57. GLYCOLYSIS IN CELL-FREE EXTRACTS OF BRAIN

By A. GEIGER

From the Physiological Laboratory, The Hebrew University, Jerusalem

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THE elucidation of the intermediary processes in muscle glycolysis accomplished in recent years has been largely due to the fact that active cell-free extracts of muscle could be prepared. Most of the known intermediary products of muscle glycolysis have been isolated from such extracts. On the other hand our methods are hardly adequate for dealing with intermediary processes in living cells. Thus no substantial progress has been made in elucidating the intermediary processes of brain glycolysis, for in brain the destruction of cell structure causes complete loss of the glycolytic power. Extracts retain only a small fraction of the glucolytic power of the brain tissue and have therefore proved unsuitable for the examination of the intermediary products involved in brain glycolysis. It was shown recently [Geiger, 1939] that on cytolysis a powerful inhibitor of glycolysis and of yeast fermentation is liberated from brain tissue and it was assumed that the presence of this substance is responsible for the lack of glycolysis in brain extracts. It was also assumed that after eliminating the antiglycolytic substance contained in cytolysed brain, the extracts could be reactivated by addition of coenzymes.

By the procedure described below highly active brain extracts have been prepared. The glucolytic rate of these extracts surpasses by 4–7 times that of brain slices or pulp, and is about 50 times that of the former extracts.[1] After adding the coenzymes the extracts are active even in dilutions as high as 1 : 250.

The high activity of these brain extracts made possible the examination of a few intermediary processes of brain glucolysis. The results obtained on these extracts differ in many respects from those obtained on brain tissue. Part of the divergence is possibly due to the selective permeability of brain cells. Naturally, the reservations made in the interpretation of results obtained on muscle extracts also apply to the present case.

General methods

The analytical and preparative methods employed in these experiments were those described in a previous paper [Geiger, 1939]. Albino rats were used in most experiments. Pyruvic acid was estimated according to Case & Cook [1931]. Embden ester was prepared according to Ostern *et al.* [1936], Cori ester according to Cori *et al.* [1937]. The phosphoric esters were estimated by acid hydrolysis

[1] About 670 mg. lactic acid are formed by 100 g. of chopped fresh brain tissue in 1 hr. at 37°, and about 1100 mg. by the same weight of sliced brain. The maximum weight of lactic acid obtained by a corresponding amount of extract was 80 mg. As the work of Euler *et al.* [1936] was done on acetone-precipitated extracts it is difficult to compare their figures with those obtained on fresh extracts. A rough calculation shows, however, that in their experiments about 40 mg. of lactic acid were formed by an amount of extract corresponding to 100 g. of fresh brain.

according to Lohmann [1928]. In several cases the Ba salts of the phosphoric esters were precipitated. Lactic acid was determined chemically if not stated otherwise.

Preparation of active extracts

Cell-free extracts prepared with isotonic solutions had a very slight glucolytic activity. It was found, however, that from brain cytolysed with distilled water the inhibitory substance previously described did not pass into the extracts if the extraction was carried out at a slightly acid reaction. Upon testing these extracts no glucolytic activity was found even after adding all the known coenzymes of muscle glycolysis. The addition of boiled extracts of beer yeast, however, caused these extracts to glycolyse at a rate surpassing even that of brain slices. The following experiment illustrates this: Exp. 1. Four brain preparations were made: (*a*) Cerebrum of young albino rats macerated in a smooth mortar with 4 times its weight of Ringer solution. (*b*) Cerebrum macerated with 3 times its weight of distilled water and 1 part of 0·1 *M* phosphate of *p*H 5·5. (*c*) Cerebrum macerated with 4 parts of water and (*d*) cerebrum macerated with 4 parts of 0·5 % $NaHCO_3$. After 10 min. standing on ice (*b*), (*c*) and (*d*) were centrifuged. 2 ml. each of pulp (*a*) and of the extracts (*b*), (*c*) and (*d*) were made up in separate Erlenmeyer flasks with the necessary additions to 4 ml. After filling up with N_2 containing 5 % CO_2 they were incubated at 37° for 1 hr. while rocking in the water thermostat. The additions consisted of 0·2 ml. *M*/5 phosphate buffer of *p*H 7·2, 0·8 ml. of 1·3 % $NaHCO_3$, 50 mg. glucose or glycogen, 1 mg. Ap,[1] 2 mg. CrP, 2 mg. GSH, 2 mg. CoZ and 0·5 ml. of boiled yeast extract. No Mg was added. The yeast extract was made by macerating 1 part of air-dried beer yeast with 4 parts of water and by heating the centrifuged extract for 10 min. in a boiling water bath. The contents of each flask correspond to 0·4 g. of fresh brain. Table 1 shows the amount of lactic acid formed in each flask calculated for 5 ml. of extract (corresponding to 1 g. of fresh brain).

Table 1. *The effect of boiled yeast extract on glycolysis of different brain preparations*

	mg. lactic acid formed in 1 hr. at 37° by 5 ml. extract	
	From glucose g.	From glycogen g.
A. Brain pulp in isotonic NaCl	6·32	1·09
The same + boiled yeast extract	6·85	1·92
B. Brain extract in phosphate solution of *p*H 5·5	1·16	0·80
The same + boiled yeast extract	15·5	5·2
C. Water extract of brain	0·80	0·85
The same + boiled yeast extract	27·0	8·60
D. Brain extract in 0·5 % $NaHCO_3$	0·36	0·51
The same + boiled yeast extract	2·10	1·30
E. Boiled yeast extract + all coenzymes	0	0

From this experiment it became evident that some substances contained in boiled yeast extract activate brain glycolysis. These substances were also found to be present in smaller amounts in muscle and in still smaller amounts in brain.

[1] In the following pages Ap stands for adenosinetriphosphate, CrP for creatinephosphate, Hdp for hexosediphosphate, HMP for Robison ester, EE for Embden ester, CE for Cori ester, CoZ for cozymase, GSH for reduced glutathione.

It should be pointed out that the water extracts, although more active than the phosphate extracts, under the present conditions lose the greatest part of their glucolytic power after 30–40 min. incubation at 37°. It is shown below that by diluting the water extracts, a constant high rate of glucolysis can be obtained for about 1 hr. The activity of the phosphate extract remains fairly constant for a long period also if used in high concentration.

Acetone-dried preparations of brain extracts were made by pouring 1 part of extract into 10 parts of ice-cooled acetone. The precipitate was quickly filtered, washed with cold acetone and dried. It was invariably found that the extracts lost about 40–60 % of the activity after precipitation in acetone.

