INHIBITION OF PHOSPHORYLATION OF GLUCOSE IN MOUSE BRAINS BY VIRUSES AND ITS PREVENTION BY PREPARATIONS OF DIPHOSPHOPYRIDINE NUCLEOTIDE*

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It was observed previously that anaerobic glycolysis is inhibited in the brains of mice infected with the Lansing strain of poliomyelitis virus (1). The inhibition varied considerably from experiment to experiment and sometimes was absent or within the limits of experimental error. The average inhibition reported was therefore only 16 per cent, although inhibition as high as 50 per cent, as compared with the normal controls, was occasionally observed. The inconstancy of the inhibition made a study of its mechanism very difficult. The use of minced tissue also limited attempts to localize the site of inhibition since phosphorylated intermediates are not utilized if added to intact brain cells, whereas in brain extracts or cell-free homogenates difficulties of permeability are avoided and glucose phosphate esters are actively metabolized (2, 3).

Geiger (4) observed inhibition of glycolysis in homogenates of normal rat brains after cell destruction and Ochoa (3) confirmed this finding. It was considered that release of this "normal" inhibitor of glycolysis due to the inflammatory reaction in poliomyelitis might possibly be responsible for the decreased glycolytic rate in poliomyelitis-infected brains. It was decided, therefore, to study the nature of this "normal" inhibitor which was found to be present also in mouse brains. It was discovered (5) that two inhibitors of glycolysis are present in normal brain preparations. One of these is the enzyme responsible for the destruction of diphosphopyridine nucleotide (DPN). The second inhibitor was shown to be Na^+. The inactivation of DPN could be counteracted by the addition of nicotinic acid amide (NAA) as had been previously shown by Mann and Quastel (6), and the Na^+ effect was counteracted by phosphocreatine. Mouse brain homogenates were prepared which, on the addition of adenosinetriphosphate (ATP), DPN, NAA, and $MgCl_2$, and omission of Na^+, formed 7.5 mg. lactic acid per 100 mg. of wet weight of brain per hour, as compared to 0.4 to 0.6 mg. lactic acid formed by the brain minces used previously.

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At this point the study of glycolysis in mouse brains infected with neurotropic viruses was resumed (7). It is the purpose of the present report to describe experiments which show that the inhibition of glycolysis demonstrated in minced brains of mice infected with poliomyelitis is present also in homogenates of brain of infected mice; that the inhibition occurs in the steps leading to the formation of hexosediphosphate; and that a similar inhibition takes place when purified virus preparations are added to homogenates of normal mouse brain. Moreover, preparations of diphosphopyridine nucleotide have been shown to counteract the inhibitory action of Theiler FA mouse encephalomyelitis virus on glycolysis.

Materials and Methods

The preparation of actively glycolyzing brain homogenates and methods for the determination of lactic acid, glucose, and phosphorus fractions were described previously (5).

Fructose-6-phosphate was prepared from hexosediphosphate (8). The Lansing strain[1] and the Theiler FA strain[1] of poliomyelitis were purified by a method described previously (9), omitting the last fractional pH precipitations because of great losses known to occur at this stage. These preparations represent only a 60- to 100-fold purification of the virus according to total nitrogen determination, but were found suitable for metabolic studies. The PR8 strain of influenza A virus[1] was a preparation purified by red blood cell absorption and ultracentrifugation, the tobacco mosaic virus[1] was purified by ultracentrifugation. The influenza and tobacco mosaic viruses were received in a sodium phosphate buffer and were dialyzed before use against 0.08 M KCl at pH 7.0.

