CVI. THE GLYCOLYTIC ACTIVITY OF BRAIN

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I. MACERATED BRAIN TISSUE

KNOWLEDGE of brain glycolysis compares unfavourably with that available for muscle. As yet, none of the intermediary steps of brain glycolysis is known with certainty and our knowledge of the coenzymes involved in brain glycolysis is very incomplete. In view of the conflicting statements in this field, we have here investigated the effects on brain glycolysis of general experimental conditions, of coenzymes and of certain of the known intermediary products of muscle glycolysis. It will be shown here that none of the substances which are intermediary products of muscle glycolysis can be regarded as such in glycolysis with intact brain cells. No alternate indication, however, of the intermediary steps involved in brain glycolysis can be given on the basis of these experiments.

Since finishing this work, cell-free extracts of brain with high glycolytic activity have been obtained. The results obtained with extracts—which differ somewhat from those obtained with intact cells—will be described in a later paper. Consequently, we wish to emphasize that when the present work was done, only intact brain cells were found to be glycolytically active; therefore the conclusions reached in this paper are valid for intact brain cells only.

Methods. The technique used was mainly that described previously [Geiger, 1935]. All the experiments were carried out on 2–6 months old albino rats, unless otherwise stated. Glucose was determined by Shaffer & Somogyi's method [1933]. Later the more recent modification [1937] of this method was used. Cozymase was prepared by Euler's method and later according to Meyerhof & Ohlmeyer [1937]. The adenosinetriphosphate¹ was prepared according to Barrenscheen & Filz [1932]. Warburg manometers were used for the measurement of respiration. Lactic acid was determined by the method of Friedemann & Graesser [1933].

The effect of rat age and the season on brain glycolysis. Macerated brain cortex of young rats shows a higher glycolytic rate than that of old rats. Table I contains a set of parallel experiments with macerated brain cortex from rats 2–3 months old and from rats more than a year old. In all the experiments, a measured amount of macerated brain was suspended in a known amount of

¹ Henceforth adenosinetriphosphate will be abbreviated AP, creatinephosphate CRP, reduced glutathione GSH, and oxidized glutathione GSSG.

M/15 phosphate buffer containing 200 mg. per 100 ml. of glucose, 50 mg. per 100 ml. of adenosine triphosphate and 50 mg. per 100 ml. of glutathione, all at pH 7.1.

Table I

Dilution of the tissue macerate	mg. lactic acid formed by 100 g. brain per hr.	
	Old rats	Young rats
1 : 100	340	510
2 : 100	340	530
3 : 100	338	480
4 : 100	330	435
5 : 100	320	410
6 : 100	300	375

The seasonal variation in glycolytic activity is also noteworthy. Higher values were obtained with our rats during the months of July and August. 120 determinations, under otherwise identical conditions, showed an average lactic acid production of 465 mg. per 100 g. tissue per hr. in winter and spring, as compared with 613 mg. during the late summer months.

The effect of dilution of the tissue. As Table I shows, dilution increases the relative glycolytic power of macerated brain cortex. In these experiments only the concentration of the tissue was varied.

We were also able to confirm Ashford's [1934] observation that tissue slices possess a much higher glycolytic power than macerated tissue.

The effect of the composition of the medium. It was observed by Ashford [1934] that the rate of brain glycolysis is lower in phosphate buffer than in bicarbonate Ringer. We are able to confirm this. We found, however, that if AP is added to macerated brain cortex in phosphate buffer, the glycolysis reaches almost the same level as it does in bicarbonate Ringer with added AP. This is illustrated in Table II.

Table II

Reaction-media: all at pH 7.2	mg. lactic acid formed by 100 g. tissue in 1 hr.
<i>M</i> /15 phosphate buffer	370
<i>M</i> /15 phosphate buffer and AP	550
Bicarbonate Ringer	560
Bicarbonate Ringer and AP	590

Glycolysis with various brain cortex preparations. In previous experiments [Geiger, 1935] extracts of brain cortex were made which had very little glucolytic activity in comparison with that of cortex macerate. Ashford [1934] was unable to obtain active extracts of brain cortex. Mazza & Malaguzzi-Valeri [1935] as well as Euler *et al.* [1936], however, described experiments with brain extracts showing much greater glucolytic activity.

In the present experiments we were unable to obtain extracts more active than those described previously. The NaCl or KCl extracts, even after addition of activators, had only about 1/10 of the activity of the brain cortex from which they were prepared. It should be noted, moreover, that only the first extract of macerated brain is at all active. Further washings on the centrifuge with isotonic KCl or NaCl did not yield active extracts and did not impair the glycolytic activity of the brain macerate. Washing with water destroyed the activity of the macerate.

In the experiments presented in Table III, ice-cooled brain cortex was carefully macerated in an ice-cold solution of NaCl or KCl. The suspension was

centrifuged and the residue washed several times more in the centrifuge. The first extract or the first and second extracts combined were made up with buffer solution to the same volume as the residue. In this way corresponding amounts of diluted extract and of the washed tissue suspension were obtained for the experiments.

Table III

Weight of tissue, g.	mg. lactic acid formed by 100 g. tissue per hr.	
	0.35	0.425
Unwashed + AP	444	—
Washed, no addition	—	242*
Washed + AP + GSH	382†	426*
Extract corresponding to same amount of tissue	—	6
First extract + AP + GSH	41	15
Tissue and extract + AP	421	—

* Washed thrice.

† Washed once.

In later experiments it was observed that the destruction of the cell structure by distilled water, or by macerating with finely ground quartz-sand, completely destroyed the glycolytic activity of the brain. These experiments will be described in a later paper.

Lactic acid formation from glycogen. In Haarmann's [1932] and also in Ashford's experiments, the amount of lactic acid formed by macerated brain from glycogen was small as compared with that from glucose. Euler *et al.* [1936], however, obtained with brain extracts lactic acid formation from glycogen which sometimes equalled that obtained from glucose.