In all the following experiments the extracts were prepared by thoroughly grinding rat cerebrum in an ice-cooled mortar with 4 parts of cold water. The brain was finely ground before the addition of the water which was added in small portions with continuous grinding. One part of 0·1 *M* phosphate buffer of *p*H 5·5 was then added, mixed, let stand in ice for 10 min. and centrifuged. If phosphate-free extract was desired, the extraction was carried out with 4 parts of water. The extracts kept their activity on ice for 2–3 days. The glucolytic rate in the water extract is usually more than twice as high as in the phosphate extracts. A second extract of the brain pulp has a slight glucolytic activity, the third extract is inactive.

The effect of dilution of the extracts on the glucolytic rate. It was seen in the above experiment that the water extracts lose their high initial rate of glucolysis after a short incubation. On the other hand the acid-phosphate extracts had a slower but more constant glucolytic rate. Further addition of coenzymes and of bicarbonate to the water extracts could not restore their activity. It was found later that the water extracts still contain some of the glycolysis inhibitor and that the presence of this inhibitor is responsible for the quick decline of the glucolytic rates. On diluting the extracts, however, the inhibitor loses its activity and the extracts maintain a very high and constant rate of glucolysis for 1–1½ hr. at 37°. The optimum dilution under our experimental conditions was found to be 0·5 ml. of extract to 10 ml., corresponding to a dilution of 1 part of fresh brain to 100 ml. Under these conditions a maximum amount of 64 mg. of lactic acid was formed by 5 ml. of extract—corresponding to 1 g. of fresh brain—in 1 hr. at 37°. The average figure from 84 experiments was 46 mg., i.e. about 4 times the amount which can be formed by slices under the same conditions.

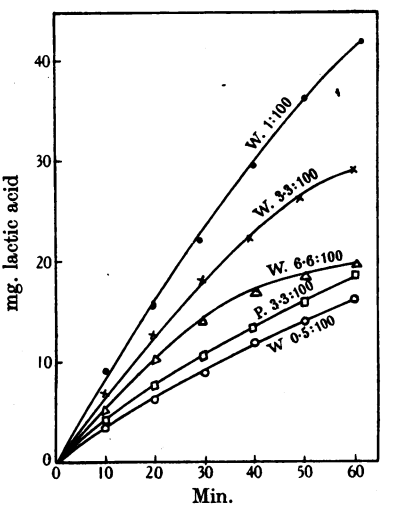

Fig. 1. The effect of dilution on the glucolytic rate in brain extracts. W, water extract; P, phosphate extract. Lactic acid is calculated in mg. for 1 g. of fresh brain. Dilutions are given in g. of fresh brain/ml. of fluid.

Further dilution of the extracts lowers the rate of glucolysis owing to the lack of a yet unknown coenzyme. The high dilution of the water extract has the further advantage that the concentration of coenzymes and of other active substances is very low. Experiments with different dilutions of brain extract are shown in Fig. 1.

Effect of dialysis on the activity of the extracts. For the experiments described in the following section it was desirable to free the extracts as far as possible from coenzymes and from preformed metabolites. On dialysis in collodion sacs and in cellophane tubes the extracts gradually lost their glucolytic power. After 8–10 hr. of dialysis a precipitate formed and the extract could not be reactivated by coenzymes and boiled yeast extract. The possibility has been considered that by dialysis a further coenzyme was eliminated. To test this possibility boiled extracts of muscle and of brain were concentrated in a vacuum and added to the dialysed extracts. In addition, fresh brain extract was dialysed and the dialysate concentrated in a vacuum. The addition of the concentrated boiled extracts and of the dialysate to dialysed brain extract did not reactivate the latter. It has to be assumed therefore that dialysis against distilled water inactivates the brain extracts irreversibly.

In later experiments it was possible to prevent the precipitation of the extracts and maintain a part of their activity by dialysis against 0·6 % NaCl solution. After 24 hr. of dialysis only a slight precipitate formed and after the addition of coenzymes the extract still retained about a third of its original activity.

Coenzymes of brain glycolysis

(*a*) *Magnesium and hexosediphosphate.* From the experiment in Table 1, it is evident that the coenzymes contained in boiled yeast extract activate brain glycolysis. In attempting to separate the active principle from other components of the yeast extract the following procedure was adopted: air-dried beer yeast[1] was ground with 4 parts of water and centrifuged after standing 15 min. The extract was heated for 8 min. in a boiling water bath. To the cool extract Pb acetate was added until no more precipitate formed; it was then filtered. An excess of mercuric acetate was added to the filtrate and the precipitate was filtered off after a few hours. The filtrate was treated with H_2S and was concentrated under reduced pressure to a fifth of its original volume. After neutralization with $NaHCO_3$ a large excess of $NaHCO_3$ and enough $Ba(OH)_2$ were added to precipitate all the carbonate. The precipitate was filtered and washed with water. The active substance remained with the $BaCO_3$ precipitate. The $BaCO_3$ was then decomposed with just enough $5N$ H_2SO_4 and the $BaSO_4$ washed once with a small amount of water. It was thus possible to concentrate the active substance contained in 1 kg. of yeast into 50 ml. The filtrate was free from CoZ, Ap and GSH. For the sake of brevity further particulars are omitted as it was found later that the boiled yeast extract can be replaced by Mg and minute amounts of Hdp. The principal active component of the purified yeast extract was found to be Mg.

One of a large number of experiments is shown in Table 2. In these experiments 2 ml. of fresh phosphate extract of brain (corresponding to 0·4 g. of fresh brain) were diluted with the necessary additions to 10 ml. and incubated for 1 hr. at 37°. The lactic acid production is calculated as usual for 5 ml. of brain extract corresponding to 1 g. of fresh brain.

The inactivity of the extracts to which no Mg was added is surprising if the rather low dilution of the extracts is considered. In muscle extracts a relatively thorough dialysis is necessary before the lack of Mg becomes apparent. In order to test the optimum amount of Mg in brain extract, the following experiments were made with more diluted water-extract. 0·5 ml. of brain extract was made

[1] We are greatly indebted to the Rishon Brewery for kindly supplying us with fresh beer yeast.

