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A DIRECT STUDY OF INTRACELLULAR GLYCOLYSIS IN EHRLICH'S ASCITES TUMOR*

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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 Britton 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 experiments 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,

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rapid killing, and two 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 microliters in size, as larger volumes of physiological salt solution contain amounts of salts that cannot be accomodated 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 experiments employed high specific activity C^{14}-glucose or high activities of P^{32}-phosphate.

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 experiments was removed to 5 volumes of cold buffer and washed twice by centrifugation in a table top International centrifuge (power on for 40 seconds). 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 volume, and used as such. All cell volumes were measured by centrifuging samples for 15 minutes in hematocrit tubes.

Buffer and Substrates

All solutions were made up to physiological tonicity and pH. The C^{14}-glucose experiments all employed sodium-phosphate, Locke's solution,[4] as suspending medium. The P^{32}-phosphate experiments employed a suspending medium containing a bicarbonate buffer but similar cation concentrations as in Locke's

solution, excepting Mg^{++} and Ca^{++}, whose concentrations 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 mM per liter 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 microcuries per milligram 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% C^{14} content for 18 hours with illumination. Following this, the photosynthesized sucrose is isolated chromatographically on paper in two 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 chromatography in two dimensions. 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 micrograms carrier phosphorus per microcurie P^{32}. Over 100 microcuries were used to label one 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 three-way pyrex stopcock (see Fig. 1). The spring loaded inner plug (a) may be rotated by hand or engaged to a motor driven gear train and rotated at 1/3 rps or slower. The hole in the plug (h) is of 25 microliter 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 aid of an Esterline Angus recorder. The volumes 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 equipped with a gas line (y) when gassing is necessary, (see Fig. 2). Also thin plastic tubes are loaded with measured microvolumes 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 shuttleber synchronized with the turning of the stopcock plug by a microswitch riding a cam at the back of the sampler. In this way microsamples were killed at as short as 1.6 second intervals in the following experiments.

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 transfered 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. Losses 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] Figure 3 shows a radioautogram of an extract of cells exposed to a C^{14} glucose and separated 30 and 20 hours 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 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 C^{14} work, counts were averaged from both sides of the paper. P^{32} samples were handled in a similar manner, excepting that the chromatograms were developed for 40 and 24 hours. Figures 4 and 5 show radioautograms of separated extracts of samples taken before and after the addition of non-active glucose to the labeled cells. The qualitative identification of all spots rests upon cochromatography in three separate solvent systems (the two 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 C^{14} runs in a doubly labeled experiment. Identification of the P^{32}-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 presterved cells (see Fig. 6, etc., for conditions) undergo a characteristic and reproducible behavior during the 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 experiments by Crane, Field and Cori.[11]

Figure 6 shows an experiment in which C^{14}-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°C, however, this first peak was found to last about 9 seconds, and was thus caught in more than one sample.) This was followed by a peak in PGA concentration and then a peak in hexosediphosphate concentration. Hexosemonophosphate rises steeply after 10 seconds.

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 P^{32} experiments which show that the concentration of sugar-phosphates is very low before the addition of glucose.) Taking, then, the packed volume of the cells used and the micrograms of glucose fed, we may calculate the concentrations of the intermediates counted. At 10.2 seconds hexosediphosphate is 1.2 mM,** and PGA is 0.2 mM, in the case of the latter

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

compound we must take into consideration the fact that it contains half the number of carbon atoms of glucose. At 47 seconds hexosemonophosphate reaches 0.7 mM concentration. It should be remembered that these concentrations are calculated for the cell as a whole, and, if there are regions of the cell impermeable to the sugarphosphates, the cytoplasmic concentration will be higher. Also, then it is seen that any given sample in these experiments contains only a fraction of a microgram of these compounds.