We repeated these experiments using both brain macerate and brain extracts, on the supposition that these conflicting results could be explained by the difference in the enzymic materials of these two preparations. Table IV shows the results.

Table IV

	mg. lactic acid formed by 100 g. brain cortex per hr.
Macerated brain suspension + AP + GSH + glucose	612
Macerated brain suspension + AP + GSH + glycogen	36
Macerated and washed twice + AP + GSH + glucose	589
Macerated and washed twice + AP + GSH + glycogen	31
Brain extract + AP + GSH + glucose	45
Brain extract + AP + GSH + glycogen	32

These experiments confirm the findings of Ashford [1933] according to which the amounts of lactic acid formed by macerated brain cortex from glycogen are small, as compared with the amounts formed from glucose.

In the extracts, the lactic acid formation from glycogen is as low as that in the macerated tissue, but since the glucose-splitting activity of the extracts is much lower than that of the macerated tissue, the relative rates of these two processes in the extract are different from those in the macerated tissue. In other words, the seemingly different behaviour of the extract and the macerate towards glycogen is merely due to the fact that under these conditions only a very small fraction of the glucose-splitting enzyme of the brain is active in the extract, while the activity of the glycogenolytic enzyme system is the same in the extract as in the original tissue.

Wilstätter & Rohdewald [1937] found that leucocytes and yeast transform glucose into glycogen prior to the formation of hexosediphosphate. In similar experiments made with brain no glycogen formation from glucose could be observed.

The inability of macerated brain cortex to form lactic acid from added hexosediphosphate. It was shown by Ashford [1933] and by Edlbacher *et al.* [1934] that brain cortex is able to produce small amounts of lactic acid from hexosediphosphate. Upon repeating these experiments with varying amounts of hexosediphosphate we found that the more hexosediphosphate added to chopped brain cortex, the greater the lactic acid formation. These experiments are contained in Table V.

Table V

	mg. lactic acid formed per hr. by 100 g. tissue	
	Exp. 1	Exp. 2
5 mg. hexosediphosphate at once	57	—
5 mg. hexosediphosphate after 1 hr.	62	—
10 mg. hexosediphosphate at once	100	121
10 mg. hexosediphosphate after 1 hr.	180	172
30 mg. hexosediphosphate at once	150	169
30 mg. hexosediphosphate after 1 hr.	380	376
60 mg. hexosediphosphate at once	—	250
60 mg. hexosediphosphate after 1 hr.	—	565

The amounts of hexosediphosphate indicated in this table were added to 10 ml. of brain suspension containing 0.4 g. of chopped brain cortex.

This singular behaviour of hexosediphosphate can be explained on the basis of the enzymic equilibrium between hexosediphosphate and dihydroxyacetonephosphate, discovered by Meyerhof & Lohmann [1934]. According to these authors zymo-hexase is present in brain in much smaller quantities than in muscle. When hexosediphosphate is added to the brain macerate, triosephosphate is formed by the action of zymo-hexase in amounts depending—*ceteris paribus*—on the amounts of hexosediphosphate present. The rate of the process is proportional to the amount of enzyme present. The triosephosphate is in turn transformed into lactic acid artificially during the precipitation of the carbohydrates by copper-lime. This is illustrated in Table VI. Lactic acid and triosephosphate determinations were carried out immediately and at various time intervals after the addition of a known amount of hexosediphosphate to the brain pulp suspension. The triosephosphate was determined in the trichloroacetic filtrates according to Meyerhof & Lohmann [1934] by determining the amount of P liberated in 2*N* alkali at room temperature in 20 min.

Table VI

	mg. lactic acid formed	mg. triosephos- phate (P_2O_5)
With hexosediphosphate: At once	0.420	0.537
7 min. later	1.680	1.740
15 min. later	1.680	1.730
30 min. later	1.600	1.700
60 min. later	1.548	1.680

From these experiments it is evident that 7 min. after the addition of hexosediphosphate a certain amount of lactic acid is already "formed" and that this amount does not increase with time. On the other hand, estimation of triosephosphate shows it to be present in amounts corresponding to the amounts

of lactic acid found after treatment with copper-lime. It can therefore be safely deduced that chopped brain forms no lactic acid enzymically from hexose-phosphate and that the amount of lactic acid found in the experiments is derived from the artificial transformation of the triosephosphate which in turn is formed from hexosediphosphate by zymohecase. The relatively long time necessary for establishing the equilibrium between hexosediphosphate and triosephosphate in the brain pulp is due to the low zymohecase content of the brain. According to Meyerhof & Lohmann [1934] the zymohecase content of brain is only 1.6% of that in voluntary muscle, and in our experiments this small amount is diluted about 30 times. From the same experiments it may therefore be concluded that the brain is unable to form lactic acid from triosephosphate.

The inability of brain to convert hexosediphosphate into lactic acid led us to consider the possibility that lactic acid formation from glycogen by the brain is also effected via glucose. In this case the rate of lactic acid formation should depend on the diastatic activity of the brain. As the experiments in Table VII show, the addition of diastase to the brain pulp greatly increases the lactic acid formation from glycogen.

Table VII

	mg. lactic acid formed by 100 g. tissue per hr.	
	Exp. 1	Exp. 2
Brain pulp + glucose	557	472
Brain pulp + glucose + diastase	573	481
Brain pulp + glycogen	13	11
Brain pulp + glycogen + diastase	153	287

In these experiments 0.4 g. of brain pulp was suspended in 10 ml. of Ringer's solution. AP and glutathione were added to each flask.

Autolysis of brain cortex. In the hope of obtaining a suitable brain cortex preparation for studying the activators of brain glycolysis—brain extracts not being suitably active—we tried to eliminate the coenzymes, at least in part, by autolysing brain cortex in the absence of glucose. A series of experiments were made under different conditions, the results of which are summarized in Table VIII.

