XCV. RÔLE OF GLUTATHIONE IN ANAEROBIC TISSUE GLYCOLYSIS.

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THE relative amounts of lactic acid produced anaerobically by animal tissues from glycogen and from glucose depend on the tissue. Skeletal muscles, while able to form a large quantity of lactic acid from glycogen, produce only a relatively small amount of lactic acid from glucose. On the other hand involuntary muscles, brain tissue, heart muscle and lungs produce far more lactic acid from glucose than from glycogen. Other tissues, such as kidney, spleen *etc.*, are able to glycolyse glycogen and glucose in about equal amount (for literature see Haarmann [1932]).

Recent evidence seems to suggest that the course of breakdown of glucose is different from that of glycogen. Thus while the presence of inorganic phosphate is essential in muscle glycolysis where glycogen is the main substrate, according to Ashford and Holmes [1929] no inorganic phosphate is necessary for lactic acid production from glucose by brain tissue. It has been later shown by Ashford [1933] that the amount of lactic acid formed by brain tissue from glycogen and from glucose when both are present is equal to the sum of the amounts formed from each substrate separately. Bumm and Fehrenbach [1930; 1931], examining the effect of different activators on the glycolytic action of different organs, suggest two ways for the breakdown of glucose one of which does not pass through the phosphoric ester stage.

There are different opinions as to the precursor of lactic acid. Neuberg and his school advocate methylglyoxal as an intermediary product. On the other side Embden and his co-workers [1933] and Meyerhof and Kiessling [1933] found that in muscle pyruvic acid is to be considered as the precursor of lactic acid. Case and Cook [1931] succeeded in isolating pyruvic acid as well as methylglyoxal from incubated muscle tissue. The quantity of pyruvic acid was always much larger than that of methylglyoxal.

In the present experiments on the rôle of glutathione in glycolysis evidence is offered in favour of the theory that the mode of anaerobic breakdown of glucose is different from that of glycogen, and that methylglyoxal can be considered as an intermediary product of glucose breakdown but not of glycogen breakdown.

METHODS.

The animals used for these experiments were albino rats from a uniformly bred strain. The animals were fed for generations on a standard diet consisting of

Caseinogen ("Physiological" Glaxo)	21 % of the total Calories
Starch 	57 %
Sucrose 	8 %
Olive oil 	14 %
Osborne and Mendel salt mixture...	40 g. to each kg. food

In addition each rat received daily 0.5 g. dried yeast; 0.1 ml. cod-liver oil and a few drops of lemon juice. Litter-mates were used for comparative experiments.

The rats were killed by stunning or decapitation. The required organs were immediately removed, weighed and quickly ground over ice in a mortar. From the finely ground organs a fairly uniform emulsion was obtained by adding slowly during grinding an ice-cooled buffer solution or a saline solution of known volume and composition. Measured quantities of these emulsions were distributed in small Erlenmeyer flasks. From finely cut skeletal muscles weighed quantities were distributed in the Erlenmeyer flasks. For the anaerobic experiments the bottles were evacuated repeatedly and filled up with nitrogen. The incubating vessels were ordinary 50 ml. Erlenmeyer flasks each provided with a rubber stopper in which a stopcock was inserted. During the experiments these bottles were slowly rocked in a water-thermostat regulated to $37^{\circ} (\pm 1^{\circ})$.

The determination of lactic acid was carried out chemically by the method of Friedemann *et al.* [1927]. The original method with permanganate oxidation was used in those experiments in which methylglyoxal was the substrate. The error due to methylglyoxal was very low when permanganate was used as oxidising agent. When no methylglyoxal was present the more recent modification of this method was used with MnO_2 . In the presence of methylglyoxal the trichloroacetic acid filtrates were used directly for lactic acid estimation. In the absence of methylglyoxal a copper-calcium precipitation of the neutralised trichloroacetic filtrates was carried out. Both methods gave uniform and reliable results.

The estimation of glutathione was carried out by Kuehnau's method [1931]. Qualitative nitroprusside tests were carried out according to Hopkins.

The methylglyoxal was estimated in the trichloroacetic acid filtrates by the method of Barrenscheen and Dreguss [1931]. This method was slightly modified in so far as Schott's sintered glass filters were used instead of centrifuging tubes. A weighed amount of repeatedly recrystallised methylglyoxalbis-2:4-dinitrophenylhydrazone, prepared by precipitating a methylglyoxal solution with 2:4-dinitrophenylhydrazine and dissolving a weighed amount of the recrystallised precipitate in nitrobenzene, served as standard for the colorimetric estimation of methylglyoxal. (The author is indebted to Dr M. Weizmann for the preparation of the 2:4-dinitrophenylhydrazine.) The volume of the nitrobenzene was adjusted so that 2 ml. of the solution corresponded to 0.1 mg. of methylglyoxal. 2 ml. of this solution made up with alcoholic potash solution to 100 ml. served as a standard. The nitrobenzene solution keeps very well and saves labour in preparing fresh standard solution for each experiment.