Further experiments were made with cells labeled by incubation with P^{32} ortho-phosphate. Figure 7 shows the kinetics of some of the P^{32} labeled compounds in such an experiment. After the addition of glucose there appear the same glycolytic intermediates found in the C^{14}-glucose experiments and their behavior is identical. There is evidence that the equilibration of P^{32} in all the organic phosphates of the cell is not complete; the activity in ATP and other nucleotides increases after 20 minutes. When samples of ADP are re-seperated 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 two 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 two phosphates are equilibrated, the concentration of ATP is 2 mM. In the case of ADP only an upper limit of 0.4 mM concentration 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 P^{32} activity during the experiment). Thus, their activity should be directly proportional to their concentrations, and these may be calculated to be similar to those obtained in the C^{14} experiments. One exception, however, is hexosediphosphate which is about 30% low: this may be a reflection of the change in the suspending medium in the P^{32} experiments where a bicarbonate buffer was employed. As well as confirming the labeled glucose experiments, the active phosphorus experiments 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' experiments, 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 two or three.

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 four periods (see Figures 7 and 8).

In the first period, lasting about 1-2 seconds, at 37°C, 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 isomerase 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 seconds. In this time the ATP

concentration 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 concentration 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 concentration of oxidized DPN[1] and ADP.

The third period, between 8 and 30 seconds approximately, is characterized by a return to normal of ATP and a sharp decrease in oxidized DPN[1] 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 concentration 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 experiment. With the decrease in the utilization of hexose monophosphate during this period, its pool size increases rapidly until it reaches 0,6-0,8 mM intracellular concentration. This is high enough to slow the rate of

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*** Although the overall concentration 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.

glucose phosphorylation by means of the familiar product inhibition of animal hexokinase. (For example, Weil-Malherbe and Bone found that mM 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 concentration. We have seen that hexosemonophosphate 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 concentration. 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 concentration 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.

SUMMARY

The use of high specific activity tracers combined with two 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 concentration 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 concentration of ATP.

BIBLIOGRAPHY

1. B. Chance and B. Hess, Ann. N. Y. Acad. Sci., 63 (1956) 1008.
2. J. A. Bassham and M. Calvin, The Path of Carbon in Photosynthesis, Prentice Hall Inc., Englewood Cliffs, N. J. 1957.
3. R. W. McKee, K. Lonberg-Holm and J. Jehl, Cancer Research, 13 (1953) 537.
4. R. W. McKee and A. J. Walker, Science 118 (1953) 133.
5. E. W. Putman and W. Z. Hassid, Jour. Biol. Chem., 196 (1952) 749.
6. A. T. Wilson and M. Calvin, J. Am. Chem. Soc., 77 (1955) 5948.
7. A. A. Benson, J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas and W. Stepka, J. Am. Chem. Soc., 72 (1950) 1710.
8. S. S. Cohen, Jour. Biol. Chem., 201 (1953) 71.
9. E. L. Bennett, Biochim. Biophys. Acta, 11 (1953) 407.
10. O. Lowry and J. Lopez, Jour. Biol. Chem., 162 (1946) 421.
11. W. Beck, Jour. Biol. Chem., 232 (1958) 251.
12. E. Racker, Ann. N. Y. Acad. Sci., 63 (1956) 1017.
13. H. Weil-Malherbe and A. D. Bone, (Biochem. J. (London), 49 (1951) 339.

