MEASUREMENT OF ANAEROBIC GLYCOLYSIS IN BRAIN AS RELATED TO POLIOMYELITIS*

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Recently, several attempts have been made to determine the effect of neurotropic virus diseases upon the metabolism of the central nervous system. Racker and Kabat (1) and Nickle and Kabat (2) reported that the rate of anaerobic glycolysis in suspensions prepared from brains of mice infected with poliomyelitis or Western equine encephalomyelitis was significantly decreased as compared with normal brain suspensions. Likewise, Victor and Huang (3) reported a decreased glycolysis with minced whole chick embryo infected with Western equine encephalomyelitis. On the other hand, Wood, Rusoff, and Reiner (4) were unable to confirm the findings of Racker and Kabat (1) although an attempt was made to duplicate all conditions.

All of these measurements of rate of anaerobic glycolysis were performed with tissue minces or homogenates, with no additions other than salts, buffers, and substrate (glucose). Since the brain preparations had $Q_{CO_2}^{N_2}$ values of 4 to 10 as compared to values up to 50 reported by Geiger (5) and Ochoa (6) for cell-free preparations from brain, it was apparent that the measurements might not have been made under optimal conditions ($Q_{CO_2}^{N_2}$ equals cubic millimeters of CO_2 per hour per milligram of dry tissue).

The $Q_{CO_2}^{N_2}$ values in the experiments with chick embryo tissue minces (3) were approximately 1 to 2. It is probable that these values likewise do not represent maximum rates, since Meyerhof and Perdigon (7) have shown that chick embryo preparations need to be fortified with coenzymes which are rapidly destroyed after treatment of the tissue. With addition of proper components, chick embryo extracts formed acid from hexosediphosphate at a $Q_L^{N_2}$ of 14. ($Q_L^{N_2}$ equals cubic millimeters of lactic acid produced per hour per milligram of tissue and is roughly equivalent to the preceding $Q_{CO_2}^{N_2}$ value. The lactic acid is expressed as gas, 22,400 c.mm. = 1 mM) Needham and Nowinski (8) have reported $Q_L^{N_2}$ values of 12.5 to 16.75 for 3 to 5 day intact chick embryos with glucose as the substrate. In view of these reports, the activities of the embryo minces of Victor and Huang (3) seem far from optimal.

It is the purpose of this report to demonstrate that only a small part of the potential anaerobic glycolytic activity has been measured in studies of the effect of virus on metabolism (at least as far as brain is concerned) and to show how maximum activity can be obtained. In addition, we wish to discuss the implications of these findings with regard to the interpretation and significance of previous studies on the effect of poliomyelitis upon the anaerobic glycolysis of brain.

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Methods

Mice of the ABC strain, weighing 10 to 13 gm., were decapitated, the entire brain was removed, weighed, and homogenized with four parts of ice-cold distilled water according to the technique of Potter and Elvehjem (9). In a few indicated experiments, the tissue was homogenized in Locke's solution or isotonic saline rather than water. The same general method has been applied to the preparation of very active glycolytic preparations from the brain and spinal cord of rats (10).

The mouse brain homogenates were tested in Warburg respirometer vessels of small capacity (7 to 9 ml.) at 37.5° C. with an atmosphere of 5 per cent carbon dioxide and 95 per cent nitrogen. The volume of the reaction mixture was 1 ml., and it contained 0.1 ml. of the brain homogenate, equivalent to 4 mg. dry weight of brain. The homogenate was placed in the side arm and the other constituents were in the main chamber. The final concentrations used were as follows: 0.028 M glucose, 0.048 M $NaHCO_3$, 0.01 M Na_2HPO_4-KH_2PO_4 buffer (pH 7.3), 0.001 M diphosphopyridine nucleotide[1] (DPN), 0.0007 M adenosine triphosphate (ATP), 0.008 M $MgCl_2$, 0.0025 M hexosediphosphate (HDP), and 0.04 M nicotinamide.

The activity of the preparations was evaluated by manometric measurement of the carbon dioxide liberated from the bicarbonate buffer by lactic acid arising from glycolysis.