The methylglyoxal was prepared according to Neuberg and Hoffmann [1930]. Another solution of methylglyoxal was obtained by distilling dihydroxyacetone with sulphuric acid according to Neuberg. In the experiments both preparations gave the same result although both contained small quantities of impurities.

Adenosinetriphosphoric acid was prepared from rabbit muscle according to Barrenscheen and Filz [1932]. Two such preparations were used for the experiments.

Three glutathione preparations were used, one of which was a B.D.H. preparation, the other two were prepared from baker's yeast according to Pirie's method [1930]. All three preparations gave identical results. Oxidised glutathione was prepared by dissolving a weighed amount of glutathione in a known volume of 0.5 % NaHCO_3 solution and shaking this solution in the presence of oxygen until the disappearance of the nitroprusside reaction.

The general procedure (if not stated otherwise) was as follows. For each

experiment at least four bottles were prepared, containing the buffer solution, the substrate and the other substances. Equal volumes of the tissue emulsion were measured into each bottle, after which the bottles were quickly evacuated repeatedly, filled up with nitrogen and put in the rocking arm of the thermostat. The time was noted when the tissue emulsion was placed in the bottles. The first two bottles contained no substrate besides that contained in the tissue suspension. After adding the tissue suspension the whole contents of one bottle were immediately precipitated by trichloroacetic acid. The contents of the second bottle were precipitated at the end of the experiment. To the third and fourth bottles a known amount of the substrate was added. The contents of one of these were immediately precipitated and the contents of the second at the end of the experiment. When the influence of an activator was tested more bottles were added to the set. The final volume, the p_H and the osmotic pressure were the same in all bottles. By this arrangement the influence of interfering substances contained in the substrates and in the tissue emulsions could be excluded from the results. By subtracting the amount of lactic acid found in the first bottle from that in the second, the lactic acid production without substrate was obtained (A); by subtracting the value of the third from that of the fourth bottle the lactic acid production in the presence of the substrate was obtained (B); the amount of lactic acid produced from the added substrate alone could be calculated by subtracting from the value (B) the value (A).

COENZYMIC FUNCTION OF REDUCED GLUTATHIONE IN THE PROCESS OF LACTIC ACID FORMATION FROM GLUCOSE.

Lohmann demonstrated [1932] that muscle extracts freed completely from reduced glutathione by dialysis retained their ability to form lactic acid from glycogen but were unable to transform methylglyoxal into lactic acid. The addition of reduced glutathione restored the ability of these extracts to form lactic acid from methylglyoxal. From his experiments Lohmann concluded that reduced glutathione was the coenzyme of the methylglyoxalase. In order to explain the fact that glutathione was not required for the process of lactic acid formation from glycogen this author assumed the mechanism of conversion of synthetic methylglyoxal into lactic acid to be different from that of the methylglyoxal, formed according to the theory of Neuberg as an intermediary product of carbohydrate breakdown.

The experiments of Lohmann may, however, also suggest that methylglyoxal does not occur at all as an intermediary product in the process of glycogen breakdown in muscle. From the work of Embden *et al.* [1933] and of Meyerhof and Kiessling [1933] the intermediary formation of methylglyoxal from glycogen in muscle is very doubtful.

On the other hand methylglyoxal may be an intermediary product in the process of lactic acid formation from glucose. In this case reduced glutathione, being a coenzyme of methylglyoxalase, would have a coenzymic function in the process of glucose breakdown.

In order to test this hypothesis we first applied Lohmann's experiments to glucose by using such types of tissues as readily metabolise glucose, *i.e.* brain tissue and involuntary muscles.