Table 2. *The effects of Mg and of hexosediphosphate on glucolysis*

	mg. lactic acid formed by 5 ml. extract	
	From glucose	From glycogen
Brain extract + all coenzymes	0·80	0·55
The same + 0·5 ml. yeast extract	18·1	6·7
The same + 4 mg. $MgCl_2$	17·9	6·8
The same + 4 mg. $MgCl_2$ + yeast extract	18·1	6·7
The same + $MgCl_2$ + Hdp	18·3	6·8
The same + $CaCl_2$ + Hdp	0·9	0·45

up to 10 ml. The resulting dilution was 1 part of fresh brain to 100 parts of fluid. Other additions were: 2 mg. Ap; 1 mg. of CoZ; 3 mg. of GSH; 3 mg. of CrP; 0·5 mg. of Hdp; 1·8 ml. of 1·3% $NaHCO_3$; 0·8 ml. of a $M/5$ phosphate solution of *p*H 7·2 and 1 ml. of a 5% solution of glucose or glycogen. The results, calculated for 5 ml. of brain extract, are contained in Fig. 2.

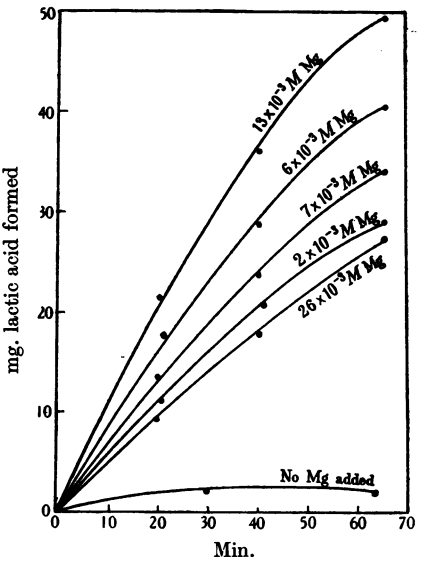

Fig. 2. The effect of Mg on the glucolytic rates in brain extract. Lactic acid is calculated for 1 g. of fresh brain. Dilution of the extract 1 : 100.

It appears that the concentration of Mg which is necessary for full activation of the brain extracts is about 3 times as high as that necessary for muscle extract. In our experiments the optimum concentration of Mg was about 13×10^{-3} M as compared with Lohmann's [1928] figures of $4{\cdot}8 \times 10^{-3}$ M for muscle extracts with glycogen and $1{\cdot}4 \times 10^{-4}$ M for Hdp. In these experiments lactic acid was estimated by chemical and by manometric methods. It was found, however, that higher Mg content made the manometric experiments unreliable. In these experiments some Mg phosphate was precipitated, and a large amount of CO_2 liberated, making measurements of glycolysis impossible. This CO_2 formation lasted for $\frac{1}{2}$–$\frac{3}{4}$ hr. It is interesting to note that addition of large amounts of coenzymes inhibited to a considerable extent the CO_2 formation and the precipitation of Mg phosphate.

Mg cannot be replaced by Ca. The addition of large amounts of Ca causes inhibition of glycolysis. Inhibition is caused also by larger amounts of Mg.

The addition of small amounts of Hdp further activated glucolysis in some experiments with highly diluted brain extract. The coenzymic function of Hdp in muscle glycolysis has been pointed out by Kendall & Stickland [1937]. Experiments with Hdp are also contained in Table 2. In all the following experiments the boiled extract of beer yeast was replaced by $MgCl_2$ and minute amounts of Hdp.

(*b*) *Creatinephosphate.* The role of CrP in muscle glycolysis has been extensively studied during recent years. It was found that its role as a coenzyme in muscle glycolysis is facultative [Parnas, 1937]. On the other hand, according

to Meyerhof *et al.* [1937], for the main route of muscle glycolysis the presence of creatine is essential.

It seems, however, that in brain CrP is a coenzyme of glucose breakdown, although it is possibly not indispensable for lactic acid formation from glycogen. In the experiments on fresh extracts the effect of CrP on glucolysis was variable. In dialysed extracts, however, its effect was conclusive. We have not yet been able to find the reason for the variable effect of CrP in fresh extracts. In the majority of experiments with fresh extracts the activating effect of CrP was unmistakable while in some other experiments it was very small or not appreciable. For this reason a large number of experiments are reported in Table 3.

Attention should be drawn to the fact that in concentrated extracts the necessity of CrP is more evident than in the diluted extracts. In the majority of the experiments, if the extracts were diluted to 1 : 100, the maximal glycolytic rate was also obtained without the addition of CrP. In fresh water-extracts of brain the activating effect of CrP was very seldom visible.

In a number of experiments the extracts were autolysed without substrates at different reactions in order to split the preformed CrP. The behaviour of these extracts with regard to CrP was not different from that of the unautolysed extracts.

It should be pointed out that in no case had CrP any activating effect on lactic acid formation from glycogen. Dialysed extracts did not form any significant amount of lactic acid from glycogen.

CrP could not be substituted by creatine or by any of the known coenzymes.

Table 3. *The effect of phosphocreatine on glycolysis in brain extracts*

Dilution of the extract: 1 g. brain to total vol. (ml.)	mg. lactic acid formed by extracts corresponding to 1 g. of fresh brain, in 1 hr. at 37°			
	From glucose		From glycogen	
	Without CrP	With CrP	Without CrP	With CrP
A. Phosphate-extracts				
15	6·7	18·5	6·1	5·8
20	5·2	19·1	6·7	6·4
20	11·5	18·1	5·5	5·9
20	16·8	17·2	5·6	5·4
100	6·5	17·8	7·6	7·9
B. Water-extracts				
15	33·0	45·0	9·4	9·1
20	32·0	31·7	9·8	9·5
100	38·2	49·3	12·5	12·3
C. Dialysed water-extracts (for 24 hr. against 0·6 % NaCl)				
15	0·8	10·6	—	—
25	0·5	16·1	—	—
50	0·8	18·6	—	—

On the other hand the necessity of CrP in brain glucolysis could be conclusively demonstrated with dialysed extracts. For this purpose water extracts of brain were dialysed for 20–25 hr. against 0·6 % NaCl solution. The experiments made with such extracts have shown that in the absence of CrP no lactic acid is formed from glucose, mannose or fructose. All these experiments gave uniform results. The lactic acid formation from glycogen in dialysed extracts was always very low and these were unsuitable for the examination of the role of CrP in lactic acid formation from glycogen.

(*c*) *Adenosinetriphosphate.* It was shown [Geiger, 1935] that Ap activates glucolysis in brain pulp. This was confirmed by Euler *et al.* [1936]. In the present experiments it was seen that Ap is a coenzyme of brain glucolysis. In diluted extracts the glycolysis comes to a standstill after a very short incubation and is restored by the addition of Ap. In Fig. 3 one experiment taken from a larger number of identical ones is reported. In these experiments 0·5 ml. of water extract of brain was made up to 10 ml. with the usual additions.

