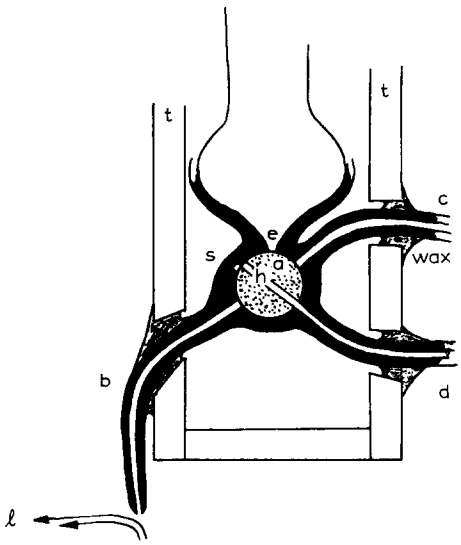

Fig. 2. Diagramatic cross-section of the Automatic Rapid Sampler (see text).

equipped with a gas line (y) when gassing is necessary, (see Fig. 1). Also thin plastic tubes are loaded with measured μvols. of substrate solution (v) and buffer rinse (w), so that substrate may be added by merely pressing a gas filled syringe (x). The tubes of ethanol are held in an electrically heated aluminum block which is moved under the sampler by a solenoid shuttlebar synchronized with the turning of the stopcock plug by a microswitch riding a cam at the back of the sampler. In this way micro-samples were killed at as short as 1.6 sec intervals in the following expts.

Analytic

Combined 80 and 20 % ethanol extracts of each sample were evaporated *in vacuo* with the aid of the "Octopus" multiple sample evaporator[6] and transferred with several rinses to the origin of a chromatogram on oxalate washed Whatman No. 4 filter paper. An aliquot is also removed and counted to determine the total activity in each sample. Loss due to sticking of sugar phosphates was shown to be less than 2 % if clean acid washed glassware was used throughout. The chromatograms were developed first in phenol solvent[7] and then in the second dimension in butanol–propionic acid solvent[7]. Fig. 3 shows a radioautogram of an extract of cells exposed to [^{14}C]glucose and separated 30 and 20 h in phenol and butanol–propionic acid solvents. The spots have been treated with ammonium sulfate fractionated polidase[8] directly on the paper by the method of WILSON[6], then eluted onto the origin of another chromatogram and re-run with carrier sugars. The identities that were found in samples from the cells exposed for short periods of time to glucose are given under

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the figure. If the chromatograms are developed for shorter periods of time the glucose and lactic acid (which must be sprayed with alkali to be retained) may also be separated and counted. In the ^{14}C work, counts were averaged from both sides of the paper. ^{32}P samples were handled in a similar manner, excepting that the chromatograms were developed for 40 and 24 h. Figs. 4 and 5 show radioautograms of separated

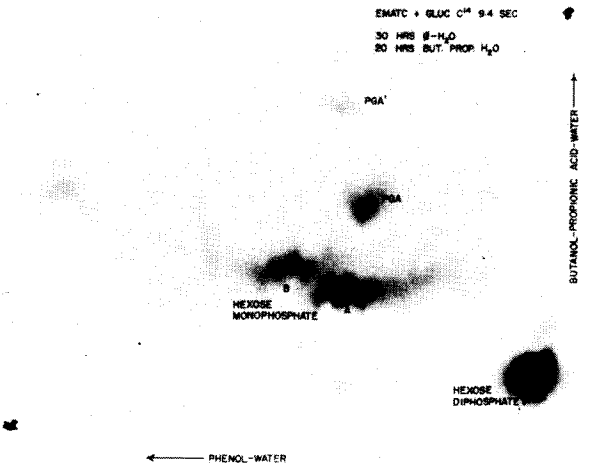

Fig. 3. Radioautogram of a chromatogram of an extract of ascites tumor cells exposed to [^{14}C]-glucose for 9.4 sec. "Hexose diphosphate" contains mostly fructose with up to a few percentage glucose and sedoheptulose and up to 1 % glyceric acid. "Hexose monophosphate" contains, during the first half min of exposure, up to 80 % glucose, 20 % fructose, 10 % mannose, 2.5 % gluconic acid and 2 % sedoheptulose (as confirmed by conversion to sedoheptulosan). It is found to be 2 spots, A and B. A contains mainly glucose, sedoheptulosan and gluconic acid, and B contains fructose, mannose and more glucose. PGA sometimes double spots, but both spots give the same spot when they are eluted and re-run with carrier.

extracts of samples taken before and after the addition of nonactive glucose to the labeled cells. The qualitative identification of all spots rests upon cochromatography in 3 separate solvent systems (the 2 above and propanol–ammonia solvent[9]). Also the nucleotides were confirmed by their retention on acid washed Darco G-60 and by electrophoresis in pH 3.4 propionate buffer. The sugar phosphates were shown to be the same compounds found in the ^{14}C-runs in a double labeled expt. Identification of the [^{32}P]nucleotides was made difficult by their "sticky" nature and their variable chromatographic behavior in the very small amounts that were present in the samples.

