Phosphorylated Intermediates in Tumor Glycolysis

IV. Glycolysis in Tumor Homogenates*

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(Received for publication September 10, 1947)

That slices of tumor tissue will produce large amounts of lactic acid has been well known since the early studies in Warburg's laboratory in 1924-30 (10, 20). That tumor glycolysis involves the same intermediate pathway as found in normal tissues has, however, been frequently questioned, perhaps most recently by Salter who re-emphasized at the Hershey Conference on Intracellular Enzymes in Normal and Malignant Tissues in 1945 his earlier views that "glycolysis in muscle and tumor extracts follows different pathways" (4). The latter paper called attention to the series of papers by Boyland and his co-workers during 1935-38 in which the opposite conclusion was reached, but reported that "tumor extracts fail to catalyse the reaction between triosephosphate and pyruvic acid which plays an important part in the accepted scheme for muscle glycolysis." It is significant, however, that the text stated "whether or not this difference is due to the absence of the specific coenzyme which is necessary for this reaction in muscle remains to be answered experimentally," although they cited the experiments by Boyland's group in which yeast cozymase (DPN) stimulated glycolysis in tumor extracts. Other discrepancies in cancer metabolism were also noted. That part of the lack of agreement might be due to the preparation of extracts was recognized by Salter in the comment that Boyland's aqueous extracts contained coenzyme-destroying enzymes that were absent from the saline extracts used by Salter and his associates. It was pointed out that tumor adenylic acid deaminase could be extracted by water but not by saline solution.

It is the opinion of the present authors that the technics employed in the manipulation of these systems is probably the chief cause of such lack of agreement as may exist. All of the published work on tumors has been done with extracts of one kind or another, and thus an ill-defined portion of the original tissue activity has been measured. Since the publication of these reports, considerable advance has been made in the preparation of tissues for the study of enzyme systems, as well as in the reconstruction of these systems. So far as we are aware, no one has attempted to study glycolysis in tumor homogenates. However, some excellent work has been done in the case of brain homogenates, and the conditions for the study of glycolysis in homogenates of this tissue have received considerable attention (1, 13, 14, 17, 18). The present study has been greatly facilitated by the studies cited.

The second source of the lack of agreement seems to be more theoretical than technical. In its simplest terms it may be stated as a question—how much does the glycolysis of tumor have to differ from that of muscle to have it constitute a "different" pathway? Potter emphasized in 1944 (11) that the metabolism of any given tissue is the resultant of the balance between the individual enzymes that it contains, and more recently (12) developed the generalization that "the junction of a tissue is the resultant of the organized action of its enzymatic components." The studies in tumor glycolysis reported below show that by every test that we have applied, the phosphorylating pathway of glycolysis occurs in tumor tissue, as in muscle, brain, embryo, and other normal tissues that have been studied. But again and again it has been our experience that small deviations in individual enzyme components of the over-all system occur. The glycolytic enzyme systems are no exception; the behavior of the total glycolytic system varies significantly from tissue to tissue. It is these differences in detail, now incompletely understood, that further the conclusion that the overall pathway is "different" and it is this conclusion with which we differ.

The chief importance of the present work is considered to be the advance it represents in methodology, which may eventually make possible the definition of those differences in detail that exist between the various tissues. The report also brings out the fact that the glycolytic system in tumor...
tissue is highly active and relatively uncomplicated so that tumor homogenates may prove to be a useful tool for the further study of the fundamental aspects of glycolysis.

EXPERIMENTAL

Preparation of homogenates.—All of the experiments were carried out with whole homogenates, i.e., no extracts were prepared and no part of the sample was discarded. The Q values may therefore be compared with the established values for slices. Since previous work involved aqueous extracts, saline extracts, and frozen extracts, all of these variations were included in the preparation of homogenates. Most of the work was done with the transplantable Flexner-Jobling carcinoma, but the Jensen sarcoma and Walker 256 carcinosarcoma were also tested. The tumors were rapidly excised from decapitated rats and placed in small beakers of ice-cold isotonic KCl (1.15 per cent) which were kept in cracked ice. The tumors were removed from the KCl, carefully blotted and trimmed of outer connective tissue and any necrotic tissue present. The sample was then weighed and added to a cold homogenizer tube containing 3 ml. of distilled water or isotonic KCl. More water or isotonic KCl was then added to make the final volume of fluid equal to 9 times the weight of the tumor sample. The homogenization was carried out in the cold and the resulting “water homogenate” or “KCl homogenate” was kept at 0°C until used. In certain cases an aliquot of the KCl homogenate was frozen in liquid air; it will be referred to as a “frozen KCl homogenate.” The KCl used for homogenization was made slightly alkaline (pH 7.7-8.1) by the addition of 8 cc. of 0.04 M KHCO3 per liter.