(a) *Experiments with tissue extracts.*

The tissue extracts used in these experiments were prepared according to Lohmann, finely cut tissue being macerated in 0.5 % KCl solution. The centrifuged extracts were dialysed against 0.5 % KCl solutions through collodion

membranes for 8–12 hours in ice-cooled vessels. At the end of the dialysis glutathione could still be detected in all the extracts. The extracts were therefore made slightly alkaline with NaHCO_3 (p_{H} 7.8) and were agitated for one hour with oxygen at room temperature. At the end of this period no more glutathione could be detected. For each substrate 5–7 bottles were set up provided with stopcocks as stated above. Each bottle contained 14 ml. of fluid, containing equal volumes of tissue extract. MgCl_2 was added in a final concentration of 8 mg./100 ml. The initial concentration of glucose or of glycogen or of methylglyoxal was 0.3 %. Phosphate buffer of p_{H} 7 was used in a final concentration of $M/15$. The concentration of reduced glutathione was 30 mg./100 ml. Adenosinetriphosphoric acid was added in a final concentration corresponding to 5 mg. P_2O_5 per 100 ml. The incubation lasted usually for 2 hours at 37° .

The tissue extracts had different concentrations according to the tissue. The proportions of tissue to 0.5 % KCl were as follows: 1 part muscle to 2 parts of KCl solution; 1 part brain to 5 parts of KCl solution; 1 part heart to 6 parts of KCl solution; 1 part uterus to 7 parts of KCl solution; 3 ml. of muscle extract or 4 ml. of the other extracts were used for each experiment. The results are summarised in Table I.

Table I.

Tissue extract	Substrate	mg. lactic acid formed from the added substrate by 100 ml. of the tissue extract		
		Without activator	With adenosine- triphosphoric acid	With adenosine- triphosphoric acid and glutathione
Muscle, rabbit	Glycogen	—	+ 89.8	+ 80.4
	Methylglyoxal	+ 4.96	+ 2.80	+ 407.0
	Glucose	− 0.66	− 0.84	+ 9.9
Muscle, rat	Glycogen	—	+ 231.0	+ 207.0
	Methylglyoxal	+ 7.3	—	+ 379.0
	Glucose	+ 0.12	− 0.31	+ 21.3
Brain, rat	Glucose	− 1.33	− 1.33	+ 20.0
	Methylglyoxal	—	+ 0.81	+ 142.4
"	Glucose	− 4.5	− 2.14	+ 18.9
	Methylglyoxal	+ 0.30	—	+ 91.0
"	Glucose	—	+ 0.1	+ 17.4
	Methylglyoxal	—	+ 3.3	+ 102.0
Liver, rat	Glucose	− 9.0	− 3.0	+ 107.0
Heart, rat	"	+ 0.09	+ 0.14	+ 28.0
"	"	− 0.8	− 1.0	+ 20.0
Uterus, rabbit	"	+ 0.35	+ 3.5	+ 12.9

The experiments reported in Table I demonstrate uniformly the inability of all the tissue extracts to form lactic acid from glucose or from methylglyoxal if no reduced glutathione is present, and the ability of the same tissue extracts to produce lactic acid from glycogen even in the absence of reduced glutathione. The addition of reduced glutathione to these extracts enables them to form lactic acid from glucose and from methylglyoxal, but it does not increase the amount of lactic acid formation from glycogen. Reduced glutathione seems, according to these experiments, to be a coenzyme common to the glycolytic enzyme system and to the methylglyoxalase, but it is not concerned in the process of lactic acid formation from glycogen.

According to these experiments methylglyoxal may be an intermediary product in the formation of lactic acid from glucose, reduced glutathione being

essential in both processes. It is, however, impossible to consider this methylglyoxal as an intermediary product in the breakdown of glycogen as this last process takes place even in the absence of reduced glutathione.

It is evident that in this experiment the lactic acid production is very slight. The inhibiting factor of the lactic acid production in these extracts is probably the oxidised glutathione. Since it was not possible to eliminate completely the glutathione from the tissue extract by dialysis we oxidised it. Thus all the extracts contained small quantities of oxidised glutathione. As will be shown later small quantities of oxidised glutathione are able to exert a strong inhibiting action on glycolysis.

(b) *Experiments with chopped tissues.*

The following experiments were made on fresh chopped tissue suspended in phosphate buffer solution. It was intended to investigate the activating effect of reduced glutathione on lactic acid production from glucose and from glycogen. As the tissue suspensions already contain preformed glutathione only the activating effect of added reduced glutathione could be investigated.

The technical details of these experiments have been described previously. Three to four rats were used for each experiment with brain tissue.

The bottles were set up as follows:

Phosphate buffer p_H 7.0	5.0 ml.
3 % solution of glucose or glycogen	1.0 ml.
Tissue emulsion, containing 0.4 g. of tissue	3.0 ml.
Adenosinetriphosphoric acid (or water)	0.5 ml. containing 0.5 mg. P_2O_5
Glutathione solution (or water) ...	0.5 ml. containing 4.0 mg. of glutathione

Adenosinetriphosphoric acid or glutathione or both were added only where it is stated in the Table. The experiments are reported in Table II.