TRANSIENT PERIODS IN ASCITES TUMOR GLYCOLYSIS AT 37°C

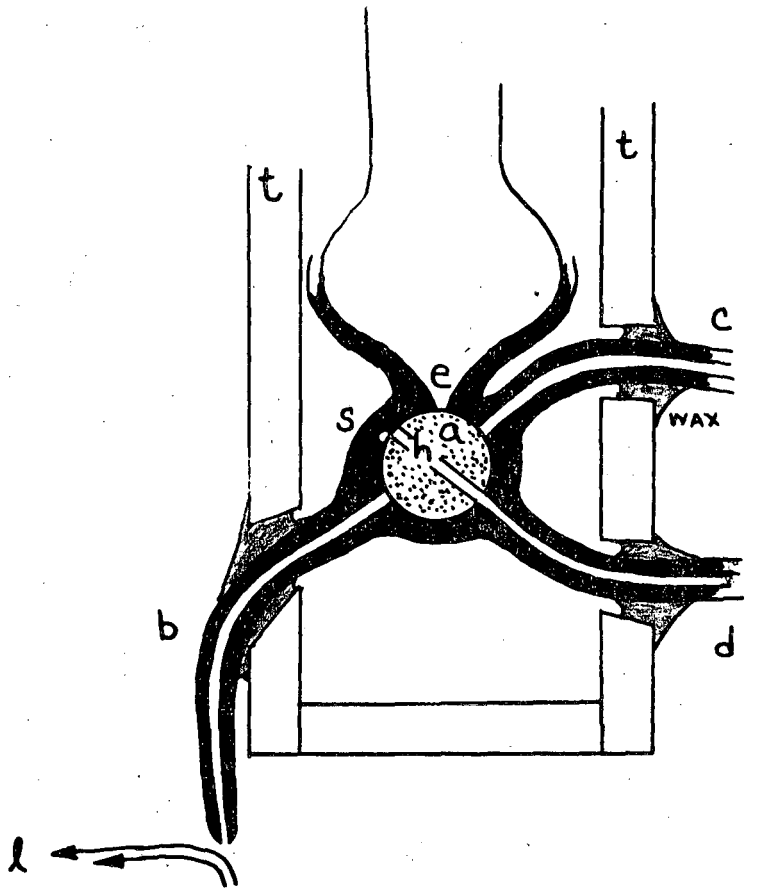
Period	Approx. times (seconds)	Extramitochondrial: ATP	ADP	Activity of: Hexokinase	Phospho-hexokinase
I	0-2	high	low	high	low
II	2-8	lowered	high	high	high
III	8-30	high	low	high	low
IV	> 30	high	low	low	low

Fig. 6. Appearance of some C^{14} labeled compounds in mouse ascites tumor cells following C^{14} glucose exposure.

About 300 micrograms of C^{14} glucose was added at zero time to 113 microliters of cells that had been incubated seven minutes in sodium phosphate Lockes.[3] The total volume was 1.1 ml, and the temperature was 37°C. Percentage of total C^{14} is plotted against time in seconds.

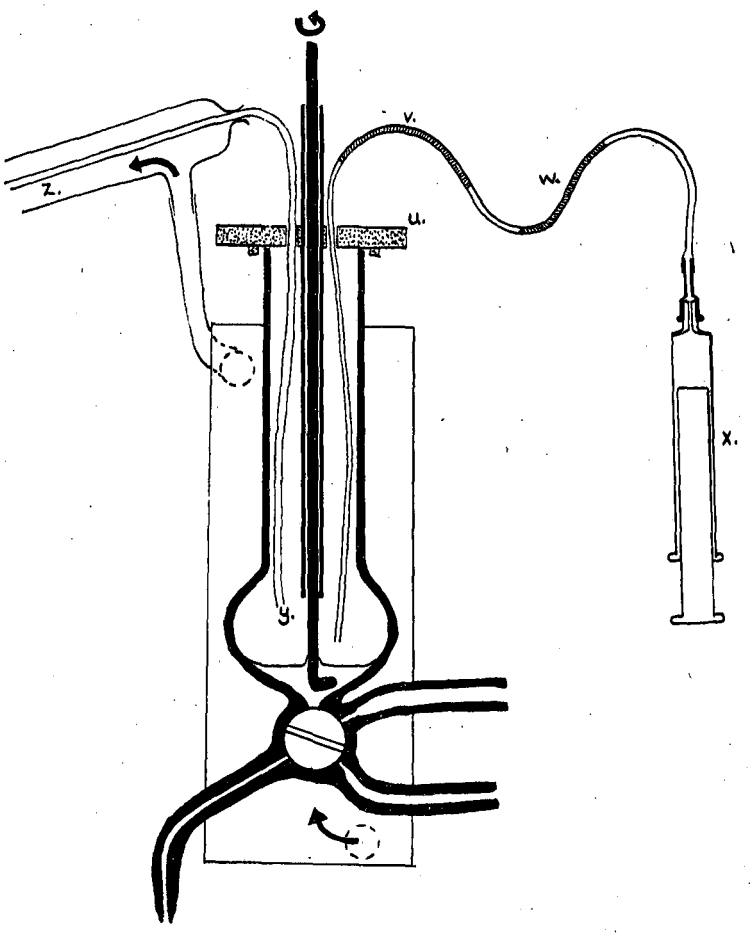
Fig. 7. Appearance and changes in some P^{32} labeled compounds in mouse ascites tumor cells as caused by addition of glucose.