Components of the complete system.—The need for adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN, Co I), Mg ions, hexosediphosphate (HDP), and nicotinamide for glycolysis in brain homogenates was demonstrated by Utter and his associates (17, 18). Racker and Krimsky (13, 14) emphasized the desirability of substituting K ions for Na ions and this has accordingly been our practice. Elliott and Henry (1) showed that pyruvate stimulated glycolysis in unfortified homogenates of brain. We have found that pyruvate stimulates glycolysis in fortified tumor homogenates. On the basis of the above-mentioned reports and some preliminary experiments we set up a “complete” system with the following components: water to make a volume of 3.0 ml. for the final reaction mixture; isotonic KCl equivalent to the volume of water homogenate if this was used, 0.3 ml. 0.1 M ammonium phosphate pH 7.6, 0.3 ml. of 0.5 M KCl in 0.5 M KHCO3 (1 M K2CO3 + 1 N HCl, equilibrated with 5 per cent CO2), 0.2 ml. of 0.01 M K-ATP, 0.3 ml. of 0.4 M nicotinamide, 0.1 ml. of 1 per cent DPN (equivalent to 670 μM DPN, and added as the K salt, from the side arm after temperature equilibration), 0.3 ml. of 0.04 M K-HDP, 0.3 ml. of 0.28 M glucose, 0.1 ml. of 0.1 M MgCl2, 0.1 ml. of M/67 K-pyruvate (freshly prepared from 1 N pyruvic acid and K2CO3), and, finally, 0.3 ml. of 10 per cent tumor homogenate. In the figures to follow, deviations from this complete system will be noted, and when concentrations other than given here are employed they will be stated as final concentrations, or as multiples of the amount in the above “complete” mixture. The reactants were pipetted in the order given, and the Warburg flasks were placed in cracked ice after the ATP addition. After the addition of the homogenate, the flasks were attached to Warburg manometers and gassed from a manifold with either 95 per cent N2 : 5 per cent CO2 or 95 per cent O2 : 5 per cent CO2 for 13 minutes. They were then placed in the bath and equilibrated for 5 minutes. The DPN was tipped in from the side arms. After 5 minutes the zero reading was taken, and the measurement of CO2 evolution begun. The nitrogen was not purified, but for the present work traces of oxygen were not considered detrimental; the tumor homogenates do not take up oxygen in air, and secondly, differences between O2 and N2 could be demonstrated without making the N2 absolutely free of oxygen.

Effect of modifications of the complete system.—With the complete system, tumor homogenates exhibited an extremely active glycolysis. When various individual components were omitted the activity was considerably lowered. Fig. 1 shows that in the complete system, glucose is of slight importance while the omission of HDP results in almost complete absence of activity: it is clear that HDP is glycolyzed in the absence of glucose but glucose is not glycolyzed in the absence of HDP. The striking effect of nicotinamide in this system is also shown in Fig. 1. Omission of this compound results in the rapid loss of glycolytic activity. In the experiments on brain glycolysis this was also the case and was related to the fact that nicotinamide prevents the breakdown of DPN (13, 14, 17, 18). This fact has not been utilized in experiments on

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2 In some of the experiments the ATP was added to the main compartment but later it was found advantageous to add the ATP from the side-arm with the DPN. In the case of the experiments on chick embryo homogenates (9) better results were obtained with the ATP in the main compartment from the beginning.
tumor glycolysis up to the present. It is of interest that we were unable to demonstrate any beneficial effect of nicotinamide in experiments on glycolysis in homogenates of chick embryos (9). Even with nicotinamide present, it is necessary to add DPN to the system, and the omission of this compound gives an inactive system (Fig. 1). This is of course understandable since the tumor tissue has been diluted 100 fold in the reaction mixture. The data in Fig. 1 show that there is essentially no difference between the KCl homogenate and the water homogenate. One might have anticipated that a difference, if any, would appear in the flasks from which nicotinamide was omitted but such was not the case.