Adenosine triphosphate used in these experiments was prepared as the barium salt from rabbit skeletal muscle by the methods of Kerr (11) or Needham (12), and had a purity of 90 per cent according to phosphorus and ribose determinations. The diphosphopyridine nucleotide was isolated from yeast according to the description of Williamson and Green (13). A yeast assay with an apozymase from baker's yeast prepared according to Govier (14) indicated a purity of 50 to 55 per cent. Sodium hexosediphosphate was prepared from the commercial dibarium salt. With both hexosediphosphate and adenosine triphosphate, the barium salts were dissolved in a minimum amount of dilute HCl and the barium was precipitated by addition of Na_2SO_4. The solutions were then neutralized with NaOH and centrifuged.

EXPERIMENTAL

The experiments emphasize two different points: (1) the necessity of the individual components of the reaction mixture for full activity of brain homogenates; (2) the effect of different solutions used in homogenization, and a comparison of activities of these preparations with and without proper activators.

A. Necessity of Phosphate Esters and Coenzymes for Full Activity

It will be shown that hexosediphosphate, adenosine triphosphate, diphosphopyridine nucleotide, and $MgCl_2$ are necessary in order to obtain full activity with brain homogenate.

The effect of individual omissions of accessory components and substrate is shown in Fig. 1. The values shown represent an average of duplicate determinations on two animals. At the 60 minute reading, the total spread of the four determinations in the most extreme case (curve 6) was about 20 per cent of the reading. In all other cases, the total spread was 10 per cent or less and there was no overlapping of points except in the three lower curves. The com-

[1] Other names: cozymase, coenzyme I.

plete system (curve 7) includes all the components as given in the section on methods. The function of these components and their interrelationship can best be understood by consideration of the over-all changes of anaerobic glycolysis and some of the individual steps as presented in Diagram 1.

Let us first consider the part played by the adenosine triphosphate (ATP). The concentration of ATP is of critical importance in determining whether or