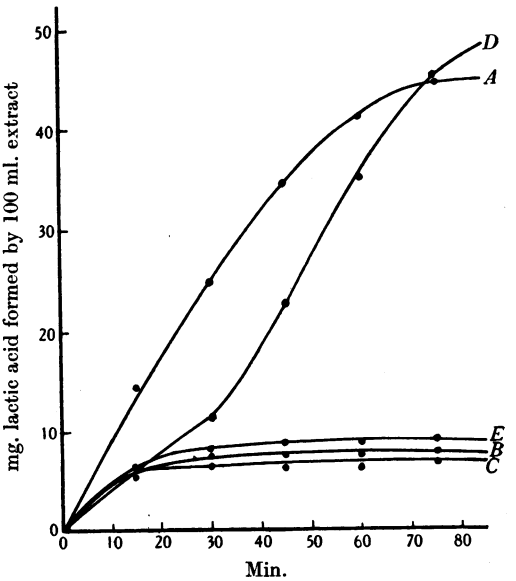

Fig. 3. The effect of cozymase, of Ap and of muscle adenylic acid. *A*, all coenzymes; *B*, without Ap; *C*, without cozymase; *D*, with adenylic acid and CrP, without Ap; *E*, with adenylic acid and phosphopyruvic acid without Ap.

The effect of Ap on glucolysis is evident even in concentrated brain extracts. Thus, in one experiment with 2 ml. of brain extract diluted to 3·5 ml., 24·6 mg. lactic acid were formed in 1 hr. with added Ap and 3·3 mg. without Ap. Under our experimental conditions about 1·5 mg. of Ap had to be added to 10 ml. in order to obtain a maximum activation.

Ap could not be replaced by yeast adenylic acid, but muscle adenylic acid together with CrP or with Hdp could replace it. In the latter case a long latent period was observed. Muscle adenylic acid with phosphopyruvic acid was less effective.

(*d*) *Cozymase.* Diphosphopyridinenucleotide (Euler) is indispensable in brain glycolysis. In all our experiments relatively large amounts of CoZ had to be added in order to obtain full activation.[1] In this respect the behaviour of brain extract was similar to that described by Boyland *et al.* [1937] for tumour extract. It is probable that the high CoZ requirement of brain extract is due to the rapid destruction of CoZ by an enzyme which is not necessarily involved in brain glycolysis. The experiment given in Fig. 3 was made similarly to those with

[1] The grade of purity of our cozymase preparation has not been tested. It was prepared according to Ohlmeyer [1936] and should be 90% pure.

Ap. For this experiment highly diluted extracts were used, but the effect of CoZ is very marked also in concentrated extracts.

(*e*) *Glutathione.* The activating effect of glutathione on brain extract has been described previously [Geiger, 1935]. As these earlier extracts had very slight activity we repeated the experiments on extracts made by our present method.

The main difficulty was encountered in obtaining extracts free from glutathione. Even after 25 hr. of dialysis the extracts still contain small amounts of glutathione. Activation of glucolysis by glutathione was seen in every experiment, but its degree was variable. The effect of glutathione on the extracts did not differ much from that on brain pulp. On the other hand dialysed extracts responded more regularly to glutathione. Euler *et al.* [1936] confirmed the activating effect of glutathione on brain glucolysis, but they attributed this action to the capacity of glutathione to combine with heavy metals. It was shown by Geiger & Magnes [1939] that this latter contention is untenable.

The results obtained with glutathione on brain extracts and on brain pulp are very similar to those of Needham & Lehmann [1937] obtained on embryo brei. Although it seems to be fairly well established that glutathione plays some part in glucolysis it is at present impossible to say whether it is a coenzyme or not. The results obtained with glutathione are contained in Table 4.

Table 4. *Effect of glutathione*

	mg. lactic acid produced by 5 ml. extract in hr. at 37° from glucose	
Dilution of extracts	With GSH	Without GSH
5 hr. dialysed, 1 : 15 dilut.	7·8	5·1
,, ,,	6·6	4·1
Not dialysed, 1 : 100 dilut.	47·5	41·3
18 hr. dialysed, 1 : 45 dilut.	14·0	10·2
5 hr. dialysed, 1 : 15 dilut.	31·0	21·0
Not dialysed, 1 : 50 dilut.	31·0	24·0
Not dialysed, 1 : 25 dilut.	29·0	18·0
22 hr. dialysed, 1 : 50 dilut.	24·5	18·0

(*f*) *The possible participation of a further coenzyme.* If the extracts are diluted in proportion of 1 part of brain to 250, the activity of the extracts is about $\frac{1}{3}$ of the 1 : 100 dilution. The addition of boiled extract of brain activates these extracts to a rate corresponding to the 1 : 100 dilution. This shows that in addition to the coenzymes described previously, probably a further coenzyme participates in brain glucolysis.

Lactic acid production from various substrates

(*a*) *Hexoses and pentoses.* The rate of lactic acid production in brain extract from various substrates is shown in Table 5. It appears that from glucose, mannose and fructose large amounts of lactic acid are produced. The rate of lactic acid production from fructose is about 10 % higher, from mannose about 10 % lower, than from glucose. Practically no lactic acid is formed from the other sugars.

The most striking fact in these experiments is the large amount of lactic acid produced from fructose. In all the previous experiments made on brain pulp it was found that no lactic acid can be produced from fructose. Needham & Nowiński [1937] have shown that embryonic tissue is also unable to convert

fructose into lactic acid. Nevertheless brain tissue can oxidize fructose within R.Q. of unity, although unable to form lactic acid from it as was shown by Loebel [1925] and by Dickens & Greville [1935]. It is therefore impossible to suppose that fructose cannot enter into the brain cells. The high rate of lactic acid production from fructose by brain extracts is therefore the more surprising. No explanation can be offered for this fact.

In Table 5 are also a few experiments with glucose and glycogen added separately and together to the same extract. They show that the lactic acid formed from glucose is equal in amount to that formed from glucose and glycogen together. The additive behaviour of these two substrates observed by Ashford & Holmes [1929] with brain pulp is not seen in brain extracts. It seems, therefore, that the glucolytic and the glycogenolytic enzyme systems in brain, though probably different, have common components.