Inorganic phosphate was measured by the method of LOWREY AND LOPEZ[10].

RESULTS

It has been shown that the glycolytic intermediates of prestarved cells (see Fig. 6, etc., for conditions) undergo a characteristic and reproducible behavior during the

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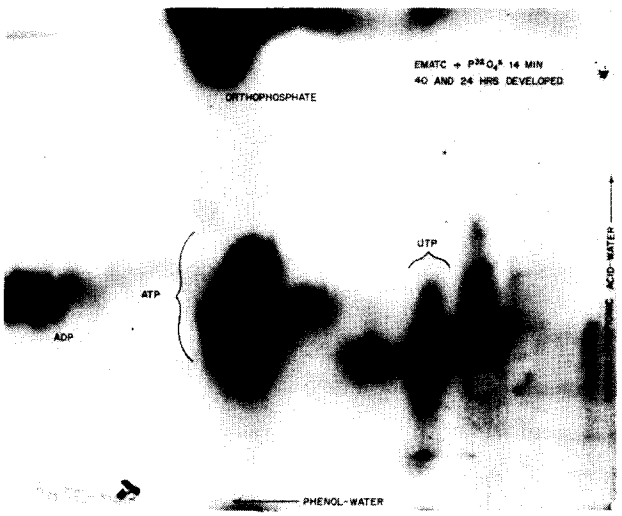

Fig. 4. Radioautogram of the chromatogram of an extract of ascites tumor cells exposed to ^{32}P for 14 min. The ATP spot is composed of ATP and ADP arising from ATP during the development in the second solvent.

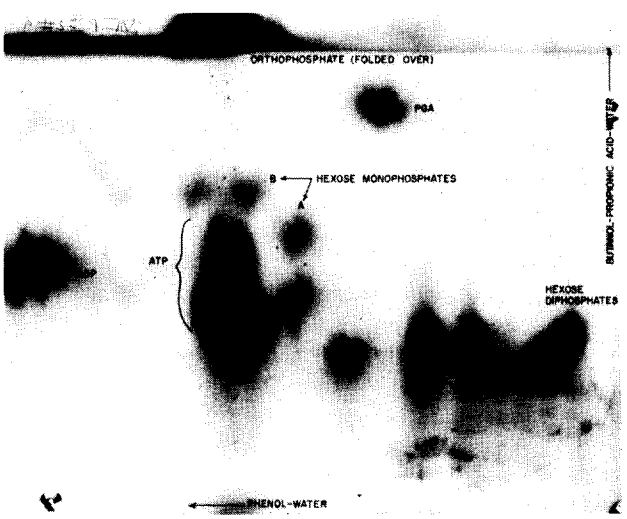

Fig. 5. Radioautogram of the chromatogram of an extract of ascites tumor cells exposed to ^{32}P for 15 min and to glucose for 5.8 sec. The sugar phosphates are the same as found in the [^{14}C]-glucose expts.

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half minute following feeding of glucose substrate. This behavior consists of transient concentration changes, and therefore in transient changes in the relative rates at which the cell conducts the various enzymic steps involved in glycolysis. That any transient behavior can be measured testifies to the rapid entry of glucose into the cell, an entry that is more rapid than the subsequent utilization. This was also found to be the case for higher concentrations of glucose than used in these expts. by CRANE, FIELD AND CORI[14].

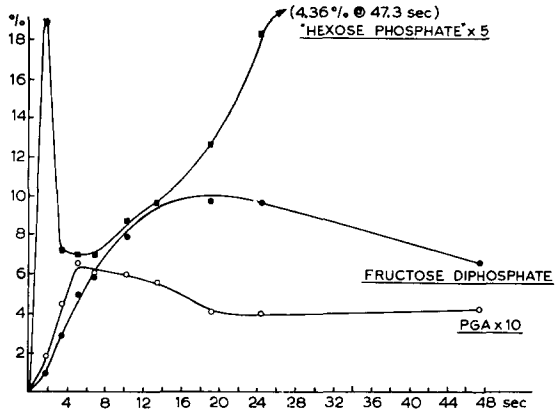

Fig. 6. Appearance of some ^{14}C-labeled compounds in mouse ascites tumor cells following [^{14}C]-glucose exposure. About 300 μg of [^{14}C]glucose was added at zero time to 113 μl of cells that had been incubated 7 min in sodium phosphate LOCKES[4]. The total volume was 1.1 ml and the temperature was 37°. Percentage of total ^{14}C is plotted against time in sec.