These experiments were made by macerating the brain in an isotonic NaCl solution, the required pH being obtained by the addition of HCl or NaHCO₃. This suspension was shaken in a water bath at 37° for a known time in an atmosphere of N₂, O₂ or air. Subsequently, equal amounts of the neutralized brain suspension were transferred in a measured amount of bicarbonate-Ringer to the experimental flasks containing glucose and the necessary additions. The flasks were then incubated at 37° for 1 hr.

Table VIII

	mg. lactic acid formed by 100 g. tissue per hr.			
	E. 105		E. 108	
	Glucose	Glucose + AP + GSH	Glucose	Glucose + AP + GSH
Before incubation	383	434	—	388
Incubated in N ₂ for 1 hr. at neutrality	137	289	56	247
Incubated in O ₂ for 1 hr. at neutrality	149	328	143	326

As Table VIII shows, autolysis in N_2 inactivates more completely than in O_2 , and brain macerate inactivated in O_2 can be reactivated more completely by the addition of AP and GSH than when inactivated in N_2 .

The rate of inactivation at different reactions was investigated in similar fashion. These experiments show that at an alkaline reaction the brain suspension becomes very rapidly inactivated and cannot be reactivated by the addition of AP and GSH. In an acid reaction the inactivation proceeds more quickly than at neutrality but reactivation can still be effected if the suspension is not autolysed for too long a time. In this connexion it is interesting to note that according to Giri & Datta [1936] brain phosphatases are inactive at neutrality and exhibit their greatest activity in the alkaline and acid ranges at about pH 5.0 and 9.0. In experiments during the late summer months we were unable to obtain as complete an inactivation by autolysis in O_2 at neutrality, even when we extended the time of autolysis to 2 hr. At the same season the rate of glycolysis of the unautolysed tissue was found to be much higher than in the experiments made in the winter, spring and early summer months.

Activators of brain glycolysis

Adenosinetriphosphate was found to activate brain glycolysis. The experiments in Table IX show the rate of glycolysis with macerated brain cortex with and without the addition of AP.

Table IX

pH	mg. lactic acid formed by 100 g. tissue per hr.	
	Glucose only	Glucose + AP
6.00	140	145
6.40	325	342
7.00	441	533
7.50	389	580
8.00	279	555

At neutrality, the addition of about 3 mg. of AP to 10 ml. of brain suspension was sufficient for maximum activation. In the acid range, AP has practically no activating effect. Maximum *activation* occurs in the alkaline range. The maximum *rate* of glycolysis, however, is found at neutrality. In these experiments the fresh brain cortex of rats was used without previous autolysis, so that the brain itself contained some AP. The experiments were made in phosphate buffer, in which, as previously shown, the original amount of AP in the brain is not sufficient to cause full activation.

In order to be the better able to test the activating effect of several substances known to be present in brain macerate, we autolysed the fresh macerate at neutrality in air for 1 hr. at 37° as previously described. Table X contains

Table X

	mg. lactic acid formed by 100 g. tissue per hr.	
	Exp. 1	Exp. 7
Before autolysis + glucose + AP + GSH	482	486
Autolysed brain cortex + glucose	34	92
Autolysed + glucose + AP	223	410
Autolysed + glucose + CRP	40	100
Autolysed + glucose + hexosediphosphate	162	226
Autolysed + adenylic acid + CRP + glucose	—	95
Autolysed + glucose + AP + hexosediphosphate + CRP	289	421
Autolysed + cozymase + glucose	62	90

experiments of this type made with a suspension of macerated and autolysed brain cortex in bicarbonate Ringer.

To calculate the degree of activation in those experiments in which hexosediphosphate was also added, we subtracted the amount of lactic acid found in the flasks to which only hexosediphosphate was added, from the lactic acid content of the other flasks, which contained hexosediphosphate and other additions.

These experiments show that AP is able to bring about a maximal activation when added alone. None of the other substances caused the same degree of activation provided that the inactivation of the brain suspension was complete enough. The activating effect of hexosediphosphate seems to depend on the degree of inactivation of the tissue. The greater the inactivation of the brain suspension the less the activating effect of hexosediphosphate alone. After a complete autolysis in N_2 the brain pulp could not be reactivated by AP. Cozymase had no activating effect on brain glycolysis.

Table XI

Additions to brain pulp	mg. lactic acid formed by 100 g. tissue per hr.			
	Fresh chopped brain in phosphate buffer		Autolysed brain	
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
Glucose only	460	440	74	90
Glucose + AP	574	700	253	312
Glucose + hexosediphosphate	618	597	126	202
Glucose + AP + hexosediphosphate	618	—	260	307
Glucose + adenylic acid	465	427	68	95
Glucose + adenylic acid + hexosediphosphate	618	707	255	318

The experiments in the foregoing table show that adenylic acid alone has no activating effect but that the combination of adenylic acid and hexosediphosphate gives as high an activation as the addition of AP. Hexosediphosphate alone may sometimes also give full activation. This is probably due to the rapid dephosphorylation of the brain AP and the slower process of deamination of the adenylic acid.

Transference of phosphorus to adenylic acid

According to the findings of Parnas and co-workers, added hexosediphosphate is broken down in muscle to phosphopyruvic acid which transmits its phosphorus to the adenylic acid.

The following experiments show that in the brain this mechanism is different. Phosphopyruvic, added together with adenylic acid, is unable to activate glycolysis.

Table XII

	mg. lactic acid formed by 100 g. brain per hr.		
	Not autolysed	Autolysed brain	Autolysed brain
Glucose without addition	460	76	132
Glucose and adenylic acid	495	69	126
Glucose and adenylic acid and hexosediphosphate	618	397	402
Glucose and adenylic acid and phosphopyruvic acid	512	79	138
Glucose and adenylic acid and phosphoglyceric acid	—	83	140
Glucose + adenosinetriphosphate	—	382	398

Another proof that a brain suspension is unable to split phosphopyruvic acid is given by experiments in which the activating effects of small amounts of pyruvic acid and phosphopyruvic acid are compared. According to Mendel *et al.* [1931] small amounts of pyruvic acid (9–40 mg. per l.) added to brain cause an increased lactic acid production. The experiments in Table XIII show that the addition of phosphopyruvic acid is unable to bring about this activation with brain, although pyruvic acid does.