All the experiments reported in Table II demonstrate that reduced glutathione strongly accelerates the rate of lactic acid formation from glucose but depresses to a small extent the rate of lactic acid formation from glycogen. The low glycolytic values observed in the experiments in which no coenzymes were added are probably due to the high dilution of the coenzymes. As Jowett and Quastel [1933] have observed, lysis and high dilution of the erythrocytes may cause a complete inhibition of the activity of their methylglyoxalase which may be restored by the addition of reduced glutathione.

In the main these experiments corroborate the previous series made with tissue extracts; reduced glutathione added to the macerated tissues brought about a strong activation of lactic acid production from glucose. The activating effect of added reduced glutathione was of about the same magnitude as that of added adenosinetriphosphoric acid. The largest activation was observed when both reduced glutathione and adenosinetriphosphoric acid were added to the tissue suspensions. The activating effect of reduced glutathione on the lactic acid production from glucose was clearly evident in all the tissues examined. In the experiments made with liver tissue the effect of adenosinetriphosphoric acid was not examined. Reduced glutathione had no activating effect at all on lactic acid production from glycogen. On the contrary a small inhibiting effect is seen in the experiments made with skeletal muscle.

Table II.

Exp. No.	Tissue	Substrate	mg. lactic acid formed by 100 g. tissue per hour			
			Without activator	With adenosine-triphosphoric acid	With adenosine-triphosphoric acid and glutathione	With glutathione without adenosine-triphosphoric acid
Brain hemispheres:						
1	0.358 g. to each bottle, total vol. 10 ml.	Glucose	55	179	500	188
2	0.358 g. to each bottle, total vol. 10 ml.	„	81	304	537	267
3	0.358 g. to each bottle, total vol. 10 ml.	„	67	288	566	300
4	0.441 g. to each bottle, total vol. 10 ml.	„	104	200	340	—
		Glycogen	14.1	8	10	—
5	0.400 g. to each bottle, total vol. 10 ml.	Glucose	60	189	370	—
		Glycogen	10	11	8	—
Skeletal muscle:						
6	0.4 g. to each bottle, total vol. 10 ml.	Glucose	—	83	123	—
		Glycogen	—	898	778	—
7	0.4 g. to each bottle, cont. total vol. 10 ml.	Glucose	—	67	112	—
		Glycogen	—	1120	908	—
8	0.4 g. tissue, total vol. 10 ml.	„	—	1404	1216	—
9	0.4 g. tissue, total vol. 10 ml.	„	—	1160	1022	—
Liver tissue:						
10	0.4 g. total vol. 10 ml.	Glucose	360	—	—	508
11	Do.	„	211	—	—	459
12	Do.	„	178	—	—	285

A somewhat unexpected result of these experiments is the activating effect of adenosinetriphosphoric acid on the lactic acid production from glucose by brain tissue. Cozymase and adenosinetriphosphoric acid are generally considered to belong to the esterifying enzyme system. But according to Ashford and Holmes [1929] glucolysis (from glucose) by brain tissue is independent of the presence of inorganic phosphate. According to Bumm and Fehrenbach [1931] cozymase does not accelerate brain glycolysis.

An attempt to explain these seemingly contradictory results will be made below.

Activating effect of reduced glutathione in relation to its concentration.

The quantitative relationships of the activating effect of glutathione on lactic acid formation from methylglyoxal have been investigated by Lohmann [1932]. He found, that the maximum activation is reached when the concentration of reduced glutathione is about 40 mg. per 100 ml., but even with 20 mg./100 ml. the maximum activation is approached. In the present experiments made on brain tissue about the same quantitative relation has been found for lactic acid formation from glucose. In Table III two such experiments out of five similar ones are reported. These experiments were made exactly in the same way as those described in Table II. To all the bottles adenosinetriphosphoric acid was added in the usual concentration.

Table III.