Fig. 6 shows an expt. in which [^{14}C]glucose was fed to glucose-starved cells. The observed behavior is entirely reproducible. There is first a rapid transient peak in "hexose monophosphates" lasting but a few seconds. (When the temperature was lowered by 15°, however, this first peak was found to last about 9 sec, and was thus caught in more than one sample.) This was followed by a peak in PGA concn. and then a peak in hexosediphosphate concentration. Hexosemonophosphate rises steeply after 10 sec.

We can be reasonably sure that the activity of each compound is proportional to the concentration of that compound within the cell. (This is substantiated by ^{32}P expts. which show that the concn. of sugar phosphates is very low before the addition of glucose.) Taking, then, the packed volume of the cells used and the μg of glucose fed, we may calculate the concns. of the intermediates counted. At 10.2 sec hexosediphosphate is 1.2 mM*, and PGA is 0.2 mM, in the case of the latter compound we must take into consideration the fact that it contains half the number of carbon atoms of glucose. At 47 sec hexosemonophosphate reaches 0.7 mM concn. It should be remembered that these concns. are calculated for the cell as a whole, and, if there are regions of the cell impermeable to the sugar phosphates, the

* The poolsize of hexose diphosphate at its maximum concn. is large relative to the rate of flux. The given concns. of the intermediates are roughly 10 times those that would be calculated for the suspension as a whole, as the cells are about 10% by vol. The glucose given to the cells is 1.7 mM and is used up in about 7 min. (Actually the rate of glycolysis is faster during the first 20–30 sec as shall be mentioned later.)

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cytoplasmic concn. will be higher. Also, then it is seen that any given sample in these expts. contains only a fraction of a μg of these compounds.

Further expts. were made with cells labeled by incubation with [^{32}P]*ortho*-phosphate. Fig. 7 shows the kinetics of some of the ^{32}P compounds in such an expt.

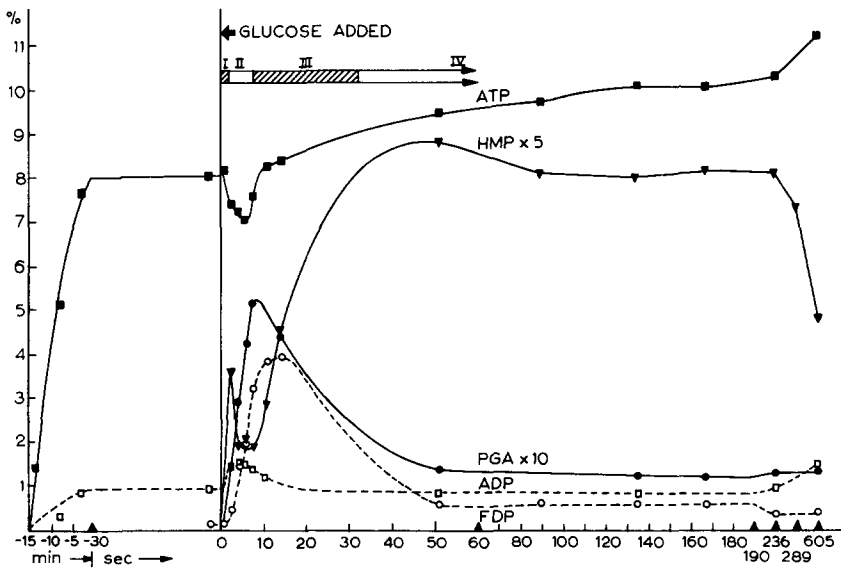

Fig. 7. Appearance and changes in some ^{32}P compounds in mouse ascites tumor cells as caused by addition of glucose. 300 μg of glucose added at zero time to 107 μl of cells that had been incubated for 15 min with [^{32}P]orthophosphate (120 μC) in bicarbonate buffer containing 8 mM lactate substrate. The concn. of inorganic phosphate was about 3.2 mM (overall) in the suspension. The total vol. of the suspension was 1.1 ml, and the temperature was 37°. Percentage of total extractable activity is plotted against the time relative to the addition of glucose.

After the addition of glucose there appear the same glycolytic intermediates found in the [^{14}C]glucose expts. and their behavior is identical. There is evidence that the equilibration of ^{32}P in all the organic phosphates of the cell is not complete; the activity in ATP and other nucleotides increases after 20 min. When samples of ADP are reseparated chromatographically, appreciable hydrolysis to active inorganic phosphate and inactive AMP occurs. Also, the increase in activity in ADP upon the addition of glucose is just half the decrease in activity of ATP. Thus it is not unreasonable to assume that most of the activity is in the last 2 pyro-phosphate groups of ATP and that the group attached to the ribose moiety is only slowly equilibrated. Because of the uncertainties in the distribution of labeled phosphorus only a rough estimate of the intracellular ATP can be made; if one assumes that the last 2 phosphates are equilibrated, the concn. of ATP is 2 mM. In the case of ADP only an upper limit of 0.4 mM concn. at its maximum may be suggested, but much of this may arise by hydrolysis of ATP during the workup of the samples.