300 micrograms of glucose added at zero time to 107 microliters of cells that had been incubated for 15 minutes with P^{32} orthophosphate (120 microcuries) in bicarbonate buffer containing 8 millimolar lactate substrate. The concentration of inorganic phosphate was about 3.2 millimolar (overall) in the suspension. The total volume of the suspension was 1.1 ml, and the temperature was 37°C. Percentage of total extractable activity is plotted against the time relative to the addition of glucose.



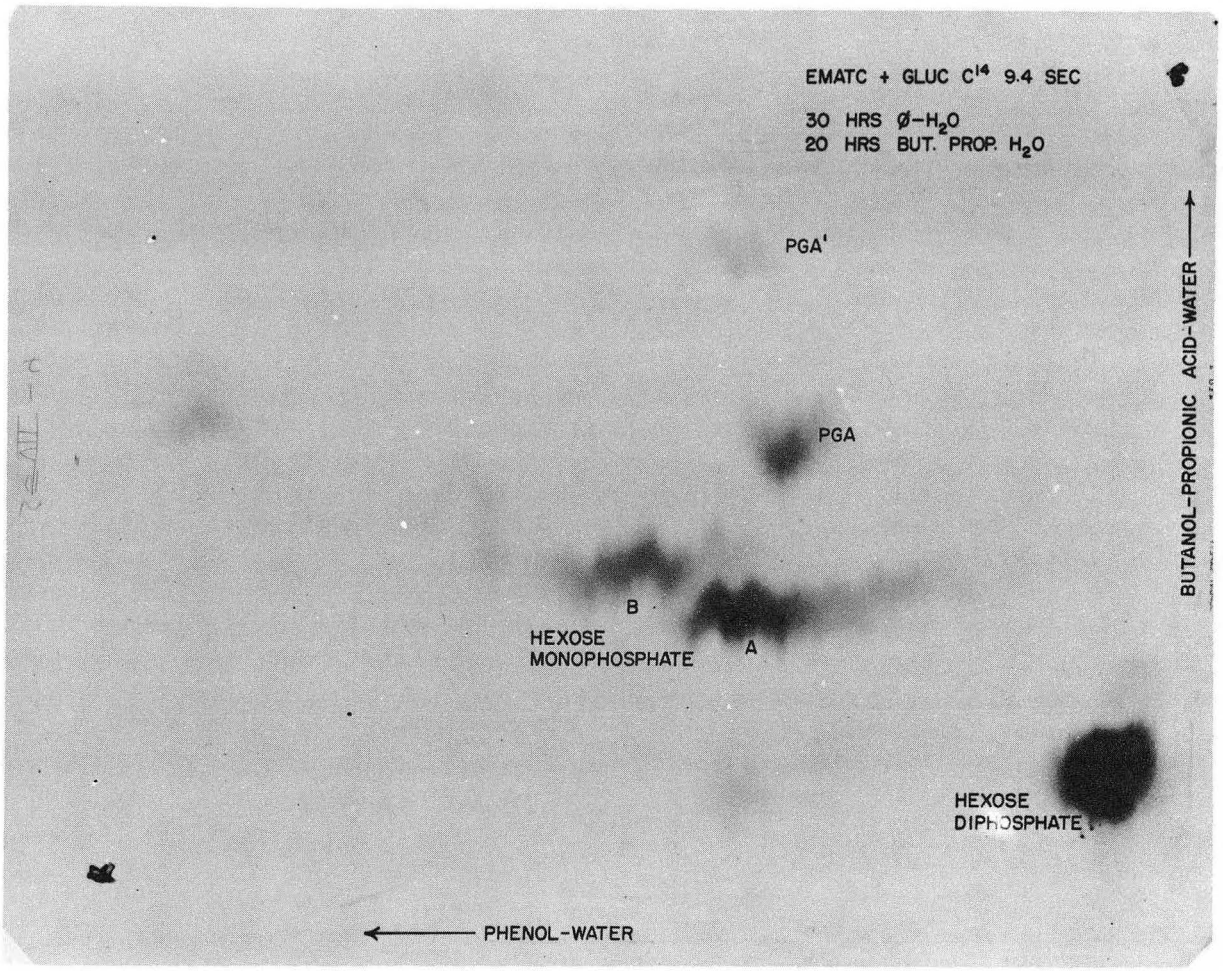
MU-11473

Fig. 1



MU-13015

Fig. 2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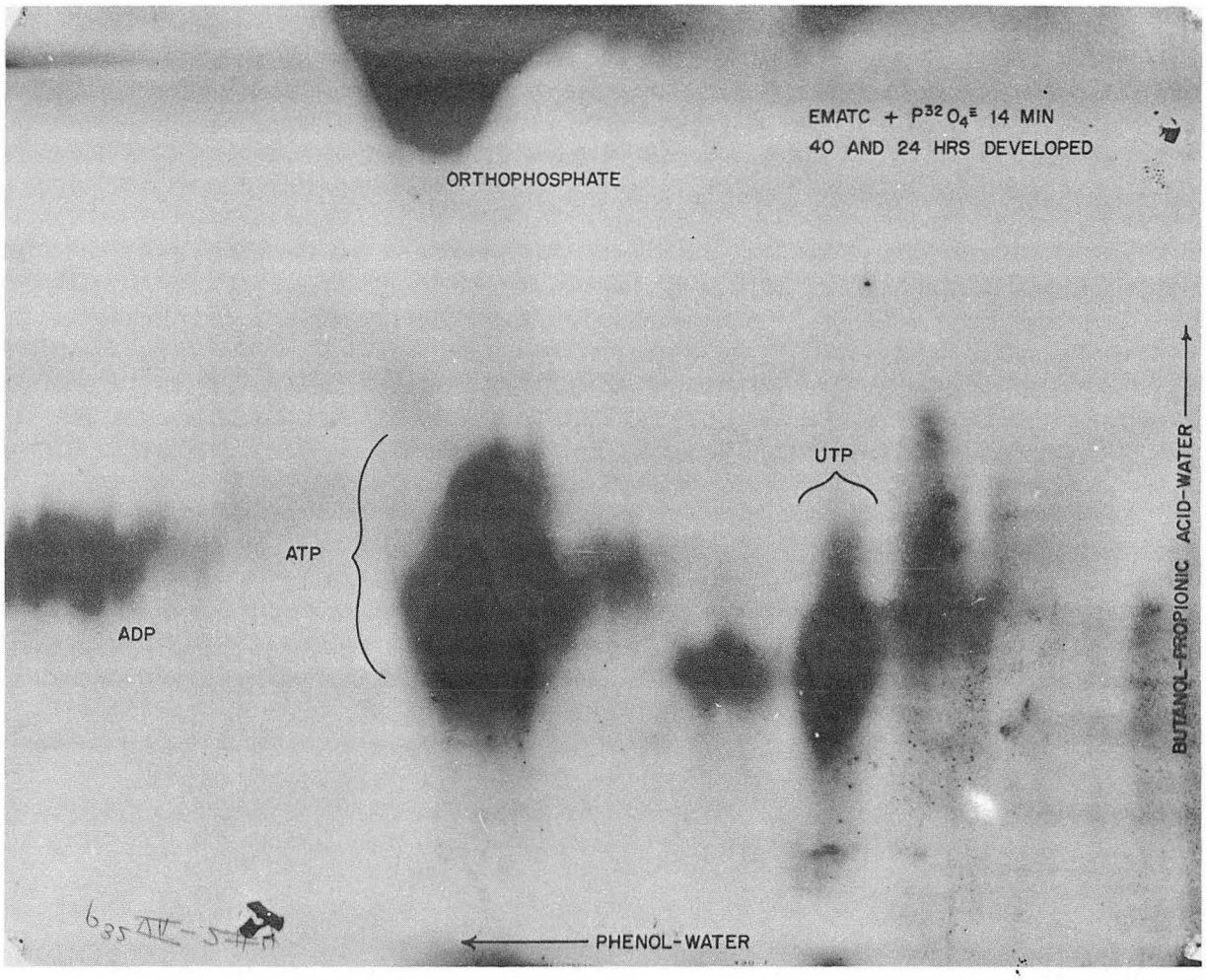
20 HRS BUT. PROP. H2O
PGA'
PGA
B
HEXOSE
MONOPHOSPHATE
A
HEXOSE
DIPHOSPHATE
BUTANOL-PROPIONIC ACID-WATER
PHENOL-WATER