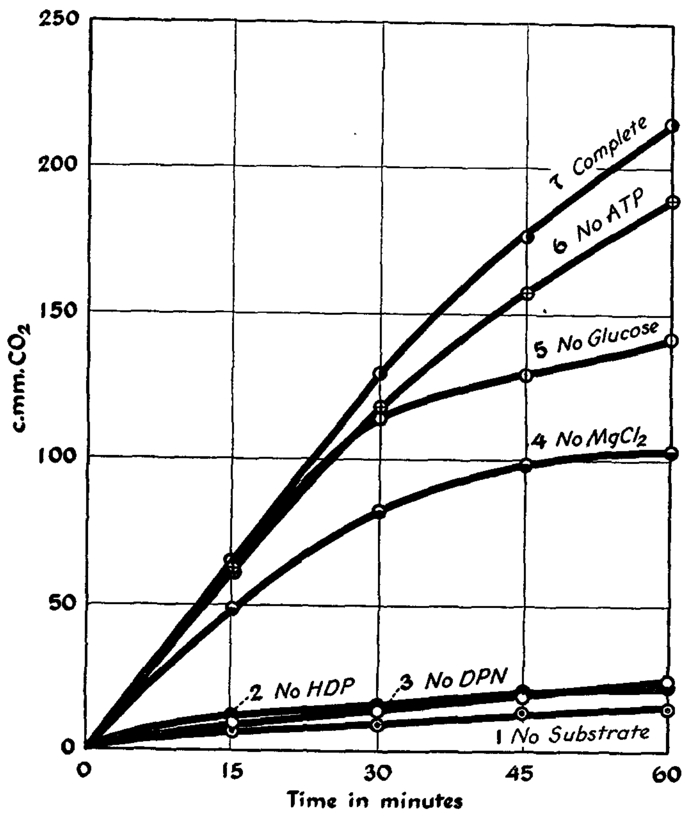

FIG. 1. Necessity of phosphate esters and coenzymes for anaerobic glycolysis

not glycolysis of glucose will occur, primarily because of its rôle in reaction 3. In the over-all conversion of glucose to lactate in reaction 1, there is a net formation of 2 molecules of ATP from adenosine diphosphate (ADP) and inorganic phosphate, and when hexosediphosphate (HDP) is the substrate (reaction 2) four molecules of ATP are formed. Therefore, if there is no destruction of ATP, and if there is sufficient ATP present for initial phosphorylation of the glucose (reaction 3) the ATP will not be a limiting factor. Actually, destruction of the ATP does occur, so that the concentration of ATP is determined by the relative rates of synthesis and destruction of ATP. Synthesis of ATP occurs in reactions 6 and 7. Removal of ATP occurs by reaction 3, and by other reactions such as formation of creatine phosphate; destruction occurs by dephosphorylations as in reaction 9. Reaction 9 is catalyzed by adenosine triphosphatase whereby ATP is converted to inorganic phosphate and ADP or adenylic acid. This reaction does not inactivate the molecule in so far as its capacity to act as a phosphate acceptor in reactions 6 and 7 is concerned, but does destroy its capacity for function in reaction 3. Therefore, if adenosine

DIAGRAM 1

Anaerobic Glycolysis

Over-all Reactions

1. Glucose + 2 ADP* + 2 $H_3PO_4 \xrightarrow{\text{DPN}}$ 2 lactate + 2 ATP
2. HDP + 4 ADP + 2 $H_3PO_4 \xrightarrow{\text{DPN}}$ 2 lactate + 4 ATP

Individual Steps

3. Glucose + 2 ATP $\longrightarrow$ HDP + 2 ADP
4. HDP $\rightleftharpoons$ 2 triose phosphates
5. Triose phosphate + H_3PO_4 + DPN $\rightleftharpoons$ 1,3-diphosphoglycerate + DPN (reduced)
6. 1,3-Diphosphoglycerate + ADP $\rightleftharpoons$ 3-phosphoglycerate + ATP
7. 3-Phosphoglycerate + ADP $\longrightarrow$ pyruvate + ATP
8. Pyruvate + DPN (reduced) $\rightleftharpoons$ lactate + DPN
9. ATP $\longrightarrow$ ADP + $H_3PO_4 \longrightarrow$ adenylic acid + 2 H_3PO_4
10. Adenylic acid $\longrightarrow$ inosinic acid + NH_3 or adenosine + H_3PO_4

* ADP = Adenosinediphosphate. Presumably, adenylic acid can be substituted for ADP in most places in this diagram.

triphosphatase is present in too great a quantity it will stop glycolysis of glucose, but not of HDP, for which reaction 3 is not essential. The reactions in 10 illustrate two conversions, the deamination of adenylic acid to inosinic acid and also the dephosphorylation to adenosine. The resulting compounds can no longer function, efficiently at least, as phosphate acceptors, and if reaction 10 is too rapid even glycolysis of HDP is stopped. For a more complete discussion of the reactions of glycolysis see the review articles of Potter, and Mann and Lutwak-Mann (15).

All the changes observed in Fig. 1 can be explained on the basis of these relationships. When ATP is omitted (curve 6), there is only a slight diminution of activity. This is as expected since there is sufficient adenylic acid in the brain preparation to act as an acceptor and the glycolysis of HDP may therefore proceed. Furthermore, the conversion of HDP to lactate (reaction 2) gives rise to ATP so there is now provision for the phosphorylation of glucose and it in turn may be glycolyzed.

Experiments with rat brain homogenates (10) indicate that omission of ATP causes a much larger decrease in anaerobic glycolysis than is reported for mouse brain in Fig. 1. It is possible that one or both conversions of reaction 10 occur in greater intensity in rat than in mouse brain, and therefore there is not enough adenylic acid to act as a phosphate acceptor for the HDP.

When HDP is omitted (curve 2), there is very low activity. In this case the destruction of ATP is presumably more rapid than its synthesis; and, since there is no reservoir of organic phosphates, such as the HDP, for renewal of ATP, glycolysis is retarded.

When HDP serves as the sole substrate (curve 5), considerable glycolysis is possible until the added amount of HDP is exhausted. Theoretically, 112 c.cm. of CO_2 could have been produced from the HDP alone. When both glucose and HDP are present (curve 7), the glycolysis uses up both glucose and HDP although some of the latter compound is continually reformed by phosphorylation of glucose (reaction 3).