The effects of variations in the amount of HDP and pyruvate are shown in Fig. 2. The final concentration of HDP in the complete system is 0.004 M. Twice as much HDP gives essentially the same rate of glycolysis, while one-third as much HDP gives a good initial rate but the reaction rate falls off as the substrate is depleted. With this low level of HDP, a glucose effect may be seen, in contrast to the situation in Fig. 1. In this reaction mixture, the tumor homogenate cannot handle glucose in the absence of HDP, but with a small amount of HDP present to maintain the phosphorylation mechanism, some glucose can be glycolyzed. The inference is that the amount of glucose that can be glycolyzed is the amount that can be phosphorylated.

According to the Embden-Meyerhof scheme of glycolysis, HDP is converted to triose phosphate and this is anaerobically oxidized by means of the conversion of pyruvate to lactate. Pyruvate can be formed from phosphoglyceric acid but at the start of the reaction no pyruvate is present to act as an acceptor of hydrogen, and some triose phosphate must be converted to a phosphoglycerol. Fig. 2 shows that by adding pyruvate the initial lag can be eliminated. The amount of pyruvate in the “complete” system is 0.0005 M, which is similar to the concentration of pyruvate in blood and may be near the “steady state” level.

**Glycolysis of glucose and hexosemonophosphates.**—The data in Fig. 2 show that when the concentration of HDP is initially low, i.e., about 0.001 M, the rate quickly falls off unless glucose is present. In such a system the rate of CO₂ production indicates the occurrence of the hexokinase reaction (ATP + glucose) and the phosphofructokinase reaction ATP → fructose-6-phosphate) according to the Embden-Meyerhof formulation. The data in Figs. 3 and 4 show that the monophosphates can be glycolyzed in the absence of HDP (in contrast to glucose) but that they are not as effective as HDP itself. Glycogen was ineffective in these experiments. The glucose-6-phosphate alone had a much slower rate of glycolysis than HDP alone; fructose-6-phosphate had approximately the same initial rate as HDP, but the rate was not maintained. When glucose-6-phosphate and HDP were combined the total CO₂ output was considerably greater than the sum of the two substrates taken separately.

**The fluoride system.**—A well-established technic for the study of the key reaction in glycolysis has been the blocking of the conversion of phosphoglyceric acid to pyruvic acid by means of fluoride. In the complete system described above, the phosphoglyceric acid does not remain as such but is converted to pyruvate. If fluoride is added to the system, phosphoglyceric acid accumulates (and can be measured in terms of CO₂ evolution) and pyruvate may be added to the system to act as a hydrogen acceptor for the oxidation of triose phosphate to phosphoglyceric acid (3). The conversion of pyruvate to lactate does not constitute acid production. For the fluoride system, with all other components of the complete system present, the amount of glycolysis is determined by the amount of pyruvate added since no pyruvate is formed. Thus the “fluoride system” differs from the “complete system” by having 10 or 20 times as much pyruvate added, in addition to the fluoride. The other components remained the same. We employed potassium fluoride, in a final concentration of 0.01 M (0.15 ml. of 0.2 M KF per flask). Fig. 5 shows that in the fluoride system, which contains a high level of pyruvate, the initial rate of CO₂ production was greater than in the complete system, but parallel experiments showed that when the pyruvate concentration was made the same in the complete and in the fluoride systems, the initial rate of glycolysis was the same in both systems. The increased rate produced by increasing the pyruvate concentration was shown in Fig. 2. It may therefore be concluded that fluoride does not inhibit the oxidation of triose phosphate or the reduction of pyruvate to lactate. The reaction stopped, however, in the flasks containing fluoride, due to the depletion of the pyruvate. In other experiments, twice as much pyruvate (0.01 M final) gave a proportionately greater CO₂ production before leveling off (see next section). Fig. 5 also shows the effect of varying the HDP concentration and the effect of glucose in the fluoride system. Not shown are the results obtained by omitting glucose from re-
action mixtures with the higher levels of HDP because the effects are less marked, as was the case in the systems without fluoride.

The reaction mixtures represented by Fig. 5 were also analyzed for lactic acid and phosphoglyceric acid. The reactions were stopped at 100 minutes by the addition of 2 ml. of 17.5 per cent trichloroacetic acid and the protein-free filtrates were analyzed for lactic acid by the method of Barker and Summerson and for phosphoglyceric acid by the method of Rapoport (see 8). The results are given in Table I. The data show that phosphoglyceric acid accumulated in the reaction mixtures containing fluoride, while lactic acid was formed in all the reaction mixtures. The theoretical 1:1 correlation between the amount of lactic acid and the amount of phosphoglyceric acid was not obtained and much further work needs to be done to determine what side reactions may be responsible for the discrepancies. At present it may be said that the coupled oxidoreduction that characterizes muscle also occurs in the Flexner-Jobling carcinoma, but the limiting reaction is not defined. The rate of CO₂ output in the fluoride system with HDP and pyruvate probably represents minimal values for the triose phosphate dehydrogenase and for lactic dehydrogenase.