ZN-2108

Fig. 3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40 AND 24 HRS DEVELOPED
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ATP
ADP
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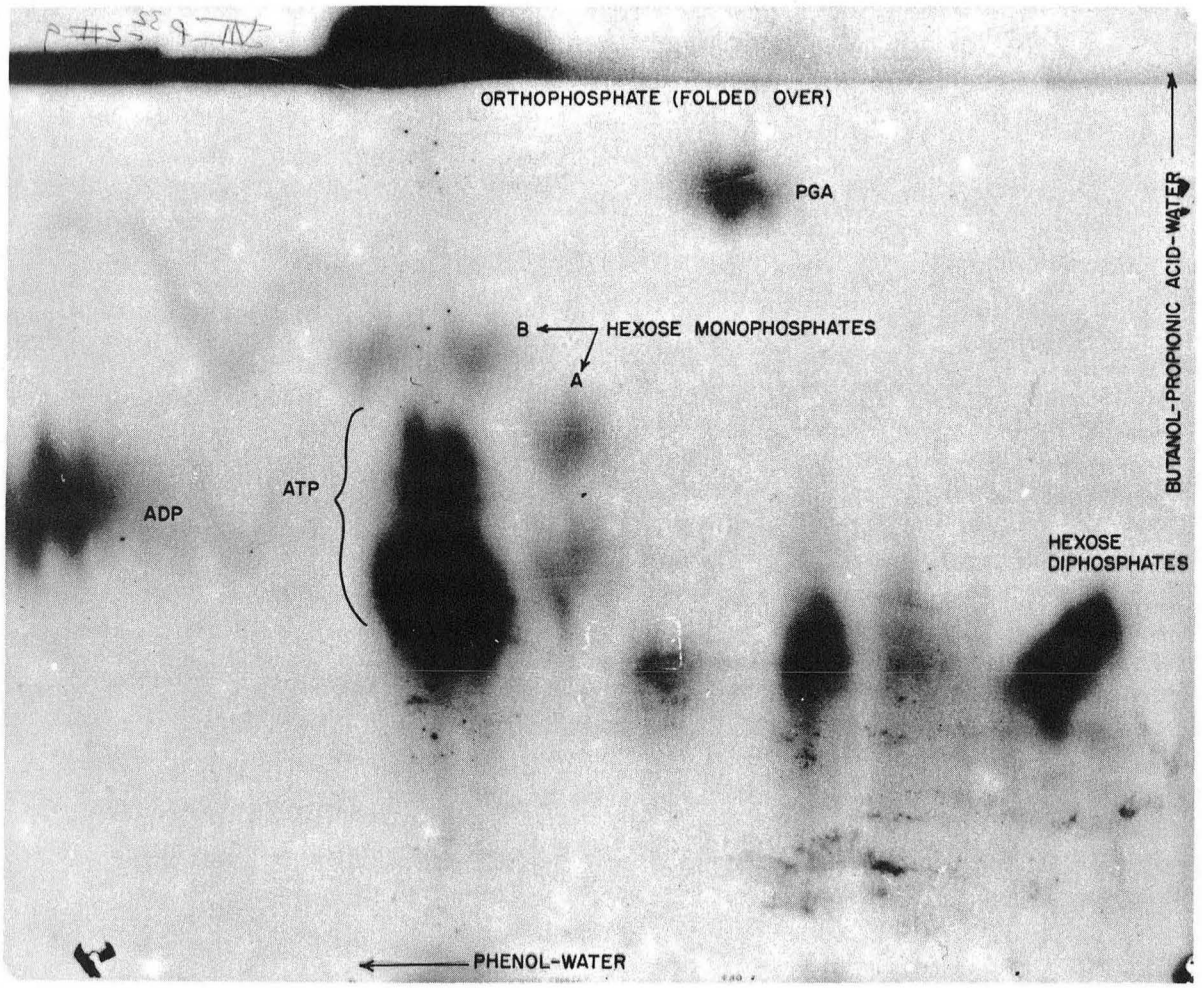
PHENOL-WATER
ZN-2109