Table 5. *Lactic acid formation from different substances in brain extracts*

The lactic acid formation is calculated for 5 ml. of extract corresponding to 1 g. of fresh brain. The values are averages from at least 4 experiments

Substance added	mg. lactic acid formed in 1 hr. at 37°
Glucose	42·1
Glycogen	14·2
Glucose + glycogen	41·0
Fructose	49·5
Mannose	34·2
Galactose	0·9
Arabinose, xylose and sorbose	0
Embden ester	3·1
Robison ester	1·6
Hexosediphosphate	0·6
Phosphoglyceric acid	3·8
α-Glycerophosphate	2·1
Pyruvic acid	1·2
Phosphopyruvic acid	1·3
Hexosediphosphate with 10^{-5} *M* arsenate	1·8
Hexosediphosphate with 10^{-3} *M* arsenate	2·9

(*b*) *Phosphorylated intermediary products of muscle glycolysis.* In Table 5 the extent of lactic acid production by brain extract from phosphorylated intermediary products of muscle glycolysis may be seen. It appears that none of these substances is able to give rise to any substantial amount of lactic acid in brain extract, and the combination of these substances also gave negative results. It must be concluded, therefore, that none of the known phosphorylated intermediary products of muscle glycolysis can be considered as such in brain glycolysis. Experiments made with Hdp in the presence of 10^{-3} *M* arsenate have shown only a very small extent of acceleration of lactic acid formation from Hdp.

The blocking of glycolysis from Hdp and monophosphate occurs probably at the triosephosphate stage. The same was found by Needham & Nowiński [1937] for embryonic tissue. This is evident from the following experiments. A flask, containing 2 ml. of brain extract, Hdp and coenzymes as usual, was incubated at 37°. Lactic acid, inorganic phosphate and triosephosphate were estimated in aliquots at the beginning, after 10 min. and after 60 min. of incubation. 0·78 mg. lactic acid, 5·6 mg. inorganic P and 0·1 mg. alkali-labile P were found at the

beginning; 1·25 mg. lactic acid, 0·62 mg. alkali-labile P and 5·8 mg. inorganic P after 10 min. of incubation; and 1·27 mg. lactic acid, 6·3 mg. inorganic P and 0·66 mg. alkali-labile P at the end of the incubation period. This experiment shows that the equilibrium under our experimental conditions between Hdp and triosephosphate is established after about 10 min. at 37° and that from this time on the lactic acid and the triosephosphate contents remain unchanged. It is evident, therefore, that no lactic acid is formed from Hdp by brain extracts and that the increase in lactic acid appearing after 10 min. of incubation is due to the artificial transformation during the analysis of triosephosphate into lactic acid by the alkaline copper-lime solution. In order to avoid errors it is advisable to estimate the initial value of lactic acid, in experiments in which Hdp is present, after 10 min. of incubation at 37°, i.e. at a time when the equilibrium between Hdp and triosephosphate is established.

Inhibition of glucolysis in brain extracts by cytolysed brain pulp

In a previous paper [Geiger, 1939] the inhibiting effect of cytolysed brain on muscle glycolysis and on yeast fermentation was described. This inhibitor being inactive towards intact cells, its action on brain glycolysis could not be tested. It is now found that cytolysed brain tissue inhibits glycolysis in brain extracts. In the experiments in Fig. 4 various amounts of washed cytolysed brain were added to 1 ml. of brain extract, and the whole was made up to 10 ml. with the usual additions.

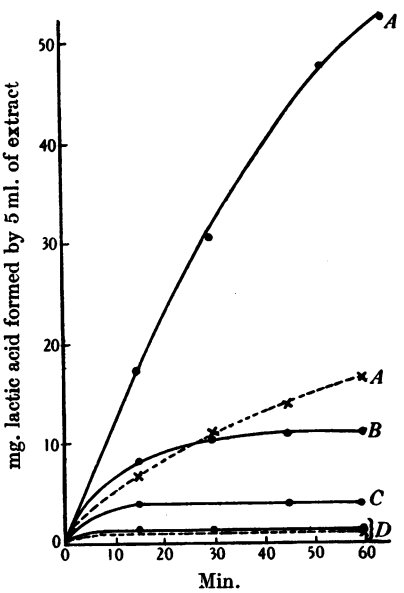

Fig. 4.

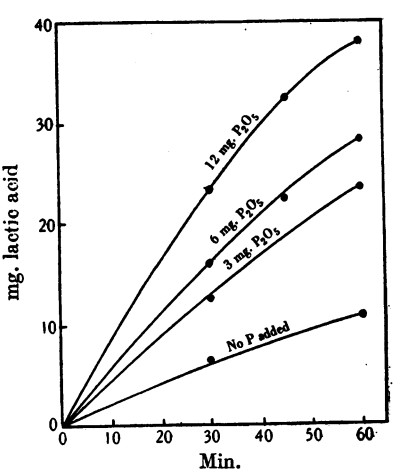

Fig. 5.

Fig. 4. The inhibiting effect of cytolysed brain pulp on the glycolysis of active brain extract. —— glucose, - - - - glycogen. *A*, brain extract alone; *B*, 0·2 g. cytolysed brain added; *C*, 0·3 g. cytolysed brain added; *D*, 0·4 g. cytolysed brain added to a total volume of 10 ml. containing 1 ml. of brain extract.

Fig. 5. The effect of the addition of different amounts of P to 10 ml. of brain extract. Lactic acid is calculated for 1 g. of fresh brain.

The role of phosphorus in glycolysis of brain extracts

(*a*) *The necessity for inorganic phosphorus.* Up to the present brain glucolysis was considered to be mainly a non-phosphorylating glucolysis. Ashford [1934] was unable to obtain any phosphorylation with glycolysing brain slices and with brei. Ashford & Holmes [1929] have also shown that in brain glucolysis the presence of inorganic P is unnecessary. The same conclusions were reached recently by us in experiments on brain pulp in isotonic solution. Euler *et al.* [1936], however, observed the disappearance of inorganic P in brain extracts of low glycolytic activity.

The present experiment shows that in contrast to the results obtained on brain tissue, glycolysis in brain extracts is dependent on the presence of inorganic phosphate. In Fig. 5 experiments are shown in which 0·5 ml. of fresh brain extract was made up to 10 ml. with the usual additions and incubated at 37°. One experiment with glucose and one with glycogen are shown in Fig. 5. The same experiment was repeated with dialysed brain extracts. The results show that there is no glycolysis at all in the absence of inorganic phosphate. The experiments with dialysed extracts are contained in Table 6. With respect to the need of inorganic phosphates, brain extract seems to differ from brain tissue.