Table XIII

	mg. lactic acid formed from 100 g. brain per hr.		
	E. 145	E. 142	E. 163
Glucose and AP	495	—	427
Glucose and AP and pyruvic acid	615	825	759
Glucose and AP and phosphopyruvic acid	512	555	481

These experiments indicate that the transference of phosphate to adenylic acid, if such a mechanism exists in brain, is not via phosphopyruvic acid but by some other route. It has been shown by Neuberg [1935], by Lutwak-Mann & Mann [1935] and by Ohlmeyer [1935] that in yeast the direct transfer of P from hexosediphosphate to adenylic acid is possible. The experiments just described suggest the existence of this mechanism in brain.

Phosphorylation

A further feature of brain glycolysis is the apparent absence of phosphoric ester formation. Ashford & Holmes [1929] and others were unable to demonstrate any phosphorylation. Euler *et al.* [1936], however, report experiments on brain extracts in which a disappearance of inorganic phosphate during glycolysis was observed.

In view of the strong activating effect of AP on brain glycolysis and of its role in muscle glycolysis as a phosphate carrier, it was felt necessary to reinvestigate this question. In our experiments made with and without the addition of AP and NaF, no phosphoric ester formation could be demonstrated. Moreover, in accordance with Ashford & Holmes' [1929] findings, a slight liberation of inorganic phosphate was found.

The role of glutathione

In a previous paper [Geiger, 1935] experiments in which GSH had a marked activating effect on brain glycolysis were described. A similar effect had been observed by Quastel & Wheatley [1932] in yeast fermentation. Euler *et al.* [1936] confirmed the observation that GSH had a slight activating effect on lactic acid production by brain extract. These authors attribute the activating effect of GSH to its capacity for combining with heavy metals.

A large number of experiments carried out by us during the years 1935–6 have shown that the activating effect of GSH—although a constant factor in every experiment—is of a very different order of magnitude in different experiments. It may happen that in two experiments which are identical in every respect, except for the rats, the activating effect of GSH is as high as 100 % in the one and not more than 10 % in the other. Table XIV contains experiments selected at random from a large number of experiments made under different conditions.

Table XIV

	mg. lactic acid formed							
	Exp. A		Exp. B		Exp. C		Exp. D	
	No GSH	GSH added	No GSH	GSH added	No GSH	GSH added	No GSH	GSH added
Brain cortex of young rats in bicarbonate-Ringer	410	500	487	512	330	462	243	533
Brain cortex of old rats in bicarbonate-Ringer	320	426	229	346	274	306	222	451
Brain cortex in phosphate buffer pH 7.2	245	355	270	290	248	296	361	381
Brain cortex in phosphate buffer with added AP	385	492	399	411	367	416	239	500
Washed twice with 0.9% NaCl, then AP added	396	466	443	470	339	510	470	485

The only factor influencing the activating effect of GSH was the H^+ concentration of the medium. The activating effect of GSH is largest as a rule at pH 6.4 and diminishes with increasing alkalinity. The behaviour of GSH towards pH is thus directly opposite to that of AP which activates more strongly on the alkaline side.

Nutrition is another factor which has some influence on the activating effect of GSH. The addition of GSH to the brain pulp of starved or vitamin B_1 -deficient rats produces a more marked activation.

The possibility that the activating action of GSH was due to its binding the traces of Cu or Fe present in the brain pulp (or possibly in the reagents used) may be discarded on the basis of the relatively large amounts of Cu which are necessary to cause inhibition. In a series of experiments various amounts of Cu and Fe were added to the experimental flasks. Table XV shows that the addition of 0.04 mg. $CuSO_4$ to 10 ml. brain suspension causes only a very slight inhibition of brain glycolysis. It is remarkable what large amounts of Cu are necessary to cause inhibition in brain macerate, as compared with muscle glycolysis. These experiments showing that small traces of heavy metals have no effect on brain glycolysis exclude the possibility that the activating effect of GSH on brain glycolysis is due to the binding of traces of Cu or Fe originally present in the macerate as suggested by Euler *et al.* [1936].

Table XV

	mg. lactic acid formed by 100 g. brain per hr.	
	$CuSO_4$	$FeCl_3$
No addition	565	581
With 0.40 mg. % heavy metal salt	520	—
With 1.00 mg. % heavy metal salt	460	567
With 2.00 mg. % heavy metal salt	470	—
With 20.00 mg. % heavy metal salt	410	—

The effect of oxidized glutathione

The statement was made by one of us [Geiger, 1935] that oxidized glutathione strongly inhibits muscle glycolysis and also inhibits brain glycolysis to a smaller extent. It appears from later experiments that this statement was erroneous. The error was caused by the presence of a relatively large amount of Cu in the glutathione preparation used. Later it was shown by Wagner-Jauregg & Rzeppa [1936] that minute amounts of Cu inhibit muscle glycolysis. It was also observed

later by us that the Cu liberated from our glutathione preparation on oxidation was sufficient to cause an almost complete inhibition of muscle glycolysis. When benzoin-oxime was added to precipitate the Cu liberated by oxidation, no inhibitory effect was observed. A recrystallized preparation of glutathione or a commercial one did not inhibit, or at most only very slightly when employed in large quantities. Table XVI shows the results of these experiments. (Benzoin-oxime, when added to the muscle preparation, had no effect on glycolysis.)