	Concentration of reduced glutathione mg./100 ml.	mg. lactic acid formed by 100 g. tissue per hour
<i>Exp. 1. Brain tissue, rats:</i>		
Glucose without added reduced glutathione	4.5	215
Substrate with added reduced glutathione	14.5	395
Substrate with added reduced glutathione	19.5	500
Substrate with added reduced glutathione	24.5	578
Substrate with added reduced glutathione	39.5	567
<i>Exp. 2. Brain tissue, rats:</i>		
Glucose without added reduced glutathione	4.0	227
Substrate with added reduced glutathione	14.0	457
Substrate with added reduced glutathione	24.0	533
Substrate with added reduced glutathione	34.0	530
Glycogen substrate, muscle tissue:		
Without added reduced glutathione	5.0	1212
With added reduced glutathione	15.0	1024
With added reduced glutathione	25.0	1075
With added reduced glutathione	45.0	971

These experiments show that the concentration of reduced glutathione necessary for full activation of the glycolysis by chopped brain tissue lies between 20 and 25 mg./100 ml.

The last experiment made on chopped skeletal muscle shows again a small but distinct inhibitory effect of reduced glutathione on lactic acid formation from glycogen.

The inhibitory effect of oxidised glutathione on glycolysis.

The indispensability of reduced glutathione for the glycolysis of glucose observed in the previous experiments offered a probable explanation of the so-called Pasteur-Meyerhof reaction.

In a recent paper Giršavicius [1933] reported that moderate access of oxygen caused rapid inactivation of glyoxalase. Bumm and Appel observed [1932] that the aerobic glycolysis of tumour tissue can be raised almost to the anaerobic level by addition of reduced glutathione. In view of the fact that reduced glutathione becomes slowly oxidised if agitated with oxygen in slightly alkaline solution it was supposed that the loss of reduced glutathione by oxidation might be responsible for the inhibitory effect of oxygen on glycolysis. In two experiments the effect of an excess of reduced glutathione in the presence of oxygen was examined. Quantities of reduced glutathione were added such that at the end of the incubation period each bottle still contained sufficient reduced glutathione to cause a full activation of glycolysis. The technique of these experiments was similar to that of the previous ones.

The flasks containing the tissue suspension, buffer solution together with glucose and the usual concentration of adenosinetriphosphoric acid, were divided into four groups. One of them to which no reduced glutathione was added was incubated anaerobically, the second one similarly prepared was incubated in oxygen. The third group to which an excess of reduced glutathione had been added was incubated anaerobically and the fourth group, similar to the third, was incubated in the presence of oxygen. At the end of the incubation period quantitative estimations of reduced glutathione were made in addition to the lactic acid estimations. Of the three experiments all giving similar results one is reported here (Table IV).

Table IV.

	mg. lactic acid produced by 100 g. tissue per hour	Final conc. of reduced glutathione in mg./100 ml.
Brain tissue without added glutathione in N_2	202	7
„ without added glutathione in O_2	82	Hopkins's reaction positive
„ with added glutathione in N_2	390	67
„ with added glutathione in O_2	76	24
„ without added glutathione in H_2O_2 *	82	0
„ with added glutathione in H_2O_2 *	72	11

* 0.2 ml. of 1 % solution of H_2O_2 was added to each 2 ml. of brain suspension (containing 0.4 g. of tissue) before it was introduced into the flasks. These flasks were filled with N_2 .

The experiments described in Table IV show that molecular oxygen inhibits glycolysis even when an excess of reduced glutathione is present. The addition of small amounts of H_2O_2 causes the same inhibiting effect as molecular oxygen. It is thus evident that the inhibiting effect of molecular oxygen on glycolysis cannot be ascribed to the absence of reduced glutathione.

The next experiments show clearly that the inhibiting factor in the Pasteur-Meyerhof reaction is oxidised glutathione. The technical arrangements of these experiments were like those of the previous ones. To some of the bottles various quantities of oxidised glutathione were added at the beginning of the experiments. All bottles but one were incubated anaerobically. At the end of the experiments the nitroprusside reaction showed that during the incubation period a part of the oxidised glutathione had been reduced by the brain tissue. One of three uniform experiments is reported in Table V. Glucose was the substrate.

Table V.

	mg. lactic acid produced by 100 g. tissue per hour	Nitroprusside test at the end of the experiment
Brain tissue without added glutathione in N_2	282	+
„ with 10 mg./100 ml. oxidised glutathione in N_2	171	+++
„ with 20 mg./100 ml. oxidised glutathione in N_2	168	+++
„ with 40 mg./100 ml. oxidised glutathione in N_2	146	+++
„ without added glutathione in O_2	162	±

In these experiments 10 mg./100 ml. of oxidised glutathione cause about the same inhibition of glycolysis as the presence of oxygen. The inhibiting effect of oxidised glutathione on glycolysis is still more marked in the experiment with muscle tissue and with glycogen as substrate.

The next experiment on finely cut muscle tissue and with glycogen was made exactly like the previous ones. One of the three experiments which all gave the same result is given in Table VI.