Table 6. *The effect of inorganic phosphate on glucolysis in dialysed brain extracts*

The extracts were dialysed for 25 hr. against 0·6 % NaCl solution. 2 ml. of extract were made up to 10 ml. with the usual additions

Phosphate added (as mg. P_2O_5)	mg. lactic acid formed by 5 ml. extract in 1 hr. at 37°
None	0·21
3	5·66
6	9·60
12	13·30
20	13·50

(*b*) *Phosphoric ester formation in brain extracts.* In a second series of experiments the question if phosphorus is esterified during glycolysis was examined. The conditions in these experiments varied with regard to the dilution of the extracts. In the experiments contained in Fig. 6, 1 ml. of brain extract was diluted with the usual additions to 3·7 ml. and incubated at 37°.

It is evident that simultaneously with lactic acid formation from glucose, fructose and mannose, inorganic phosphate disappears. No esterification of phosphate was observed during lactic acid formation from glycogen under these conditions. The esterification of phosphate runs parallel with the formation of lactic acid and ceases together with the latter. As it was seen that phosphorylation is more marked in concentrated extracts than in diluted ones, these experiments were made on concentrated extracts. On the other hand water extracts had to be used. Thus, the rates of lactic acid formation diminish after about 30 min. incubation at 37°. This drop of the glycolytic rate was due mainly to the glycolytic inhibitor present in the brain extracts.

The phosphoric ester formation was well marked in every experiment. It appears, however, that the amount of phosphoric ester found is influenced by the dilution of the extracts. In several experiments with different dilutions of the same brain extract more phosphoric ester was found with the less diluted extract

with identical amounts of lactic acid. In these experiments, which are contained in Table 7, 1 ml. of brain extract was diluted to 3 and to 10 ml. Fructose and glucose were used as substrates.

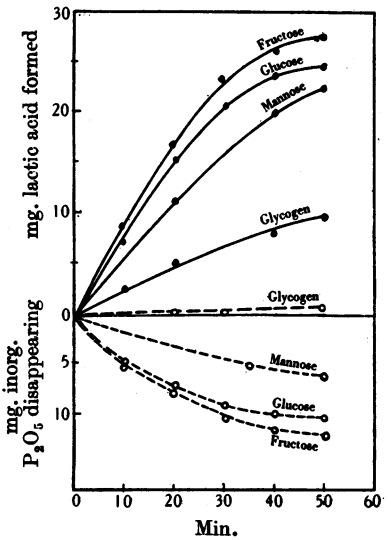

Fig. 6.

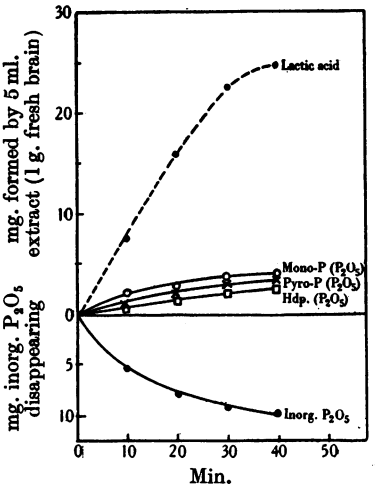

Fig. 7.

Fig. 6. The disappearance of inorganic P in brain extract during lactic acid formation from different substrates. The dotted lines show the changes in inorganic P, the continuous ones the lactic acid produced by extract from 1 g. of fresh brain.

Fig. 7. The distribution of the P compounds formed during glucolysis. In this experiment the substance was glucose.

Table 7. *The effect of dilution of the extracts on phosphoric ester formation*

mg. lactic acid formed and phosphorus esterified, respectively, by 5 ml. of brain extract

Dilution of the extract	1 : 3	1 : 10	1 : 3	1 : 10
Substrate	Glucose		Fructose	
In 30 min.	21·2, 11·7	25·9, 4·0	23·3, 12·0	28·7, 6·22
In 60 min.	24·4, 12·7	45·0, 6·2	25·0, 13·5	49·2, 6·95

It was rather surprising to find no phosphoric ester formation with glycogen. It was thought possible that in this case the formation of phosphoric esters is a slower process than the dephosphorylation. It was tried, therefore, to alter the relative rates of these two processes by changing the temperature. In experiments with glycogen at 20° a small but regular disappearance of inorganic P was seen. The lactic acid formation at this temperature was also very small. One of these experiments is given in Table 8. By comparing the relative amounts of phosphoric ester to lactic acid formed at different temperatures, we find that relatively larger amounts of phosphoric esters accumulate at lower temperature. The lack of phosphorylation of glycogen at 37° must be therefore only apparent, the low rate of phosphorylation and the relatively high rate of dephosphorylation masking the actual esterification. It is possible that the apparent lack of phosphorylation in intact brain cells may also be explained in the same way.

Table 8. *The phosphorylation of glucose and of glycogen at different temperatures*

	Glucose				Glycogen			
	At 37°		At 22°		At 37°		At 22°	
	mg. lactic acid formed	mg. P_2O_5 esterified	mg. lactic acid formed	mg. P_2O_5 esterified	mg. lactic acid formed	mg. P_2O_5 esterified	mg. lactic acid formed	mg. P_2O_5 esterified
In 30 min.	12·1	—	2·9	2·0	3·6	0	1·2	0·5
In 60 min.	21·4	7·7	5·9	3·8	7·1	0	2·3	0·9
In 90 min.	—	—	8·8	5·4	9·9	0	3·2	1·4

(*c*) *The nature of P-esters formed from glucose and from fructose.* As in the present paper it is not intended to deal in particular with the phosphorylation cycles, no attempt was made to isolate and to identify the P-esters formed during glucolysis: this will be the subject of a following paper. For the present it was attempted merely to obtain an idea of the nature of these phosphoric esters as far as is possible by the usual indirect methods such as acid hydrolysis and the solubility of the Ba salts. In a series of experiments the time relationship between the formation of the different P-esters and of lactic acid was examined. Glucose and fructose gave identical results in this respect. All the experiments were made on two different dilutions of brain extracts. In the experiments contained in Fig. 7, 2 ml. of brain extract were made up with the usual additions to 8 ml. The figures are calculated for 5 ml. of extract.

The average figure from 14 experiments shows that about 24% of the disappeared inorganic P are converted into the pyrophosphate fraction (max. 37%, min. 18%), 21% into Hdp, 6% into phosphopyruvic acid and about 45–50% into a phosphoric ester which is not hydrolysed in 3 hr. in *N* acid. For brevity's sake single experiments are omitted.