The normal or infected brains were homogenized with distilled water as previously described (5). Since it was found that nearly as much lactic acid is produced aerobically as anaerobically, and since the virus effect was also observed aerobically, it was no longer necessary to maintain anaerobic conditions. Brain homogenate from normal or infected mice was pipetted in amounts corresponding to 6 mg. dry weight of brain into test tubes 20 mm. in diameter, which were then covered with glass bulbs and subjected to preliminary incubation. In experiments where the effect of purified virus preparations on glycolysis was tested, the virus was added to homogenates of normal mouse brain and a corresponding amount of 0.08 M KCl was added to the control tubes. Usually glucose was added also to the brain homogenate before preliminary incubation since it preserved the glycolytic activity. At the end of this preliminary incubation period, which was usually 20 minutes at 37°C., the glycolytic reaction was started by the addition of ATP, DPN, and the other reagents required. Distilled water was added to bring the final volume to 2 ml. The final concentrations of the reagents added were: ammonium phosphate buffer, 0.01 M, pH 7.6; potassum bicarbonate, 0.008 M; $MgCl_2$, 0.0035M; adenosine triphosphate, 0.001 M; diphosphopyridine nucleotide, 0.0004 M; nicotinic acid amide, 0.012 M; glucose, 0.012 M. This mixture was then incubated at 37°C. for 1 hour and samples for chemical determination were removed at the end of this period.

RESULTS

Inhibition of Glycolysis in Homogenates of Infected Mouse Brains.—Homogenates of mouse brains infected with the Lansing strain or with the FA strain

[1] We wish to thank Dr. P. K. Olitsky for the Lansing strain, Dr. J. L. Melnick for the Theiler FA virus, and Dr. W. M. Stanley for his generous gift of purified influenza and tobacco mosaic virus preparations.

of the Theiler encephalomyelitis virus, showed an inhibition of glucose utilization as compared with homogenates of normal mouse brains. The data are summarized in Table I. The recorded *average* inhibition of 15 per cent with the Lansing strain and of 31 per cent with the Theiler strain, as shown in Table I, does not represent a true picture of the data. With the Lansing strain, as in the case of the original observations with minced brain preparations, some homogenates from infected mouse brains showed no inhibition while in others inhibition was as high as 50 per cent. With the Theiler strain also, some brains showed no inhibition while in others inhibition as great as 70 to 80 per cent occurred.

TABLE I

Inhibition of Glucose Utilization in Homogenates of Mouse Brains Infected with Neurotropic Viruses

Virus strain	Substrate	No. of mouse brains		Average inhibition
		Normal	Infected	
				per cent
Poliomyelitis, Lansing	Glucose	36	36	15.0
Poliomyelitis, Lansing	Hexose diphosphate	36	36	3.5
Encephalomyelitis, Theiler FA	Glucose	20	24	31.5
Encephalomyelitis, Theiler FA	Hexose diphosphate	5	5	4.5

These experiments were carried out before the importance of temperature during the preparation of the homogenates was realized. All of the experimental procedures of excising and grinding of the brains were done at room temperature and the reagents were added without preliminary incubation of the infected or normal brain. It was later found, however, that with homogenates of infected brains this variation could be avoided by preliminary incubation of the brain at 37°C. for 20 minutes before the reagent mixture (containing DPN, ATP, NAA, $MgCl_2$, and buffer) was added. This preliminary incubation results invariably in a marked inhibition of glycolysis in the infected brains as compared to the simultaneously studied normal control brains.

Since inhibition could thus be observed in cell-free homogenates of infected brains it became possible to study the localization of this inhibition in the Meyerhof-Embden scheme of glucose breakdown in tissues.

Localization of Inhibition of Glycolysis in Infected Mouse Brains.—Inhibition was apparent not only when glucose was used as substrate but also with glucose-6-phosphate or fructose-6-phosphate. No inhibition was found in infected brains when hexosediphosphate was added. Because of the greater inhibition observed with the Theiler virus, this strain was chosen for balance studies. The results of these balance studies are recorded in Table II.

Inhibition of glycolysis was shown to occur not only by decreased formation

of lactic acid but also by reduction of phosphate esters and decreased disappearance of glucose (Table II). Since the utilization of glucose-6-phosphate and fructose-6-phosphate was also impaired, both phosphorylation steps leading from glucose to hexosediphosphate seem to be inhibited in infected mouse brains.