Mg^{++} is known as a necessary component of several phosphate-transferring enzymes involved in anaerobic glycolysis. Omission of $MgCl_2$ from the reaction mixture (curve 4) has a considerable effect. Geiger (5) has previously reported that Mg^{++} is necessary for activation of cell-free brain extracts.

Before the important oxidation step of anaerobic glycolysis can occur (reaction 5) diphosphopyridine nucleotide (DPN) must be present in adequate quantities. Without added DPN (curve 3), very little glycolysis occurs with brain homogenates. Rapid destruction of DPN by brain preparations and other tissues has been shown by Mann and Quastel (16) and by Handler and Klein (17). It is, therefore, certain that the preformed DPN of the brain will be rapidly destroyed after homogenization or mincing. This makes it necessary to add a new supply of DPN to initiate normal rates of glycolysis. Geiger (5) found similar measures necessary for activation of cell-free brain extracts.

Since the enzymes attacking DPN are present in the brain homogenates, it is often necessary to inhibit the action of these enzymes to prevent complete destruction of the added DPN. It has been reported (15, 16) that nicotinamide has such an effect.

B. Effect of Nicotinamide upon Anaerobic Glycolysis

The necessity of nicotinamide addition in mouse brain homogenates depends upon the concentration of DPN initially present. Fig. 2 illustrates this point. When 0.0005 M DPN is added to the homogenate, the activity falls off rapidly (curve A) unless 0.04 M nicotinamide is added as a protective measure (curve B). However, when the DPN concentration is raised to 0.001 M (curves C and

D), added nicotinamide is actually inhibitory, at least during the 1st hour of glycolysis. Again these results are in contrast with experiments with rat brain homogenates, where nicotinamide addition is essential regardless of DPN concentration (10). This can be interpreted as an indication that the enzymes causing destruction of DPN are more powerful in rat brain preparations than in those from mice.

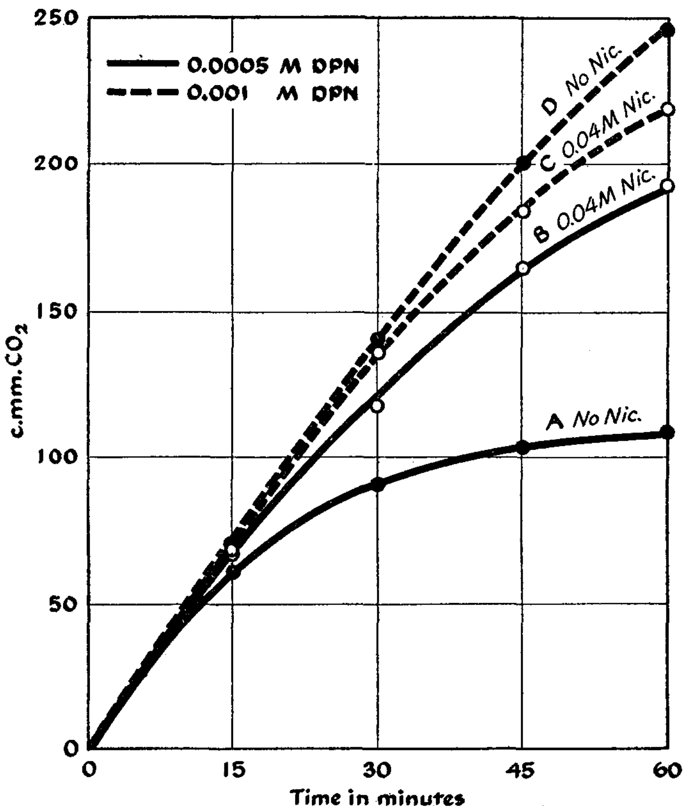

FIG. 2. Effect of nicotinamide on anaerobic glycolysis

C. Homogenation in Various Solutions and Comparison of Activities with and without Activators

A comparison of results obtained with Locke's solution, isotonic saline, and distilled water is given in Table I. Four parts of liquid to one of brain were used in the saline and distilled water preparations, and nine parts with Locke's solution. The results have been reduced to $Q_{CO_2}^{N_2}$ values on a dry weight basis, and therefore are independent of different concentrations of brain in the homogenates.