**The effect of freezing.**—Fig. 6 shows the effect of freezing the Flexner-Jobling homogenate. When HDP was the substrate, the frozen homogenate was just as active as the fresh homogenate, with or without fluoride. However, when the level of HDP was cut down to the critical concentration, so that the conversion of monophosphate to diphosphate became limiting, it was apparent that freezing was deleterious. It cannot be decided from these data whether the freezing inactivated an enzyme involved in the conversion or whether the freezing increased the activity of the phosphatases that act on the hexose phosphates and on ATP.

**The effect of oxygen.**—Figs. 7 and 8 show the effect of oxygen upon the glycolysis of homogenates of Flexner-Jobling carcinoma, in the presence and absence of fluoride. The flask were gassed simultaneously and the mixtures were assayed together. Glucose was not added to any of the flasks in the experiments shown because the interpretation is more direct when glucose-6-phosphate is considered. Experiments with glucose and fructose-6-phosphate gave results quite similar to those obtained with glucose-6-phosphate, but the latter were more striking. Fig. 7 shows that with the complete reaction mixture, using 0.004 M HDP as substrate, glycolysis was very strongly inhibited by oxygen as compared with nitrogen. With the priming level of HDP (0.001 M) glycolysis quickly stopped in both O₂ and N₂. With the priming level of HDP plus 0.004 M glucose-6-phosphate, the rate of glycolysis was no better than with the HDP alone where oxygen was used, but with nitrogen a good rate was attained. The experiments with priming levels of HDP ± glucose-6-phosphate suggest that the inhibitory action of oxygen was exerted somewhere between glucose-6-phosphate and HDP, but this interpretation is weakened by the fact that the oxygen effect was obtained with HDP alone when high levels were used. That the site of action is above HDP is, however, indicated by the data of Fig. 8, which was obtained from the same experiment, using the same type of reaction mixtures except that fluoride and pyruvate were added.

The data in Fig. 8 show that in the presence of fluoride, the oxygen effect was not obtained with HDP alone, but occurred only in the case of glu-
Figs. 1-2

Figs. 3-4
cose-6-phosphate. Further experiments will be required to determine the exact mechanism of the fluoride prevention of the oxygen effect when HDP is used, but it seems possible that in the absence of fluoride the HDP is partially dephosphorylated so that the reaction mixture consists of glucose and hexose monophosphates in addition to HDP, while in the presence of fluoride, phosphatase activity is inhibited and the system represents essentially HDP glycolysis when this is the sole substrate added. Thus the data in Fig. 8 support the view that the oxygen effect represents the blocking of the conversion of hexose monophosphate to the diphosphate.

**Data on other tumors.**—All of the experiments reported so far have been carried out with the Flexner-Jobling rat carcinoma. However, both the Jensen rat sarcoma and the Walker No. 256 rat carcinoma were tested in the complete system and with various omissions, and comparable results were obtained. In Table II the \( Q_{n_2} \) values for the three tumors, obtained by the homogenate technic, are compared with those obtained by the slice technic. According to these data, the glycolytic rates attained in the homogenates are nearly twice as high as the previously recorded rates for slices. In the case of brain tissue, slices yielded values of 15 to 19 and homogenates yielded values of 54 to 58 (see 17, 18). It is recognized, of course, that in the homogenates we have added the accessory co-factors and have also added nicotinamide to prevent DPN breakdown. The \( Q \) values for the homogenates in Table II probably are determined by the amount of lactic dehydrogenase or triose-phosphates attained in the homogenates are nearly twice those obtained by the slice technic.