This stands in contrast to the previously reported inhibitory effect of Na^+ (5) which, as we have shown, affects only the first step of glucose phosphorylation (formation of glucose-6-phosphate). In addition, phosphocreatine, which was shown to counteract the sodium inhibition does not alter the inhibition caused by the virus. For these reasons Na^+ can be eliminated as the cause of the inhibition of glycolysis in the brains of infected animals.

TABLE II

Inhibition of Glucose Utilization in Homogenates of Mouse Brain Infected with the FA Strain of Theiler Encephalomyelitis Virus

Mouse brain	Change in 1 hr.		
	Glucose	Lactic acid	P (ester)*
	mg.	*mg.*	*mg.*
Normal	−1.91	+1.75	+0.196
Infected	−0.9	+0.96	+0.100

* This represents the P values obtained by subtracting the acid-soluble P fraction after 7 minutes' hydrolysis in N HCl from the total acid-soluble P.

The lack of inhibition of glycolysis in the infected brains in the presence of hexosediphosphate as shown in Table I, makes it highly unlikely that diphosphopyridine nucleotidase can be responsible for the inhibition in infected brains, since DPN is required for the oxidation of glyceraldehyde-3-phosphate, through which lactic acid is formed from hexosediphosphate.

Effect of Purified Virus Preparations on Glycolysis of Homogenates of Normal Brain.—With exclusion of the two "normal" inhibitors, Na^+ and diphosphopyridine nucleotidase, the possibility was considered that the virus itself might be involved in the inhibitory effect observed. Partially purified preparations of the Lansing strain of poliomyelitis virus and of the Theiler FA strain of mouse poliomyelitis virus were added, therefore, to homogenates of normal mouse brain and the effect on glycolysis measured. The results are summarized in Table III.

As shown in Table III, preparations of the purified neurotropic viruses inhibited glycolysis in homogenates of normal mouse brain and the inhibition was similar to that observed in infected brain, namely, inhibition in the presence of glucose or fructose-6-phosphate, but no significant inhibition with hexosediphosphate as substrate.

To investigate the specificity of the inhibition observed with the Lansing strain of the poliomyelitis virus and the Theiler FA strain, two non-neurotropic viruses were tested, namely influenza A virus (PR8 strain) and tobacco mosaic virus. These two virus preparations, which were of much greater purity than the poliomyelitis virus preparation, caused inhibition similar to that encountered with poliomyelitis virus.

TABLE III

*Effect of Purified Virus Preparations on Glucose Utilization by Normal Brain Homogenates**

Virus preparation		Inhibition		
		Substrate		
Strain	Concentration	Glucose	Fructose-6-phosphate	Hexose-diphosphate
		per cent	*per cent*	*per cent*
Poliomyelitis, Lansing	—	21	13	3‡
Mouse encephalomyelitis, Theiler FA	—	41	14	0
Influenza A PR8	0.03 mg./ml.	40	33	5‡
Tobacco mosaic	0.045 mg./ml.	20	—	0
Sodium chloride	1.3 mg./ml.	70	2‡	0

* With the Lansing strain of poliomyelitis virus glycolysis was measured by reduction of methylene blue using Thunberg tubes. In all other cases glycolysis was determined by lactic acid production.

‡ These figures do not represent significant inhibition since they fall within the limits of experimental error.

It is evident from these results that the inhibition of glycolysis observed in mouse brain homogenates is not specific for the poliomyelitis virus. Furthermore, the type of inhibition produced by the three different viruses is apparently the same.

For purposes of comparison, the effect of Na^+ on lactic acid production is also recorded in Table III. With glucose as substrate, Na^+ inhibited lactic acid production by 70 per cent, whereas no inhibition occurred with fructose-6-phosphate or hexosediphosphate.