Table XVI

	mg. lactic acid formed by 100 ml. muscle extract in 1 hr.
50 mg. % GSSG containing Cu	181
50 mg. % GSSG containing Cu + benzoin-oxime	1120
50 mg. % recrystallized GSH oxidized	1210
50 mg. % commercial GSH oxidized	1220
Without GSSG	1180

DISCUSSION

The experiments described in this paper show that the glycolytic process in the brain differs markedly from that in muscle. One difference is that cytolysis of brain cortex with distilled water causes complete inhibition of glycolysis. Another is the difference in the behaviour of the two systems towards inhibitors such as fluoride, iodoacetate or oxalate. All these substances inhibit both brain and muscle glycolyses. However, in the case of brain, when any of these substances is present, no phosphorylated intermediary products are formed, and glucose is not attacked at all. No phosphoric ester formation can be demonstrated in the course of brain glycolysis.

Copper ions, which according to Wagner-Jauregg & Rzeppa [1936] completely inhibit muscle glycolysis in concentrations of 10^{-5} *M*, do not inhibit brain glycolysis in concentrations up to 10^{-3} *M*. In this connexion Bauer's findings [1936], according to which Cu^{++} inhibits the action of zymohexase, may be of interest. Since, as has been shown in this work, brain cannot form lactic acid from hexosediphosphate or triosephosphate, the selective inhibition of the action of zymohexase would not inhibit brain glycolysis.

There is definite proof that the way of breakdown of glucose and the intermediary products involved depart in many respects from the scheme set up for muscle glycolysis. The present paper shows that no lactic acid can be formed in brain from added hexosediphosphate, phosphoglyceric acid or phosphopyruvic acid. Brain is unable even to hydrolyse phosphopyruvic acid. It was demonstrated by Johnson [1936] that α -glycerophosphate and pyruvic acid cannot be regarded in brain as possible precursors of lactic acid.

It may be added that the absence of lactic acid formation in brain from added zymophosphates cannot yet be regarded as a conclusive proof against phosphorylation. As has been shown here, only intact cells are able to form lactic acid from glucose. Cytolysed brain suspensions, as will be shown in a later paper, are unable to attack glucose. It is therefore possible that the problems presented by living yeast—in which case phosphorylation is still under discussion—also apply to brain. Dickens [1936] has claimed that the entry of glucose into brain cells is accompanied by phosphorylation.

On the other hand, recent work by Needham & Lehmann [1937] and Needham & Nowiński [1937] has shown that hexosediphosphate does enter into embryonic cells, although practically no lactic acid is formed from it. The work of Needham

and his collaborators shows in many respects a striking similarity between embryonic and brain glycolyses.

It is however difficult to explain the activating effect of adenosinetriphosphate in a non-phosphorylating glycolysis. All our experiments show the absence of phosphorylation and the inability of brain to form lactic acid from phosphorylated compounds. On the other hand, adenosinetriphosphate which is considered as a transporter of P, activates the glycolysis in inactivated brain in some cases by as much as 300–400 %.

SUMMARY

1. The glycolytic rate of brain from adult rats is lower than from that of young animals.
2. The glycolytic rate in brain pulp increases with increased dilution of the tissue by isotonic saline solutions.
3. Higher glycolytic rates were obtained in Ringer's solution than in a phosphate buffer solution of corresponding *pH*. If adenosinetriphosphate is added to the phosphate solutions the glycolytic rate attains that reached in Ringer's solution.
4. Extraction with water destroys the glycolytic activity of brain. Repeated washing of brain pulp with isotonic solutions does not diminish its glycolytic power.
5. Only small amounts of lactic acid are formed by brain from glycogen. Brain extracts possess the same glycogenolytic activity as brain tissue. Owing to the low glycolytic activity of these extracts, the relationship—glycogenolysis: glucolysis is different in the extracts from what it is in the tissue.
6. No lactic acid is formed from hexosediphosphate by brain. The apparent lactic acid formation from hexosediphosphate is due to the slow formation of triosephosphate, which in turn is transformed into lactic acid during the analysis.
7. Brain pulp when incubated at 37° in N₂ or O₂ without addition of glucose becomes partially inactivated. The glycolytic power of the autolysed pulp may be partly restored by adenosinetriphosphate and glutathione. Adenosinetriphosphate may be replaced by a combination of adenylic acid and hexosediphosphate. Brain pulp inactivated at *pH* 8.0 cannot be reactivated by these substances.
8. Adenosinetriphosphate may be regarded as a coenzyme of brain glycolysis. Under the present experimental conditions, cozymase and creatinephosphate have proved to be ineffective.
9. It is indicated that P may be transferred by brain from hexosediphosphate to adenylic acid but not from phosphopyruvic acid.
10. Brain tissue is unable to dephosphorylate phosphopyruvic acid.
11. No phosphoric ester formation from inorganic phosphate can be demonstrated with intact brain cells.
12. The activating effect of glutathione on brain glycolysis is very irregular. It is however demonstrated that this activating effect is not connected with the presence of heavy metals.
13. A former statement, according to which oxidized glutathione inhibits glycolysis, was erroneous. The inhibition in these experiments was due to the presence of small amounts of Cu in the glutathione preparation.
14. It is suggested that the way of breakdown of glucose in brain departs in every respect from the way of breakdown of glycogen in muscle.

II. EFFECT OF CYTOLYSIS, THE PRESENCE OF A GLYCOLYTIC INHIBITOR

In the first part of this paper it was reported that brain cortex loses all its glycolytic power if suspended in distilled water or if ground thoroughly with quartz sand. Case [1929] found that only intact brain cells are able to activate lactic acid formation from glucose by muscle extract. Dickens [1936] found that, following destruction of brain cells by grinding with sand, glucose oxidation ceases. Similar observations have been reported by Jowett & Quastel [1937].