**TABLE II: Anaerobic Glycolysis in Tumors. Homogenate Technic Compared with Slice Technic**

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Homogenate</th>
<th>Slice</th>
</tr>
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<tbody>
<tr>
<td>Flexner-Jobling rat</td>
<td></td>
<td></td>
</tr>
<tr>
<td>carcinoma</td>
<td>42.5, 43.7, 53.1, 55.0, 27-37 (12)</td>
<td></td>
</tr>
<tr>
<td>Jensen rat sarcoma</td>
<td>55.4, 56.2, 66.4</td>
<td></td>
</tr>
<tr>
<td>Walker 256 rat carcino-sarcoma</td>
<td>56.2</td>
<td></td>
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</tbody>
</table>

**DISCUSSION**

The data presented above show that tumor tissues have the Embden-Meyerhof type of phosphorylating glycolysis. Taken with the preceding papers in this series, in which in vitro studies led to the same interpretation (5, 6, 7), it may now be said that every approach has led us to this conclusion. The various enzymatic components of the over-all glycolytic mechanism may vary in amount, but it seems clear that glucose is glycolyzed via the hexose monophosphates, HDP, and phosphoglyceraldehyde. There were no data to indicate that glucose could be converted to lactic acid by any other mechanism.

Glycolysis was obtained in both KCl and water homogenates provided the accessory factors were added. The results with nicotinamide indicate the handicap that previous investigators had to deal with in terms of DPN destruction, which was marked in both types of homogenates, but was effectively counteracted by the nicotinamide.

The results with frozen homogenates and with oxygen both indicate a labile enzyme which is connected with the conversion of the hexose monophosphates to hexose diphosphate. This enzyme may be the one involved in the conversion of fructose-6-phosphate to HDP, known as phosphofructokinase (also called phosphohexokinase). The enzyme has been reported to be sensitive to acidity as mild as pH 6.4 (19), to small amounts of iron and to viruses (15), and to O-R potentials above 0.05 mv. (2). The latter observation may be the explanation of the oxygen effects noted in Figs. 7 and 8, since it seems unlikely that the oxygen effect can be ascribed to oxidative removal of lactic acid: experiments in which the reaction mixture was altered to permit CO2 absorption, with air in the gas phase, gave negligible rates of oxygen uptake, and other experiments in this laboratory show that the oxidation of pyruvic acid by these tumors is not accompanied with the production of CO2.
also negligible. Nor would it be possible to ascribe the oxygen effect to the lowering of the phosphate concentration by oxidative phosphorylation (11), although the tumor homogenate will be a useful tool with which to study the phosphate effect. Szöreinyi (16) has emphasized the role of H$_2$O$_2$ in the Pasteur reaction but placed the site of action at the phosphorolysis of glycogen and at the stage of triose phosphate oxidation. It is quite possible that some H$_2$O$_2$ may accumulate in the tumor homogenates in 95 per cent oxygen. Much further work must be done to determine the nature and significance of the oxygen effect.

SUMMARY

1. Homogenates of Flexner-Jobling rat carcinoma, Jensen rat sarcoma and Walker 256 rat carcinosarcoma gave glycolysis rates of 43 to 85, as compared with the Q$^{18}_c$ values of 27 to 42 previously recorded for slices of these tumors.

2. The high rates of glycolysis were obtained in homogenates fortified with all of the known accessory factors of the Embden-Meyerhof glycolytic scheme, plus hexosediphosphate (HDP) and nicotinamide. Omission of either of the latter two compounds gave rates which were very low and such systems rapidly stopped completely.

3. Glucose in the absence of hexosediphosphates was active as a substrate, but when critical levels of HDP were present (about 0.001 M) glucose could be glycolyzed.

4. Glucose-6-phosphate and fructose-6-phosphate were superior to glucose but not as effective as HDP. When 0.001 M HDP was present the monophosphates were effectively glycolyzed.

5. In the presence of 0.01 M fluoride and 0.01 M pyruvate a coupled oxidoreduction occurred, forming phosphoglyceric acid and lactic acid. This reaction occurred at a rate equal to that which occurred in the absence of fluoride, and is the reaction that characterizes the Embden-Meyerhof glycolytic scheme.

6. Frozen homogenates were as active on HDP as the fresh homogenates, but less active on the monophosphates.

7. With fluoride and pyruvate present, glycolysis was as rapid on HDP in O$_2$ as in N$_2$, but with the monophosphates, or with fluoride absent, oxygen inhibited glycolysis. This effect was considered in terms of the Engelhardt-Sakov mechanism, which is the oxidative inhibition of phosphofructokinase.

8. The data support the view that the Embden-Meyerhof scheme of phosphorylative glycolysis operates in tumors.

REFERENCES


12. POTTER, V. R. Commonwealth Lectures, unpublished University of Louisville, Louisville, Ky.


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