Table VI.

	mg. lactic acid produced from glycogen by 100 g. tissue per hour	Nitroprusside test at the end of the experiment
Muscle tissue without added glutathione in N_2	1824	+
„ with 10 mg./100 ml. oxidised glutathione in N_2	810	+++
„ with 20 mg./100 ml. oxidised glutathione in N_2	132	+++
„ with 40 mg./100 ml. oxidised glutathione in N_2	74	+++
„ without added glutathione in O_2	1762	+

These experiments on muscle tissue show a powerful inhibitory effect of oxidised glutathione. In the presence of 40 mg. of oxidised glutathione inhibi-

tion of glycolysis is nearly complete. It is significant that in these experiments the inhibition of glycolysis caused by oxygen is very small in comparison with that caused by oxidised glutathione. It is to be observed too that in all the bottles at the end of the incubation a strong nitroprusside reaction was observed which indicated that a part of the added oxidised glutathione was reduced during the incubation period. The smallness of the inhibitory effect of oxygen in these experiments may thus be due to the reducing power of the tissue which is probably able to reduce quickly the oxidised glutathione slowly formed by the action of molecular oxygen.

With brain tissue and with glucose as substrate equal inhibition is caused by oxygen and by oxidised glutathione.

According to these experiments the inhibiting effect of molecular oxygen on glycolysis can be explained by the formation of oxidised glutathione which is directly capable of inhibiting glycolysis.

The rôle of the inorganic phosphorus in glycolysis of glucose.

According to Ashford and Holmes [1929] brain glycolysis from glucose proceeds in the absence of inorganic phosphates. It was therefore disconcerting to find that adenosinetriphosphoric acid activates the glucose breakdown by brain tissue. It was therefore decided to repeat the experiments of Ashford and Holmes and to investigate the effect of adenosinetriphosphoric acid and of reduced glutathione on tissue emulsions freed from inorganic phosphates.

At first the effect of the borate buffer alone on glycolysis was investigated. A borate buffer of p_H 8.1 was prepared according to Sørensen.

The bottles were set up as follows:

Phosphate or borate buffer p_H 8.1	...	...	5.0 ml.
2 % glucose solution	...	...	1.5 ml.
Brain emulsion in 0.9 % NaCl solution	...	...	2.0 ml. containing 0.3 g. of brain tissue
1.2 % $CaCl_2$ solution (or water)	...	...	1.0 ml.
Adenosinetriphosphoric acid solution (or water)	...	...	0.5 ml.

One of the two parallel experiments is reported in Table VII.

Table VII.

	mg. lactic acid produced by 100 g. tissue in 1 hour
Phosphate buffer	320
Phosphate buffer + adenosinetriphosphoric acid	540
Borate buffer	18
Borate buffer + adenosinetriphosphoric acid	29
Borate buffer + adenosinetriphosphoric acid + $CaCl_2$	130

These experiments show that borate itself strongly inhibits glycolysis. Adenosinetriphosphoric acid does not counteract this inhibition but strangely enough $CaCl_2$ does. To investigate this effect of $CaCl_2$ more experiments were set up with different amounts of $CaCl_2$.

The bottles were set up as follows:

Borate buffer p_H 8.2	...	...	...	5 ml.
3 % glucose solution	...	...	...	1 ml.
1.2 % $CaCl_2$ solution (or water)	...	...	...	3 ml.
Adenosinetriphosphoric acid (or water)	...	...	...	0.5 ml.
Reduced glutathione solution (or water)	...	...	...	0.5 ml.

The experiments were made anaerobically in every respect like the previous experiments. One of the two similar experiments is reported in Table VIII.

Table VIII.

	mg. lactic acid produced by 100 g. tissue per hour
Borate buffer without CaCl_2	28
„ with 0.5 ml. CaCl_2	51
„ with 1.0 ml. CaCl_2	111
„ with 2.0 ml. CaCl_2	174
„ with 3.0 ml. CaCl_2	175
„ with 2.0 ml. adenosinetriphosphoric acid	196
„ with 2.0 ml. adenosinetriphosphoric acid + glutathione	297

These experiments show that CaCl_2 counteracts the inhibiting effect of borate and that in the presence of borate and of CaCl_2 and thus in the absence of inorganic phosphate the activating effect of reduced glutathione is still very evident. These experiments also show that under the experimental conditions described so many factors introduce complications that it is difficult to estimate the rôle of the inorganic phosphorus in brain glycolysis. It seems, however, in agreement with Ashford and Holmes [1929] that the presence of inorganic phosphorus is not essential in this process.