It was ascertained that the pyrophosphate fraction did not include CE. For this purpose the trichloroacetic acid filtrate was made just alkaline to phenolphthalein and an excess of Ba-acetate was added. The filtrate was treated according to Cori. On the first Ba-precipitate as well as on the alcohol precipitate acid hydrolysis was carried out according to Lohmann. The results show that about 95% of the easily hydrolysable fraction is pyrophosphate. About 21% of the difficultly hydrolysable fraction (not hydrolysed in 3 hr.) was precipitable by Ba at *p*H 8·5; the other 75% was precipitated at the same *p*H after the addition of 2 volumes of alcohol.

(*d*) *Effect of glycolysis inhibitors on phosphorylation.* The addition of fluoride to glycolysing brain extracts causes the accumulation of phosphoglyceric acid as in muscle extracts.

In the presence of *M*/40 fluoride no lactic acid was formed and only a very small amount of inorganic phosphate was esterified. On adding large amounts of Ap, however, a larger amount of a difficultly hydrolysable ester, presumably phosphoglyceric acid, accumulated. *M*/100 fluoride caused a 96% inhibition of lactic acid formation. One experiment of this type is reported in Table 9.

In the following experiments the effect of pyruvic acid was investigated in the presence of fluoride and of glucose. In all these experiments large amounts of Ap were present. Under these conditions lactic acid is formed and P is transformed into a difficultly hydrolysable ester, presumed to be phosphoglyceric acid.

Table 9. *The effect of fluoride on brain extracts*

Substrate	Glucose	Glucose	Glucose	Glycogen
Fluoride conc.	0·3 *M*	0·3 *M*	0·3 *M*	0·3 *M*
mg. Ap P_2O_5 added	1·70	0·78	0·14	1·88
mg. inorg. P_2O_5 esterified	2·72	1·02	0·08	2·12
mg. P_2O_5 transformed into difficultly hydrolysable ester	4·02	1·41	0·11	3·24
mg. Ap P_2O_5 found at the end of exp.	0·41	0·18	—	0·53
Duration of exp. (min.)	30	30	30	30

The lactic acid formation from glucose is inhibited to about 50% by the addition of 0·03 *M* pyruvate. The same concentration of pyruvate, however, did not inhibit the lactic acid formation from glycogen. The pyruvic acid used in these experiments was redistilled in vacuum from the commercial product. No lactic acid was formed and no phosphates were esterified with pyruvate and with fluoride in the absence of glucose or glycogen. These facts are illustrated in Figs. 8 and 9.

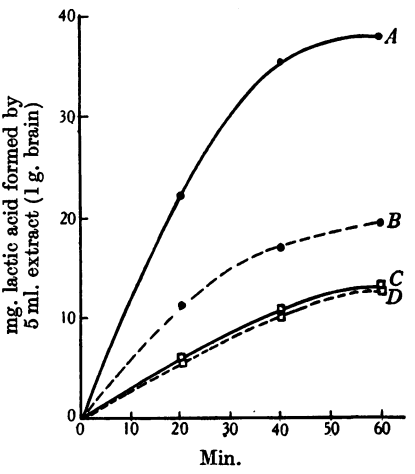

Fig. 8.

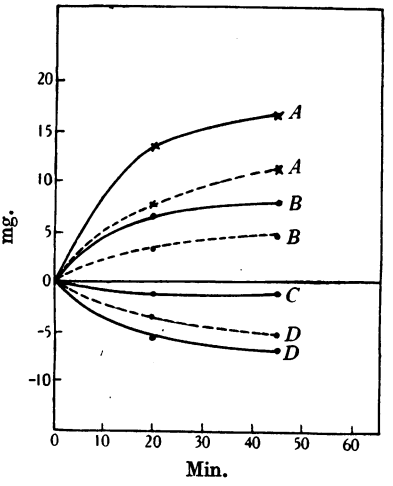

Fig. 9.

Fig. 8. The effect of pyruvic acid (0·03 *M* Na pyruvate) on lactic acid formation from glucose and from glycogen. *A*, glucose; *B*, glucose and pyruvate; *C*, glycogen; *D*, glycogen and pyruvate.

Fig. 9. Formation of lactic acid and of phosphoglyceric acid in the presence of *M*/40 fluoride and of pyruvic acid. From —— glucose and from - - - - - glycogen. *A*, lactic acid formed; *B*, phosphoglyceric acid formed; *C*, Ap disappeared; *D*, inorganic P disappeared. The amounts are calculated for 5 ml. of extract corresponding to 1 g. of fresh brain.

The experiments described here suggest a similar course of esterification in brain extract to one of those found by Meyerhof & Kiessling [1935, 1, 2] in muscle extract. It is impossible, however, to adopt at present any scheme which includes the intermediary formation of Hdp, or of triosephosphate, as brain extract does not transform either of these substances into lactic acid.

The effect of iodoacetate. The accumulation of Hdp from added glucose can be demonstrated in brain extracts in the presence of 0·0002 *M* iodoacetate and of large amounts of Ap. These experiments show that about 50% of the added Ap is converted into a phosphoric ester which is completely hydrolysed by *N* acid in 180 min. at 100°: it is probably Hdp. At the same time an equal amount

of the Ap-phosphorus is split off. No lactic acid was formed under these conditions. One experiment of this type is contained in Table 10.

Table 10. *The accumulation of hexosediphosphate in the presence of 0·0002* M *iodoacetate*

5 ml. of brain extract

	At start mg.	After 30 min. incub. at 37° mg.
Inorganic P_2O_5	11·32	14·36
Ap.-P_2O_5	7·78	1·24
Triose-P_2O_5	0·18	0·74
Hexosediphosphate-P_2O_5	0·29	2·81
Difficultly hydrolys.-P_2O_5	0·31	0·95

It is evident from this experiment that brain extract may form Hdp from glucose. On the other hand, however, it was shown that Hdp cannot be converted into lactic acid by brain extract. It cannot be concluded therefore from this experiment that Hdp plays any significant role in brain glycolysis. The relatively very slow transformation of Hdp into triosephosphate in brain seems to indicate also that Hdp is not connected with the main route of glucose breakdown.