The preparation in Locke's solution was tested by the procedure of Racker and Kabat (1). They prepared brain by mincing or homogenizing it in Locke's solution of salts, bicarbonate, and glucose. A ratio of nine parts of liquid to one part of brain was used. The homogenate was quickly placed in the previously prepared Warburg vessel containing the additional glucose and bicarbonate buffer, and the readings were started as soon as gassing and equilibration was finished. Results obtained by this method are shown under column I.

TABLE I

Effect of Homogenization in Different Solutions on Activity Measured with and without Activators

Values are expressed as $Q_{CO_2}^{N_2}$

Homogenization liquid	1 Racker and Kabat's set-up	2 Buffers and glucose only	3 Complete system
Locke's solution	7.7	2.0	36.8, 35.8
	6.5, 5.3	7.0	37.0, 37.0
Isotonic saline	—	6.3, 4.8	38.5, 40.0
	—	4.8, 4.5	53.3, 52.8
Distilled H_2O	—	3.5, 2.8	54.8, 57.8
	—	3.3, 4.0	54.8, 56.5
	—	—	56.0, 53.8

Column 1: Contains 0.2 ml. of brain (equivalent to 4 mg. dry weight) in Locke's solution, which was added to 0.04 ml. of 0.28 M glucose and 0.075 ml. of 0.48 M $NaHCO_3$ and diluted to 1.0 ml. This gives a final glucose concentration of 0.014 M and $NaHCO_3$ concentration of 0.036 M.

Column 2: Contains brain (4 mg.), glucose (0.028 M), $NaHCO_3$ (0.048 M), and KH_2PO_4-Na_2HPO_4 (0.01 M—pH 7.3) in a total volume of 1.0 ml.

Column 3: Contains brain, glucose, $NaHCO_3$, KH_2PO_4-Na_2HPO_4 as in 2, plus ATP, DPN, $MgCl_2$, HDP, and nicotinamide (see methods section for concentrations) in a total volume of 1.0 ml.

With the isotonic saline and distilled water preparations, no special attempt was made to minimize the time between death of the animal and the initial readings. These preparations, as well as the brain homogenized in Locke's solution, were tested in a mixture containing the complete complement of accessory factors (column III), and in another set of experiments (column II) with glucose and buffers only.

The values obtained by Racker and Kabat's method (column I) are comparable to those previously reported (1, 2, 4). The results in columns II and III show that regardless of the liquid used in the homogenization, addition of accessory factors is necessary. The most consistent and highest $Q_{CO_2}^{N_2}$ values were obtained when homogenization was done in distilled water. Homogenization in distilled water is reported by Potter (15) to result in disruption of 80 to 90 per cent of the cells of most tissues, while homogenization in other solutions is less effective. In homogenates fortified with accessory compounds, the activity has some relationship to the degree of disruption of the cells, probably because there is no permeability barrier between the coenzymes and enzymes in disrupted cells. The plasmolytic effect of distilled water may also serve to remove structural constraints which limit the activity of the enzymes in intact cells to less than their potential maximum. The higher activity of the distilled water preparations is therefore not surprising.

The activity of the brain homogenates is largely independent of time elapsed between homogenization and testing, at least up to a period of several hours, providing the preparations are kept at 0–5° C. and all necessary accessory factors are added at the time of testing. The implication is that, despite the lability of coenzymes after disruption of the cell, the enzyme structure itself is fairly stable. It is possible that changes occur during the initial 20 to 30 minutes after death of the animal, since no measurements were possible during this period. The degree of stability of enzymes in homogenates is important in enzyme assays since it is necessary to assume that no great change occurs if proper coenzymes and substrate are supplied.

The fortified distilled water homogenates exhibit $Q_{CO_2}^{N_2}$ values of 54 to 58 during the 1st hour of activity. There was some decrease in activity in successive 15 minute periods (*cf.* curve 7 of Fig. 1); the $Q_{CO_2}^{N_2}$ was approximately 65 over the first half hour. A large number of experiments with both rat and mouse brain homogenates has indicated that glycolytic activities of the order mentioned can be obtained very consistently.