A further fact of interest is that while the activating effect of adenosinetriphosphoric acid disappears under these conditions, the activating effect of glutathione is not altered. This phenomenon may suggest two mechanisms of glucose breakdown in the brain, one of which does not need the presence of inorganic phosphate and is activated by reduced glutathione and a second one which only proceeds in the presence of inorganic phosphate and of adenosinetriphosphoric acid. This may explain also why adenosinetriphosphoric acid activates brain glycolysis independently of glutathione.

A comparison of the glyoxalase activities of brain and muscle tissue.

In the previous pages it was suggested as possible that methylglyoxal may be an intermediary product of the breakdown of glucose but not of glycogen. It is to be expected accordingly that those tissues which produce lactic acid from glucose must be able to transform a correspondingly large quantity of methylglyoxal into lactic acid. On the other hand if methylglyoxal is not an intermediary product in lactic acid formation from glycogen those tissues in which lactic acid formation from glycogen is predominant should be unable to form a correspondingly large quantity of lactic acid from methylglyoxal.

The rats selected for these experiments were kept under strictly identical conditions at least for three generations. The technique of these experiments was described in the general part. The contents of the bottles were as follows:

Phosphate buffer solution p_H 6.95	...	...	...	5 ml.
Methylglyoxal 0.7 %	...	...	...	3 ml.
Tissue suspension containing 0.4 g. tissue	...	...	...	2 ml.

In the experiment with muscle tissue 0.4 g. of finely cut muscle was weighed into every bottle and 1.6 ml. of water were added. The methylglyoxal solution was accurately neutralised before addition. Four bottles were set up for each experiment as described above. Table IX shows the result.

Table IX shows that muscle tissue which is able to form on the average 1176 mg. lactic acid per 100 g. tissue per hour from glycogen and 101 mg. from

Table IX.

	mg. lactic acid produced by 100 g. tissue in 1 hour		
	From methylglyoxal	From glucose	From glycogen
Muscle tissue	242	—	1160
	278	—	1022
	236	108	—
	197	83	—
	221	123	—
	215	79	1212
	309	—	1024
	302	112	1462
	Average	251	1176
Brain tissue	1487	326	28
	1328	308	19
	1384	320	—
	1222	—	14
	1247	340	18
	1055	296	—
Average	1287	318	19

glucose, forms only 251 mg. lactic acid from methylglyoxal. As a general rule it is to be expected that the conversion of an intermediary product should proceed at least with the same velocity as that of the initial product. Actually the transformation of glycogen into lactic acid in the muscle proceeds 4-5 times as rapidly as that of methylglyoxal. Thus methylglyoxal cannot possibly be an intermediary product in the breakdown of glycogen.

Brain tissue, on the other hand, which produces lactic acid mainly from glucose, is able to convert a large quantity of methylglyoxal. For 318 mg. of lactic acid formed from glucose, the brain tissue is able to form 1287 mg. of lactic acid from methylglyoxal.

DISCUSSION.

The experiments contained in the first part of this paper demonstrate that the enzyme system which produces lactic acid from glycogen is unable to form lactic acid from glucose. While reduced glutathione is essential in the process of lactic acid production from glucose, glycogen can be split into lactic acid in its absence. Reduced glutathione added to tissue suspensions greatly accelerated the lactic acid formation from glucose but inhibited it to a small extent when glycogen was the substrate.

By the fact that reduced glutathione is a common coenzyme of the methylglyoxalase and of the glycolytic enzyme system, the probability of methylglyoxal being an intermediary product in the formation of lactic acid from glucose is strengthened. The concentrations of reduced glutathione necessary for the full activation of the glycolysis (of glucose) and of methylglyoxalase are similar.

On the other hand it is hardly possible to consider methylglyoxal as an intermediary in the process of lactic acid formation from glycogen, as this process proceeds also in the absence of glutathione. The experiments of Embden *et al.* [1933] of Meyerhof and Kiessling [1933] and of Meyerhof and McEachern [1933] excluded the intermediary formation of methylglyoxal from the lactic acid production from glycogen and hexosediphosphate by muscle. It is also known that muscle tissue splits mainly glycogen, glucose only to a small extent.

On the basis of these facts the hypothesis is ventured that glucose breakdown in the cells proceeds with the intermediary formation of methylglyoxal,

in accordance with the theory of Neuberg, while the breakdown of glycogen proceeds as postulated by Embden *et al.* and Meyerhof *et al.* through pyruvic acid. A recent paper of Barrenscheen and Bereschkovsky [1933], according to which tissues other than muscle are unable to produce pyruvic acid from phosphoglyceric acid, also points in this direction.