Effect of Time of Preliminary Incubation and of Temperature on Inhibition of Glycolysis in Brain Homogenates.—It was observed that the order in which the reagents were added affected the magnitude of the inhibition. This phenomenon, therefore, was studied further, using partially purified preparations of Theiler FA virus and highly purified preparations of influenza A and tobacco mosaic viruses. With all three virus preparations it was found that if the brain homogenate was first incubated with the virus before the other reagents were added, the inhibition of glucose utilization was proportional to the length of

the preliminary incubation time. These data are plotted in Fig. 1. When the incubation time was kept constant, the inhibition caused by the viruses was proportional to the concentration of virus added, as shown in Fig. 2.

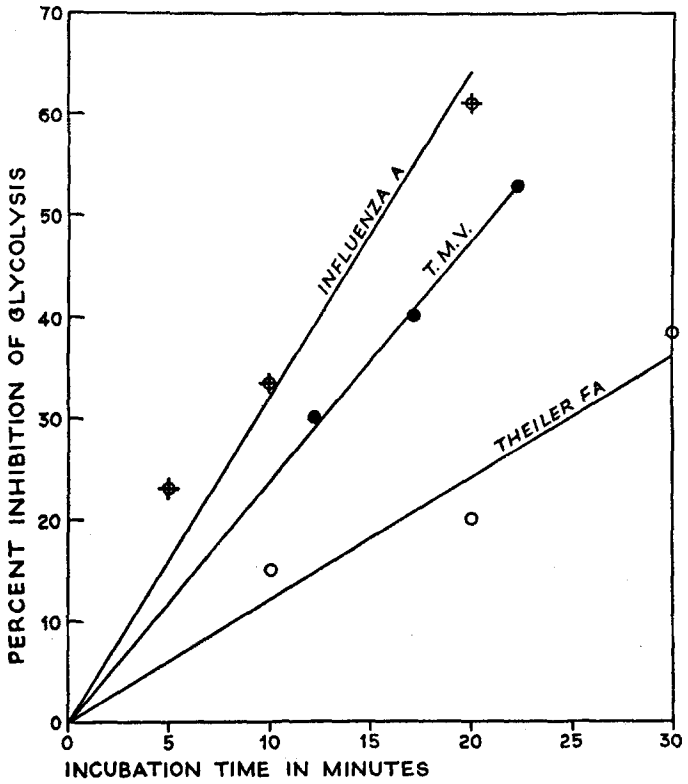

FIG. 1. Effect of time of preliminary incubation on inhibition of glycolysis by influenza A virus (30γ/ml.), tobacco mosaic virus (90 γ/ml.), and the Theiler FA strain of mouse encephalomyelitis virus. The preparations of Theiler FA virus were only partially purified, so that the actual amount of virus added could not be determined.

Increasing the temperature of preliminary incubation also brought about a striking increase in the inhibition, as shown in Table IV, by the Theiler FA strain and tobacco mosaic virus. When the virus preparations were added to normal brain homogenates and preliminary incubation carried out for 20 minutes at 0°C., no inhibition was observed. With the same amount of the viruses incubated for 20 minutes at various temperatures up to 40°C., progressively greater inhibition resulted as recorded in Table IV. With tobacco mosaic virus, inhibition as great as 77 per cent was not frequently encountered. This

will be discussed later. When the Theiler FA virus was added to normal brain homogenates the inhibition was invariably high when preliminary incubation was carried out at temperatures between 35 and 40°C.