It appears, however, that the necessity of intact cell structure for glucose breakdown is not limited to the brain cells. Irving [1927] stated that the integrity of kidney cells is essential for the utilization of glucose. The same view was also held by Case [1929]. The glycolytic activity of tumour cells is completely destroyed by freezing, grinding and by extracting with water according to Barr *et al.* [1928]. The same observation was made by the author on rat uterus (unpublished). According to Needham & Nowinski [1937] the destruction of cell structure abolishes glycolysis in the embryo.

Young [1929], Penrose & Quastel [1930] and Yudkin [1937] showed that cytolysis of certain bacteria results in the destruction of glucose dehydrogenase and of lactic dehydrogenase.

Brain extracts retain only a very small fraction of the glycolytic activity of the original tissue, the bulk of the activity remains in the tissue, and is not removed by further extractions [Part I]. Extraction with distilled water completely destroyed the glycolytic activity in the brain tissue.

Boyland *et al.* [1937] recently succeeded in activating a cell-free extract from "Crocker 180" tumour by adding relatively large amounts of cozymase and adenylic acid. These extracts produced lactic acid from glucose as rapidly as did the original tissue. These authors believe that tumour extracts during extraction are inactivated by nucleosidases liberated on destruction of the tumour cells. It appears therefore that the lack of glycolytic activity in tumour cell extracts is due to the destruction of coenzymes.

The earlier part of the present experiments was undertaken to ascertain the effects of cytolysis of brain tissue on the glucose breakdown and to study the nature of the inhibition. In the later part an inhibitor of glycolysis which is liberated from the brain on cytolysis is described.

The effect of suspension in water, of grinding with sand and of freezing, on the glycolytic power of brain cortex

Brain cortex ground in a smooth-surfaced mortar and suspended in isotonic NaCl solution has only slightly lower glycolytic activity than slices prepared from the same brain. If, however, the brain is ground with the addition of fine quartz sand the activity of the resulting suspension decreases considerably, the decrease in activity depending on the thoroughness of grinding. A complete loss of activity results from suspending the brain pulp in distilled water or from repeated freezing and thawing of the brain pulp [Table I]. For these experiments rat brain hemispheres were hashed with scissors. Aliquot parts of the hash were treated in the manner indicated in Table I and were then suspended in four parts of water or in isotonic NaCl solution. After standing at room temperature for

10 min. the suspensions were distributed in Erlenmeyer flasks containing bicarbonate-Ringer, glucose, glutathione and AP. Lactic acid was determined chemically.

Table I

	mg. lactic acid formed by 100 g. brain in 1 hr.
Ground in smooth mortar with 0.9% NaCl	712
Ground with sand with 0.9% NaCl	384
Thoroughly ground with sand with 0.9% NaCl	95
Frozen and thawed twice with 0.9% NaCl	64
Ground in smooth mortar, suspended in distilled water	35
Ground in smooth mortar, suspended in 4% NaCl	375
Ground in smooth mortar, suspended in 12% sucrose	621
1:5 0.9% NaCl extract of brain	67
1:5 water extract of brain	4

In these experiments the amount of glucose which disappeared roughly corresponded to the amount of lactic acid formed. There was no phosphoric ester formation, but liberation of some inorganic phosphate was observed. It is to be noted, that, while the extracts prepared with isotonic solutions possessed some activity, no glycolysis at all was observed in the water extracts.

Dickens [1936] stated that after the destruction of brain cells by grinding with sand, Robison ester could still be oxidized, although glucose oxidation was no longer appreciable. Therefore in the following experiments (Table II) several substances were tried as glycolytic substrates in cytolysed brain. The experiments were made in the same way as those in Table I. One of these experiments is contained in Table II.

Table II

	mg. lactic acid by 100 g. in 1 hr.	ml. O ₂ consumed by 100 g. in 1 hr.
Cytolysed brain with glucose	71	36
Intact brain pulp with glucose	680	720
Cytolysed brain with glycogen	43	—
Intact brain pulp with glycogen	39	—
Cytolysed brain with Robison ester	14	11
Intact brain with Robison ester	16	—
Cytolysed brain with hexosediphosphate*	0	0
Intact brain pulp with hexosediphosphate	0	0
Cytolysed brain with α -glycerophosphate and pyruvic acid	33	—
Cytolysed brain with lactate	—	46
Intact brain pulp with lactate	—	745
Cytolysed brain with succinate	—	900
Intact brain pulp with succinate	—	1095

* The initial values were taken after 10 min. incubation at 37°.

The respiration of brain is greatly restricted on cytolysis. The O₂ uptake with glucose and with lactic acid is very small. No more O₂ was taken up with Robison ester than with glucose; the oxidation of succinic acid however was practically unaffected by cytolysis. The glyoxalase activity of brain was also unimpaired after cytolysis. The glycolytic activity of cytolysed brain could not be restored by the addition of adenosinetriphosphate and of cozymase. No appreciable decoloration of methylene blue could be observed with cytolysed brain in the presence of AP, or adenylic acid or cozymase. Boyland & Boyland [1935] stated that tumour extracts oxidatively deaminate adenosinetriphosphate and cozymase.

The presence of a glycolysis inhibitor in cytolysed brain

Cytolysed brain cells or an alkaline brain extract inhibit the glycolysis of muscle extracts and the fermentation of yeast extracts. This is the more remarkable since Case [1929] found that the addition of intact brain cells to muscle extracts enabled the latter to form lactic acid from glucose.

It was found by Winfield & Hopkins [1915] that pancreas contains a substance which inhibits lactic acid formation from glycogen in muscle. It was later demonstrated by Case & McCullagh [1928] that this substance is amylase. The pancreatic inhibitor did not inhibit lactic acid formation from hexosediphosphate, or from glucose when hexokinase was added to the muscle extract. The same observation was made by Barr *et al.* [1928]. As the following experiments show, the antiglycolytic substance in brain is not amylase although it is probably an enzyme.