Fig. 4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PGA
B
HEXOSE MONOPHOSPHATES
A
ATP
ADP
HEXOSE
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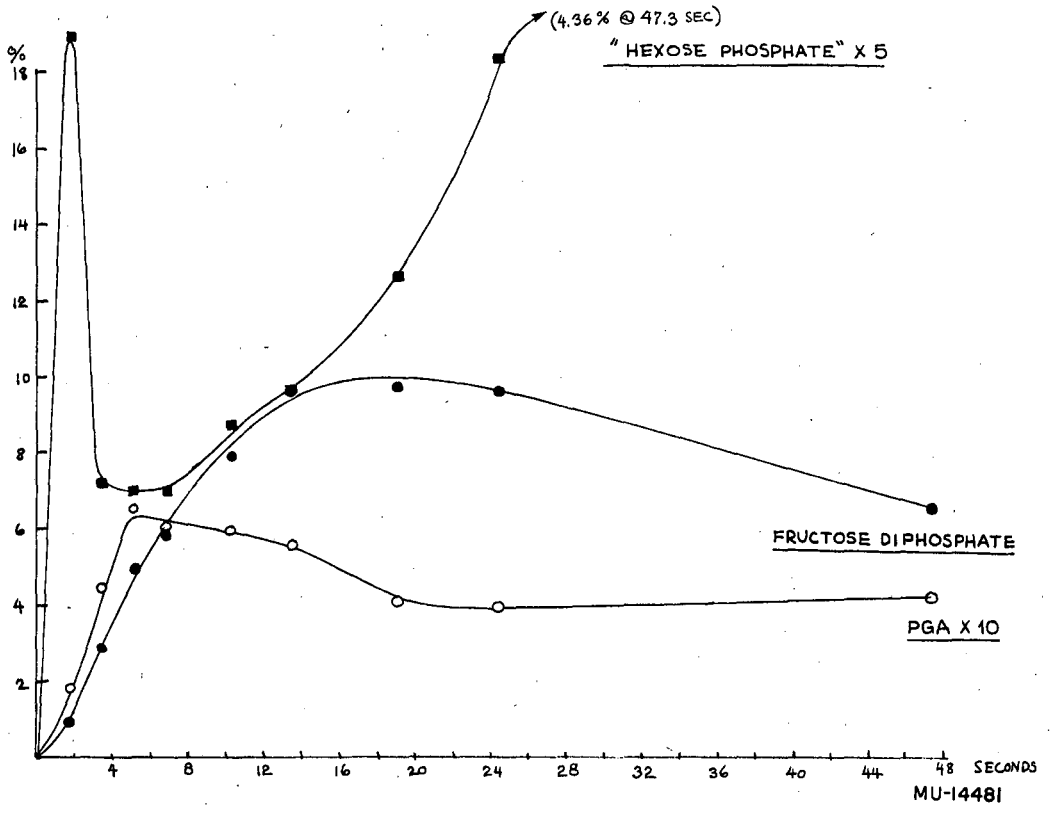
PHENOL-WATER