With homogenates of brains of animals infected with Theiler FA strain, similar results were obtained. When infected brain was removed immediately

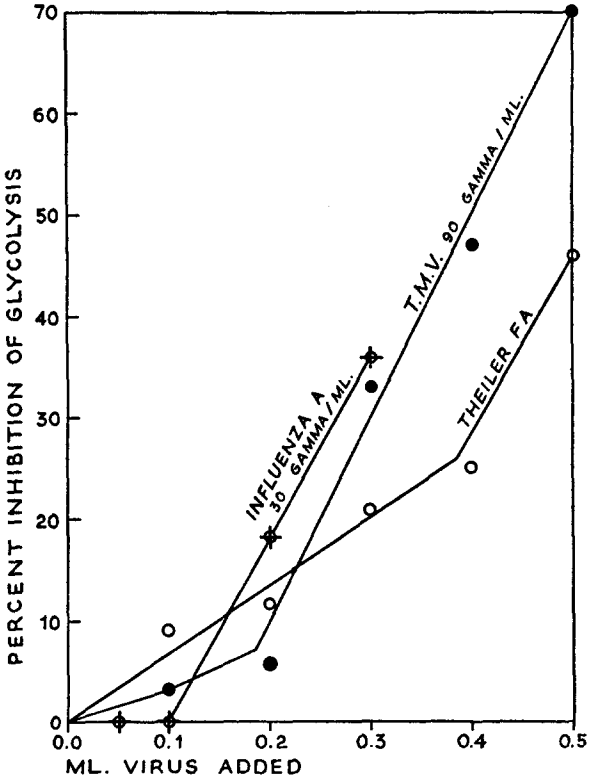

FIG. 2. Effect of concentration of virus preparations on inhibition of glycolysis by brain homogenates.

after the animals were sacrificed, grinding carried out in the cold, and the reagents added immediately, little or no inhibition was observed. When, however, the brain homogenate was incubated at 37°C. for 20 minutes before the reagents were added, the inhibition of glycolysis in the infected brains was uniformly high (Table IV). Inhibition ranging between 45 and 85 per cent was found in every infected mouse brain tested, with an average of 73 per cent.

Because of the effect of time and temperature on the degree of inhibition, the brain homogenate in all subsequent experiments was subjected to preliminary incubation at 37°C. for 20 minutes.

Presence of a Substance in Normal Mouse Brains Counteracting the Inhibition of Glycolysis by Virus Preparations.—The addition of purified virus preparations to homogenates of normal mouse brain of the same mouse strain (CFW) resulted in variable inhibition from one experimental animal to the next. This was particularly marked with the non-neurotropic viruses. In a typical experiment, the results of which are shown in Table V, homogenates of the brains of four mice were tested simultaneously. Although the same amount of tobacco mosaic virus was added to the homogenate of each brain, considerable variation in inhibition of glycolysis was encountered. Similar marked variation was also encountered with influenza virus. This variability has made the

TABLE IV

Effect of Temperature on Inhibition by Virus Preparations of Glycolysis in Homogenates of Normal Mouse Brains and in Infected Brains

Virus preparation	Temperature of incubation*	Inhibition of lactic acid production
	°C.	*per cent*
Tobacco mosaic	21	6
	28	22
	35	77
Theiler FA	0	3
	20	10
	30	13
	40	70
Infected brain (Theiler FA strain)	0	8
	37	65

* Incubation time was 20 minutes in all instances.

results of experiments with non-neurotropic virus preparations difficult to evaluate. Sometimes homogenates of several mouse brains were tested and no significant inhibition by the virus was observed, while on a few occasions inhibition as high as 77 per cent was found, as shown in Table IV. Although with neurotropic virus preparations, some variation in the amount of inhibition was also encountered in the different mouse brains, the degree of inhibition was significant in every experiment.

The occurrence of differing degrees of inhibition of glycolysis in individual mouse brains suggested either differences in the susceptibility to the effect of the virus or the presence in brain of a substance, in varying concentrations, counteracting the effect of the virus. When the latter hypothesis was tested, a heat-stable substance, capable of preventing the effect of the Theiler FA virus on glycolysis in brain, was demonstrated in extracts of normal mouse brain as shown in Table VI.