The inhibition by cytolysed brain of lactic acid formation in muscle extract from different substances

Preliminary experiments have shown that small amounts of fresh brain pulp suspended in distilled water, when added to muscle extract, inhibit lactic acid formation from glycogen. Extracts of cytolysed brain, made with distilled water,

Fig. 1. The effect of cytolysed brain on glycolysis in muscle extract. *A*, no cytolysed brain; *B*, 0.2 ml. cytolysed brain susp.; *C*, 0.4 ml. cytolysed brain; *D*, 0.6 ml. cytolysed brain added to 2.5 ml. muscle extract.

showed very slight inhibitory action. Starting with small amounts of cytolysed brain it was observed that the inhibition of glycolysis was progressive. The rate of inhibition depended on the amount of cytolysed brain added, as shown in Fig. 1. In all experiments in which complete inhibition was observed the final

glycogen content of the reaction mixture was still high. This last fact indicates that the inhibiting factor is not amylase. The experiments are described in Tables III, IV and V. Brain was cytolysed by suspending the pulp in 5 vol. of distilled water. The extract from fresh muscles of rabbits or rats was prepared according to Meyerhof. In a large number of the experiments acetone-dried muscle extracts were used. The dry muscle powder was extracted with isotonic NaCl solution and was cleared by centrifuging. The glycolytic rate was determined chemically as well as by manometric methods. In several cases the glycogen content of the flasks was also determined at the end of the experiment. In all the experiments where lactic acid was determined chemically, 1 ml. of muscle extract was made up with *M*/15 phosphate buffer of pH 7.2, or with bicarbonate-Ringer, and with other additions to 5 ml. Glutathione and AP were added to each flask. For brevity's sake only one experiment from a larger number of uniform results is given.

Table III

	mg. lactic acid formed in 1 hr.	mg. glycogen found at start of exp.	μ l. CO ₂ evolved in 1 hr.	mg. glycogen found at end of exp.
Muscle extract:	(1) Glycogen			
Without brain	2.74	156	122	38
With 0.04 g. cytolysed brain	1.21	138	46	29
With 0.06 g. cytolysed brain	0.18	129	0	24
	(2) Hexosediphosphate			
Without brain	1.89	—	108	—
With 0.04 g. brain cytolysate	1.50	—	91	—
With 0.08 g. brain cytolysate	1.04	—	35	—
With 0.12 g. brain cytolysate	0.29	—	0	—
	(3) α -Glycerophosphate			
With α -glycerophosphate	1.63	—	68	—
With 0.08 g. brain cytolysate	0.81	—	31	—
Cytolysed brain	0.44	—	11	—
	(4) α -Glycerophosphate and pyruvic acid			
Muscle extract	2.28	—	132	—
With 0.08 g. brain cytolysate	1.25	—	63	—
With 0.12 g. brain cytolysate	0.64	—	26	—

The amounts of cytolysed brain stated in the table were added to each ml. of muscle extract.

As Table III shows, the inhibitor cannot be amylase as there is still a considerable amount of glycogen left when inhibition is complete, and, as lactic acid formation is inhibited from both hexosediphosphate and from α -glycerophosphate. Larger amounts of cytolysed brain are necessary to inhibit lactic acid formation from hexosediphosphate than from glycogen.

Inhibition of lactic acid formation from glucose in muscle extract in the presence of hexokinase

Cytolysed brain also inhibits lactic acid formation in muscle extracts from glucose in the presence of hexokinase. In these experiments acetone-dried muscle extract was used. The hexokinase was prepared according to Meyerhof [1927].

Table IV. *Inhibition of lactic acid formation from glucose in the presence of hexokinase*

	mg. lactic acid formed in 1 hr.
Muscle extract, glucose, AP and hexokinase	7.42
As above, with the addition of 0.1 g. cytolysed brain	0.66

Inhibition of fermentation in yeast extract

Fermentation in yeast extracts is also strongly inhibited by cytolysed brain. In one experiment, for instance, 289 μ l. CO_2 were evolved without the addition of cytolysed brain, and only 20 μ l. in the presence of cytolysed brain.

Effect of cytolysed brain on glycolysis of intact brain cells and on the fermentation in living yeast

Glycolysis and fermentation of intact cells cannot be inhibited by cytolysed brain, hence the inhibitory substance cannot pass through cell membranes. An experiment of this type is described in Table V.

Table V

	μ l. CO_2 evolved
Brain pulp in Ringer solution	123
As above, with 0.3 g. cytolysed brain	132
Living beer yeast	186
Living beer yeast with 0.3 g. cytolysed brain	194

Effect of coenzymes

By the addition of adenosinetriphosphate, of cozymase, of glutathione and of creatinephosphate it was attempted to reactivate muscle extracts which had been inactivated by cytolysed brain. Different amounts of the coenzymes were used. The maximum amount of cozymase added to 2.2 ml. fluid was 10 mg. of a pure product. In no case could any reactivation be observed. In these experiments, as in the former ones with brain cytolysate alone, an increased CO_2 output was observed after adding cozymase. Chemical analysis showed, however, no corresponding increase in lactic acid formation. For the sake of brevity these experiments are not described here.

The facts described in this section indicate a difference in behaviour between tumour cells and brain tissue. Cozymase and adenylic acid do not reactivate cytolysed brain although they did reactivate tumour extracts in the experiments of Boyland *et al.* [1937]. On the other hand brain cells do not form lactic acid from hexosediphosphate, while according to Boyland & Boyland tumour extracts do, after the addition of adenylic acid and cozymase.

Preparation of antiglycolytic extracts from cytolysed brain

Cell-free aqueous extracts of cytolysed brain possess very slight antiglycolytic activity. Repeated washing of cytolysed brain with water only slightly diminishes its inhibitory power. Acid extraction has no inhibitory effect. On the other hand, alkaline extracts show a considerable inhibiting activity. Experiments were made as follows: finely ground pulp of fresh brain cortex was suspended in four parts of distilled water or in 0.1 *N* acetic acid or in 0.6% NaHCO_3 . After 15 min. at room temperature, part of the suspension was pipetted off and the remaining part centrifuged. The clear supernatant extract was pipetted off and the residue filled up to its original volume. In a few cases this last procedure was repeated several times. To test the inhibitory effect of brain extracts, they were added to muscle extract containing glucose, hexokinase and AP. The experiment in Table VI was made manometrically. The total volume of fluid in the manometer vessels was 3.2 ml.