The sugar phosphates, however, with their rapid turnover and negligible initial concentrations are almost certainly of the same specific activity as the inorganic phosphate (which contains about 75 % of the ^{32}P activity during the expt.). Thus, their activity should be directly proportional to their concn., and these may be

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calculated to be similar to those obtained in the ^{14}C expts. One exception, however, is hexosediphosphate which is about 30 % low: this may be a reflection of the change in the suspending medium in the ^{32}P expts. where a bicarbonate buffer was employed. As well as confirming the labeled glucose expts., the active phosphorus expts. show directly the sudden transient increase in ADP inferred by CHANCE AND HESS from their measurements of cytochrome *b* reduction and oxygen uptake[1]. In these workers' expts., the temperature appears to have been uncontrolled and was presumably at room temperature, and thus the behavior that they observed is slower than ours by a factor of 2 or 3.

TABLE I

TRANSIENT PERIODS IN ASCITES TUMOR GLYCOLYSIS AT 37°

Period	*Approx. times (sec)*	*Extramitochondrial:*		*Activity of:*	
		ATP	*ADP*	*Hexokinase*	*Phospho-hexokinase*
I	0–2	high	low	high	low
II	2–8	lowered	high	high	high
III	8–30	high	low	high	low
IV	> 30	high	low	low	low

DISCUSSION

Our explanation of the observed behavior is based upon one of the less favored theories used by CHANCE AND HESS in the discussion of their findings; namely that hexokinase activity is limited by product inhibition, and that phosphohexokinase activity is limited by an ATP inhibition[1]. To discuss this it is useful to divide the observed behavior following the feeding of glucose into 4 periods (see Fig. 7 and Table VIII).

In the first period, lasting about 1–2 sec, at 37°, the extramitochondrial ADP is low. This is a continuation of the period before the addition of glucose, and little has changed in the cell excepting that hexose monophosphates are rapidly being made. Phosphohexose isomerate is of relatively high activity, and fructose and mannose phosphates have been shown to be present directly.

The second period lasts from 2 to about 8 sec. In this time the ATP concn. in the cytoplasm* may have dropped appreciably, but not enough to slow glucose phosphorylation. However, it may have been dropped enough to release a hypothetical partial inhibition of phosphohexokinase (which if it exists may depend upon the fact that ATP binds Mg^{++} ion, a cofactor[1]). It is interesting to note that BECK has recently shown that addition of ATPase to leukemic cell homogenates already containing additional ADP speeds overall glycolysis[11]. During this second period there is an increased rate of utilization of hexosemonophosphate, the concn. of which is kept low, and large amounts of hexosediphosphate pile up. This is in spite of the fact that during the same time PGA formation may also be speeded by the increased concn. of oxidized DPN[1] and ADP.

* Although the overall concn. of ATP falls only 14 % during period 2, the cytoplasmic (extramitochondrial) concentration[12] may be incompletely equilibrated with the ATP in the mitochondria and thus may have fallen by an appreciably larger amount.

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The third period, between 8 and 30 sec approx., is characterized by a return to normal of ATP and a sharp decrease in oxidized DPN and ADP. These cause a tapering off of PGA excesses by slowing its formation from hexosediphosphate. There is also a decrease in the rate of phosphohexokinase action which we suggest results from increased amounts of cytoplasmic ATP. This decrease eventually exceeds the decrease in hexosediphosphate utilization and hexosediphosphate concn. falls. With the bleeding of the hexosediphosphate pool, the formation of lactic acid slows by a factor of 4, as has been shown in a separate expt. With the decrease in the utilization of hexose monophosphate during this period, its pool size increases rapidly until it reaches 0.6–0.8 m*M* intracellular concn. This is high enough to slow the rate of glucose phosphorylation by means of the familiar product inhibition of animal hexokinase. (For example, WEIL-MALHERBE AND BONE found that m*M* glucose phosphate inhibits rat brain hexokinase 3–4 fold[13].

The fourth period is characterized by a slow controlled rate of glucose phosphorylation as governed by hexosemonophosphate concn. We have seen that hexomonophosphate in turn is controlled by its rate of removal through rephosphorylation, a step which seems to be governed by or in accordance with the ATP concn. ATP is generated in several places in the metabolic machinery, one place being the oxidation of triosephosphate to PGA, a step which in itself is dependent upon the concn. of oxidized DPN and ADP. It is thus not accurate to say that any given step is rate limiting when the system has reached the steady state. Rather we have a system in which several steps are of related activity, controlled by feedback from a later step or steps.

ACKNOWLEDGEMENTS

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