Normal mouse brains were homogenized by grinding and then extracted with five volumes of ice cold water or 0.16 M KCl. Centrifugation was carried out at 0°C. and the supernatant heated either at 56°C. or at 100°C. for 30 minutes. Of this heated extract, 0.2 ml. was added to the virus-brain mixture before pre-

TABLE V

*Variability of Inhibition of Glycolysis Caused by Purified Tobacco Mosaic Virus with Homogenates of Four Different Normal Mouse Brains**

Mouse	Inhibition of lactic acid production
	per cent
1	0
2	24
3	35
4	14

* Tobacco mosaic virus in final concentration of 90 γ/ml. was added to each mouse brain homogenate.

TABLE VI

Effect of a Heat-Stable Factor Present in Mouse Brain on Inhibition of Glycolysis of Normal Mouse Brain by Theiler FA Virus

Addition*	Lactic acid production	Inhibition of lactic acid production
	gamma	*per cent*
Glucose	2000	
Glucose, Theiler FA virus	1123	44
Glucose, brain extract heated at 56°C. for 30 min.	2257	
Same as above plus Theiler FA virus	2171	4
Glucose, brain extract heated at 100°C. for 30 min.	2143	
Same as above plus Theiler FA virus	2000	6

* These substances were added before preliminary incubation at 37°C. for 20 minutes. At the end of this period the other necessary reagents were added.

iminary incubation was started. From Table VI it can be seen that this heat-stable extract, although it has little effect on glycolysis in the control brain homogenates, completely counteracts the effect of the virus.

It has been observed that two different samples of diphosphopyridine nucleotide (DPN) prepared from yeast have the same effect as the heat-stable factor in brain (Table VII). When these preparations of DPN were added to the virus-brain glucose mixture before preliminary incubation at 37°C. for 20 minutes, inhibition caused by the Theiler FA virus was prevented. In the

control vessel without virus, addition of DPN before preliminary incubation had no effect on glycolysis. The same phenomenon was observed when brains infected with the Theiler FA virus were used. As can be seen from Table VIII, an infected brain showing inhibition of 63 per cent glycolyzed at the same rate as normal controls when 250 γ of a preparation of DPN were added before preliminary incubation.

It was noted, however, that addition of the DPN preparation was without effect unless glucose was also present during preliminary incubation (Table VII).

TABLE VII

Effect of Preparations of Diphosphopyridine Nucleotide on Inhibition of Glycolysis of Normal Mouse Brain by Theiler FA Strain of Mouse Encephalomyelitis Virus

Additions*	Lactic acid production	Inhibition of lactic acid production
	gamma	*per cent*
Glucose	2114	
Glucose, Theiler FA	521	75
Glucose, DPN	2371	
Glucose, DPN,‡ Theiler FA	2343	1
DPN	1589	
DPN, Theiler FA	771	51

* These substances were added before preliminary incubation at 37°C. for 20 minutes. At the end of this period the other reagents necessary for glycolysis, including additional DPN were added.

‡ One-fourth to one-half the amount of DPN required for optimal glycolysis reversed the effect of the virus.

Glucose was usually added during preliminary incubation at 37°C. because of its stabilizing effect on the glycolytic enzyme system of normal brain at this temperature as shown in Table VII. In the presence of glucose and DPN, lactic acid production was 2371 γ during the experimental period, whereas when glucose was omitted, lactic acid production was only 1589γ. However, glucose in the absence of DPN preparation did not counteract the effect of the virus on glycolysis (Table VII). It was therefore concluded that both the DPN preparation and glucose are necessary to prevent the inhibitory effect of the virus on phosphorylation of glucose.

In order to test whether DPN itself, a split product of DPN, or an impurity in our DPN preparation was responsible for the effect on virus inhibition, the following experiments were carried out: the brain mixture was incubated with all the available split products of DPN, singly or in combination, namely, nicotinic acid amide, phosphate, adenylic acid, and adenosinetriphosphate

(which is rapidly split by the brain). None of these substances counteract the virus inhibition. In addition, DPN was heated for 5 minutes at 100°C., in 0.02 N KOH to prepare "cophosphorylase" (10). It was found that this procedure which splits DPN into nicotinic acid amide, ribose, and adenosinediphosphate ("cophosphorylase"), completely destroys the effect of the DPN preparation on the virus inhibition.