Pyruvic acid

In several experiments attempts were made to demonstrate the formation of pyruvic acid. In most of these the trichloroacetic acid filtrate of brain extract was precipitated with 2:4-dinitrophenylhydrazine. In other experiments the trichloroacetic acid filtrate was titrated with bisulphite. In no experiment was it possible to obtain positive evidence of pyruvic acid formation. On the other hand the formation of small amounts of phosphopyruvic acid could be demonstrated by estimating this substance according to Meyerhof & Kiessling [1935, 1]. Methylglyoxal formation could not be observed under the present experimental conditions.

The effect of several factors on the rate of glycolysis in brain extracts

The *p*H-optimum for glycolysis in brain extracts is 7–7·5. For obtaining the maximum rate the addition of small amounts of K is also necessary. On the

Table 11. *The effect of several factors on glycolytic rates in brain extracts*

In these experiments 1 ml. of 1 : 5 water-extract of brain was diluted to 10 ml. with the usual additions. The resulting lactic acid production is calculated for amounts of extracts corresponding to 1 ml. of fresh brain. Glucose was used as substrate.

	mg. lactic acid produced in 1 hr. at 37°
The effect of Ca	
Without Ca	51·6
With 0·005 *M* Ca	47·2
With 0·01 *M* Ca	20·5
The effect of *p*H	
*p*H 6·0	39·7
*p*H 6·5	44·0
*p*H 7·0	48·4
*p*H 7·5	33·3
*p*H 8·0	26·2
The effect of O_2 and of K	
In Ringer-solution (anaerobic)	48·1
In Ringer-solution (in O_2)	34·0
In 0·1 *M* KCl solution (anaerobic)	25·6
In 0·1 *M* KCl solution (in O_2)	18·0

other hand Ca and Na inhibit glycolysis, Ca doing so even in very low concentrations.

Aerobic experiments have shown that O_2 inhibits glucolysis in the extracts by about 30–40%. The addition of KCl in concentrations up to 0·1 *M* did not change this behaviour, but the glycolytic rates in the presence of such amounts of K were very low.

Experiments made at different temperatures show the dependence of the glycolytic rate upon temperature. It seems however that the temperature factor in the case of phosphorylation is smaller than that in glycolysis.

Some of the experiments mentioned under this heading are contained in Table 11.

Discussion

The preparation of glycolytically active brain extracts depends mainly on two factors. The first is the presence of a certain concentration of Mg, the second a relatively high dilution of the extract. The presence of a glycolysis inhibitor in the extracts becomes apparent very soon if concentrated extracts are used. By observing these two conditions it is easy to prepare brain extracts with high and constant glycolytic rate.

Although it is not yet possible to advance any hypothesis concerning the path of glucose breakdown in brain, it is evident that several significant differences exist between the glycolytic processes in brain and in muscle.

The glycolytic substrates in brain are different from those in muscle. In brain extracts practically no lactic acid is formed from any of the phosphorylated intermediary products of muscle glycolysis. The rate of lactic acid formation from glycogen in brain extracts is about a third of that of glucose. In addition to the known coenzymes of muscle glycolysis, CrP and possibly also glutathione have coenzymic functions in brain glycolysis. The optimum concentration of Mg in brain is about 3 times as high as in muscle.

On the other hand, the formation of phosphoric esters during glycolysis is a phenomenon common to brain and to muscle extracts. In the presence of glucose, fluoride and of pyruvic acid there is an accumulation of phosphoglyceric acid in brain extracts as well as in muscle extracts. In the presence of iodoacetate and of large amounts of Ap the accumulation of Hdp can be observed in brain extracts. It must be admitted that the evidence presented here is somewhat contradictory. It is difficult to explain the failure of brain extract to form lactic acid from such substances, which are formed by the extract from glucose. To explain these discrepancies it may be assumed that the phosphorylated products which are found in the brain extract in the presence of fluoride or iodoacetate do not represent intermediary products of glucolysis, but only a by-way. On the other hand, nothing is yet known about the nature of the phosphorus compounds which are formed during glucolysis in brain extracts. It seems that they consist mainly of compounds which hydrolyse less readily than Hdp.

The experiments also show that the differences formerly observed between brain and muscle glycolysis are not due to differences in permeability, for they still exist when muscle extracts and brain extracts are compared.

An interesting problem is presented by the peculiar behaviour of fructose in brain tissue and in brain extracts. Lack of permeability cannot be held responsible for the failure of brain tissue to form lactic acid from this sugar, since it is oxidized like glucose. On the other hand brain extract forms lactic acid from fructose at a higher rate than from glucose.

SUMMARY

The preparation is described of brain extracts, the glycolytic activity of which exceeds that of brain slices by about 4 times.

Dialysis against water destroys the activity of extracts irreversibly. By dialysing against 0·6 % NaCl solution the loss of activity on dialysis is avoided.

Magnesium has to be present in a concentration of 0·013 *M* in order to obtain optimum glucolysis. Lower or higher concentrations diminish the rate of glucolysis. Hexosediphosphate causes a small increase of the glycolytic rate if added in minute amounts.

Phosphocreatine has to be added to dialysed brain extracts in order to obtain glycolysis. This substance cannot be replaced by creatine, or by any of the coenzymes. In fresh extracts the addition of phosphocreatine is only necessary when glucose is the substrate: from glycogen lactic acid is formed without the addition of phosphocreatine. In fresh extracts the effect of creatinephosphate is inconstant.

Glutathione activates brain glycolysis, but its effect is subject to marked variations.

Adenosinetriphosphate and cozymase are both coenzymes of brain glycolysis. It is probable that a yet unknown coenzyme also participates in brain glycolysis.

Large amounts of lactic acid are formed from fructose, glucose and mannose. The rate of lactic acid formation is about a third of that of glucose. Little or no lactic acid is formed from other sugars. Practically no lactic acid is formed from any of the phosphorylated intermediary products of muscle glycolysis. In the presence of arsenate very small amounts of lactic acid are formed from hexosediphosphate.

The presence of inorganic phosphate is necessary for glycolysis in brain extracts. Inorganic phosphate is esterified during glycolysis of all the lactic acid-forming carbohydrates.

The inorganic phosphate esterified during glycolysis is converted mainly into a monophosphate, and to a smaller extent into a pyrophosphate and hexosediphosphate. In the presence of fluoride and of pyruvic acid an ester, probably phosphopyruvic acid which is formed from glucose, accumulates. Extracts poisoned with iodoacetate transfer P from adenosinetriphosphate to glucose, forming hexosediphosphate. Small amounts of phosphopyruvic acid are formed during glycolysis.

The effect of several other factors on glycolysis is described.

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