DISCUSSION

The foregoing presentation of data demonstrates quite clearly that previous investigations of the metabolism of brain in poliomyelitis (1, 2) have been conducted in such a manner that only a very small portion of the total enzymic activity of the preparation has been measured. The activity has been limited, not by concentration of enzymes but by lack of the accessory factors which are essential, as illustrated in Diagram 1. The question arises whether such studies have any real significance, in so far as concerns interpretations bearing on the possible effect of poliomyelitis on brain metabolism.

The thesis followed in the poliomyelitis studies has been that the virus disturbs the metabolism of the central nervous system, in some aspect which is susceptible to *in vitro* measurement. If metabolic aberrations of a kind which could be detected by *in vitro* methods did occur, the changes would be most likely to appear in one or more fo the following: (*a*) permeability or intracellular organization; (*b*) concentration of coenzymes, substrates, or other essential components excluding enzymes; (*c*) concentration or nature of enzymes. Item (*c*) could include an increase or decrease in the normal enzymes, an appearance of abnormal enzymes, a disturbance of their relative proportion, etc.

For normal metabolism it is certain that there must be a proper balance of the different enzymes; *e. g.*, enzymes for sugar breakdown, enzymes for synthesis of coenzymes, enzymes for phosphate ester synthesis, etc. In addition, the enzymes which destroy coenzymes must be controlled. In the intact normal cell this control and balance of enzymes is obtained by processes largely unknown at present, but certainly involving membrane permeability, and probably the concentration of energy-yielding substrates. Factors (*a*), (*b*), and (*c*) are not independent *in vivo*, but can be separated to a certain extent during *in vitro* experiments.

It is our belief that the previous experiments (1, 2) on changed rates of anaerobic glycolysis in poliomyelitis have not been well designed to detect the alterations listed above. Let us consider the points in order:

(*a*) Permeability and intracellular organization: No conclusions concerning these points can be drawn from previous work because mincing or homogenizing tissue is at present unsuitable for this type of study, since the changes in permeability and organization caused by these treatments are unknown and uncontrolled.

(*b*) Concentration of accessory compounds: Coenzymes, phosphate esters, and other substances necessary for glycolysis are subject to immediate attack after removal of the tissue from its natural environment, and especially after disruption of the cell. Since no attempt was made in the poliomyelitis studies (1, 2) to add accessory factors or to control their breakdown, no conclusion can be drawn from the experiments concerning the *in vivo* concentration of the factors. The glycolytic rates as measured with homogenates probably were determined by the concentration of accessory factors existing at the time of the measurements. Actually such differences as have been reported have not been confirmed upon repetition of the experiments (4). Even if the experiments were confirmed, the glycolytic measurements would not have any necessary correlation with the original *in vivo* concentration of the accessory substance, since the results would depend on at least two factors, the original concentration of the substance, and the rate at which it is destroyed and/or formed.

Clearly, a direct method of assay of the accessory factor under conditions in which the *in vivo* concentration of the factor is preserved would be the desired method. It should be noted that a preliminary study of the concentration of ATP in normal and poliomyelitic brains of mice has been made by Kabat (18) in which an attempt was made to prevent the breakdown of ATP. He reports an increased amount of the phosphate ester in the infected brains. This interesting observation needs to be confirmed and the method extended to other co-factors.

(*c*) Enzyme concentration: Enzyme assays are valid only if conditions are so maintained that enzyme concentrations are established as limiting factors, unless an indirect and more complicated method is used in which the concentration of all other components must be known and controlled. Since the concentration of accessory factors has in previous studies of poliomyelitis been neither known nor controlled, it is obvious that the measurements were not a reliable indication of the enzyme concentration of poliomyelitis-infected as compared to normal brains.

It is, thus, clear that no one of the determinations which are subject to *in vitro* study has been accurately measured as yet in poliomyelitis. Comparative studies of normal and poliomyelitis-infected cotton rats are now in progress, using the present method of enzyme assay, with proper precautions as to control of accessory components of the system.

SUMMARY

Experiments with mouse brain homogenates show that the anaerobic glycolysis of such preparations can be increased tenfold by addition of appropriate coenzymes and phosphate esters. The previously reported alterations in anaerobic glycolysis during poliomyelitis, as measured with low activity preparations, are believed to be of doubtful value in so far as the changes may be related to any specific phase of metabolism. In order to obtain this type of information, the experiment usually must be designed specifically to measure the desired factor.

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