The above evidence would appear to indicate that the DPN itself is the active substance which counteracts the virus inhibition of glycolysis, since the split products of mild alkaline hydrolysis are inactive. The possibility remains, however, that split products of enzymatic hydrolysis of DPN may be active, since according to Handler and Klein (11), when DPN is acted upon by brain tissue the split products are different from those resulting from alkaline hydrolysis. The possibility of the presence of an alkaline-labile substance, other than DPN, in our preparations from yeast or in brain extracts, must also be considered.

TABLE VIII

Effect of Preparations of Diphosphopyridine Nucleotide on Inhibition of Glucose Utilization in Infected Brains

Virus strain	Additions*	Inhibition of lactic acid production
		per cent
Theiler FA	Glucose	63
Theiler FA	Glucose + DPN	0

* These substances were added before preliminary incubation, as in the experiments recorded in Table VII.

DISCUSSION

Wood and his collaborators (12–14) were unable to confirm our original findings of impaired glycolysis in minced brains of mice infected with the Lansing strain of poliomyelitis virus. Later (15) they found, however, diminished glycolysis in brain extracts of cotton rats infected with the Lansing strain.

It is most unlikely that inhibition of glucose utilization in infected mouse brain homogenates to an extent of over 70 per cent, as reported in this paper, is representative of the process in the intact brain of the infected animal. During infection only a fraction of the total nerve cells are invaded by the virus while the other cells apparently remain intact. In the experimental procedure described, however, the brain is homogenized, with disruption of the cell structure and the virus can then come in contact with and act upon the enzyme systems of all the cells, whether infected or uninfected.

If glucose utilization within the infected cells is depressed to an extent similar

to that in brain homogenates, this effect could very likely play an important part in the process of neuron destruction. The sensitivity of the central nervous system to a relatively small reduction in its glucose supply is well known, as for example in the case of the cerebral effects of hypoglycemia in insulin shock.

On addition of preparations of DPN and glucose, the inhibition of glycolysis in the infected brain or in the normal brain-virus mixture is prevented. We have no evidence as yet that DPN itself is responsible for this effect. It is possible that either an impurity in our DPN preparations or an enzymatic split-product of DPN is the active substance.

The mechanism by which the virus inhibits the phosphorylation of glucose is not known. The breakdown of glucose, however, is the principal source of energy in the brain and phosphorylation of glucose represents the first step in the chain of reactions leading to the production of energy rich phosphate bonds. Possibly, a better understanding of the inhibition of phosphorylation by the virus may throw some light on the mechanism by which the energy metabolism of the host cell is utilized for the synthesis of virus protein.

SUMMARY

1. Inhibition of glucose utilization in homogenates of brains of mice infected with poliomyelitis virus (Lansing strain) is reported. The inhibition occurs with glucose, or fructose-6-phosphate as substrate. No inhibition occurs in the presence of hexosediphosphate.

2. Brain homogenates of mice infected with the Theiler FA strain of mouse encephalomyelitis virus show inhibition ranging between 45 and 85 per cent (average 73 per cent).

3. Purified preparations of the Lansing and the Theiler FA strain invariably inhibit glycolysis when added to homogenates of normal mouse brain. A similar, but much less consistent inhibition is provoked by adding high concentrations of non-neurotropic viruses (influenza A and tobacco mosaic virus) to normal mouse brains.

4. The magnitude of inhibition caused by the purified virus is a function of the virus concentration and depends on temperature and time of incubation of the virus-brain mixture.

5. The inhibition of glycolysis in the brains of mice infected with Theiler FA virus and in normal brain-Theiler FA virus mixtures is prevented by the addition of preparations of DPN and glucose.

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