Table VI

	$\mu\text{l. CO}_2$ evolved in 1 hr.
Muscle extract, glucose, hexokinase, AP	118
With 0.2 ml. of cytolysed brain suspension (1:5)	0
With 0.5 ml. of water extract of cytolysed brain	109
With 1.5 ml. of water extract of cytolysed brain	85
With 1.5 ml. of acid extract of cytolysed brain	109
With 0.4 ml. of alkaline extract of cytolysed brain	35
With 0.6 ml. of alkaline extract of cytolysed brain	3

As Table VI shows, extracts possessing about a third of the inhibitory action of the original suspension may be obtained by extraction with 0.5% NaHCO_3 solution.

The effect of heating and of drying

Heating of the cytolysed brain suspension or extract for 3 min. to 100° completely destroys its inhibiting power. On the other hand, in the heated suspension the presence of a substance which reactivates aged muscle extracts can be demonstrated. This activator can be best demonstrated on rat muscle extract which has been kept for 2 days previously in a refrigerator. One such experiment is shown in Table VII. It seems that this activator is not AP and not cozymase, as neither of these substances possesses the same activating effect as boiled brain extract. Precipitation and drying with acetone destroy a great part of the inhibiting effect of the extract. The inhibitory substance does not pass through collodion membranes.

Table VII. *Activating effect of heated brain extract*

	mg. lactic acid produced in 1 hr.
Aged rat muscle extract with AP and cozymase	39
Same as above with the addition of 0.4 ml. heated brain extract	81

The effect of pH on inhibition

The inhibitory effect of cytolysed brain on muscle glycolysis is greatest at pH 7.0–7.5. The experiment in Table VIII was made in *M*/15 phosphate buffer. The amount of cytolysed brain used was chosen so as to cause about 50–70% inhibition.

Table VIII. *Effect of pH on inhibition*

	mg. lactic acid formed in 1 hr.
pH 6.5 without inhibitor	1.42
with cytolysed brain	0.83
pH 7.0 without inhibitor	3.35
with cytolysed brain	1.31
pH 7.5 without inhibitor	4.70
with cytolysed brain	2.24
pH 8.0 without inhibitor	5.70
with cytolysed brain	4.14

DISCUSSION

The facts described in this paper prove that intact cell structure is necessary for brain glycolysis. By destruction of the cells a substance is liberated which inhibits glycolysis. No indication can be given of the nature of the inhibitory

substance although it is fairly well established that it is not amylase. It appears also that the inhibitor is different from the nucleosidase of tumour, described by Boyland & Boyland [1935]. While the nucleosidases reduce methylene blue in the presence of adenosinetriphosphate and of cozymase, no such reduction can be observed with cytolysed brain. The inhibitory action of cytolysed brain cannot be counteracted or the system reactivated by adenosinetriphosphate or by cozymase. On the other hand these substances reactivate tumour extract according to Boyland *et al.* [1937]. The glycolysis inhibitor of brain cannot be antiglyoxalase, since cytolysed brain possesses a considerable glyoxalase activity. Several facts, however, indicate the enzymic nature of the brain inhibitor. It is destroyed when heated for 2–4 min. to 100°. It does not pass through a collodion membrane. It loses part of its activity when dried and it has a pH optimum. The inhibition caused by cytolysed brain in muscle extract is a gradual one. The time at which the inhibition is complete depends on the amount of cytolysed brain present. This last fact indicates an enzymic destruction of a component of the glycolytic system.

As the inhibitory substance is indiffusible through collodion membranes it is evidently unable to penetrate cell membranes. In fact, cytolysed brain inhibits glucose fermentation in yeast extract, but it is unable to inhibit the fermentation of living yeast, and it does not inhibit glycolysis in a suspension of intact brain cells. These facts make it clear that extraction of the glycolytic system from brain causes at the same time liberation of an inhibitory substance which destroys the glycolytic activity.

SUMMARY

1. Brain tissue loses its glycolytic power if the cell structure is destroyed by grinding with sand, or freezing and thawing or by suspending it in water. The cytolysed brain cannot convert any of the zymophosphates into lactic acid.

2. The cytolysed brain tissue does not oxidize glucose. Lactate oxidation is also greatly reduced. Cytolysis has no effect upon succinate oxidation.

3. The glycolytic activity and the respiration of the brain which are lost by cytolysis cannot be restored by cozymase or by adenosinetriphosphate or glutathione.

4. The addition of small amounts of cytolysed brain to a glycolysing muscle extract inhibits the glycolysis of the latter. Lactic acid production, whether from glycogen, from hexosediphosphate, from α -glycerophosphate and pyruvic acid, or from glucose with hexokinase, is inhibited by cytolysed brain.

5. Cytolysed brain inhibits the glucose fermentation of yeast extract.

6. Cytolysed brain does not inhibit the lactic acid formation by intact brain cells or the fermentation of living yeast.

7. Neither cozymase, adenosinetriphosphate, glutathione nor creatine-phosphate reactivates muscle extracts inhibited by cytolysed brain.

8. In boiled extract of brain the presence of a substance can be demonstrated which restores the glycolytic power of aged muscle extracts. The activator is neither cozymase nor adenosinetriphosphate.

9. With hypotonic NaHCO_3 a cell-free extract of the glycolysis inhibitor can be prepared from cytolysed brain.

10. The inhibitor is destroyed by heat, and is partly destroyed by dehydration with acetone. It does not diffuse through collodion membranes.

11. The optimum pH for inhibition is 7.0–7.5.

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