ZN-2110

Fig. 5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%
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14
12
10
8
6
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2
FRUCTOSE DIPHOSPHATE
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8
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16
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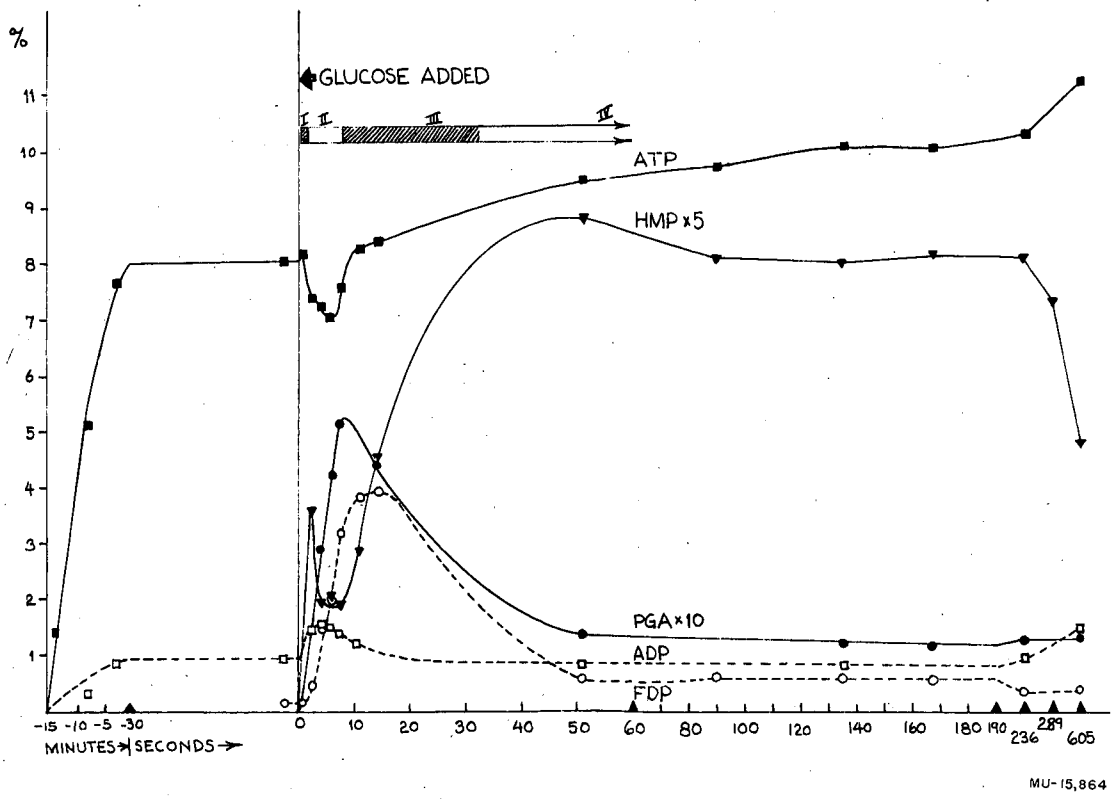
MU-14481

Fig. 6


%
GLUCOSE ADDED
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ATP
HMP x5
PGA x10
ADP
FDP
MINUTES
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-15 -10 -5 -30 0 10 20 30 40 50 60 80 100 120 140 160 180 190 236 289 605
MU-15,864

Fig. 7