The inhibiting action of oxidised glutathione is of peculiar interest as offering an explanation of the Pasteur-Meyerhof reaction. It was found formerly by Lipmann [1933] that oxidising agents such as iodine and quinone inhibited the glycolysis of muscle extracts. The glycolysis of these extracts was also reversibly inhibited by O_2 in the presence of a redox indicator, 2:6-dichlorophenolindophenol. Lipmann therefore concluded, that the Pasteur reaction is an oxidative inhibition of the glycolysis, as the glycolytic ferment of the cells becomes inactivated by oxygen transmitted by the autoxidisable respiratory enzyme.

According to the present experiments oxidised glutathione may be considered as the transmitter of oxygen to the glycolytic enzyme. Reduced glutathione can be easily oxidised by molecular oxygen in a weakly alkaline medium. In its turn, oxidised glutathione proved to be a powerful inhibitor of glycolysis.

Since in muscle glycolysis from glycogen no reduced glutathione is necessary and since the inhibitory effect of oxidised glutathione persists also in the presence of reduced glutathione it is evident that reduced glutathione in this case may act only as an intermediary by transmitting oxygen to the glycolytic enzyme.

On the other hand in the glycolysis of glucose the oxidation of glutathione has a double significance. First, by oxidation of reduced glutathione the glycolytic enzyme system is deprived of one of its coenzymes, and secondly, the presence of oxidised glutathione inactivates the glycolytic enzyme. It is to be noted that in the present experiments the quantitative aspect of the inhibition of these two types of glycolysis by oxidised glutathione has not yet been sufficiently considered. It seems, however, that a more complete inhibition can be obtained by oxidised glutathione in the breakdown of glycogen than in that of glucose.

No explanation can be offered for the observed actions of borate and of calcium on glycolysis.

The experiments on the glyoxalase activity of muscle and brain tissue provide further evidence in favour of the theory that methylglyoxal cannot be considered as intermediary in glycogen breakdown but is a possible intermediary in glucose breakdown.

SUMMARY.

1. Tissue extract from which reduced glutathione is removed cannot convert glucose into lactic acid. The addition of reduced glutathione restores this ability to the extracts.
2. Reduced glutathione added to tissue suspensions accelerates the rate of lactic acid formation from glucose but inhibits to a small extent that from glycogen.
3. Oxidised glutathione even in low concentration inhibits lactic acid formation from glucose as well as from glycogen.
4. Borate inhibits brain glycolysis, $CaCl_2$ restores it to a small extent.
5. It is concluded that reduced glutathione is a coenzyme of the glycolytic enzyme system and that lactic acid production from glucose may proceed through methylglyoxal as an intermediary, while this possibility is excluded in the case of glycogen.
6. An explanation is offered for the Pasteur-Meyerhof reaction.

REFERENCES.

- Ashford (1933). *Biochem. J.* **27**, 923.
— and Holmes (1929). *Biochem. J.* **23**, 748.
Barrenscheen and Bereschkovsky (1933). *Biochem. Z.* **265**, 159.
— and Dreguss (1931). *Biochem. Z.* **233**, 305.
— and Filz (1932). *Biochem. Z.* **250**, 281.
Bumm and Appel (1932). *Z. physiol. Chem.* **210**, 79.
— and Fehrenbach (1930). *Z. physiol. Chem.* **193**, 238.
— — (1931). *Z. physiol. Chem.* **195**, 101.
Case and Cook (1931). *Biochem. J.* **25**, 1319.
Embden, Deutike and Krafft (1933). *Klin. Woch.* 213.
Friedemann, Cotonio and Shaffer (1927). *J. Biol. Chem.* **73**, 335.
— and Graeser (1933). *J. Biol. Chem.* **100**, 291.
Giršavicius (1933). *Biochem. J.* **27**, 537.
Haarmann (1932). *Biochem. Z.* **255**, 203.
Jowett and Quastel (1933). *Biochem. J.* **27**, 486.
Kuehnau (1931). *Biochem. Z.* **230**, 353.
Lipmann (1933). *Biochem. Z.* **265**, 133.
Lohmann (1932). *Biochem. Z.* **254**, 332.
Meyerhof and Kiessling (1933). *Biochem. Z.* **264**, 60.
— and McEachern (1933). *Biochem. Z.* **260**, 417.
Neuberg and Hofmann (1930). *Biochem. Z.* **224**, 491.
Pirie (1930). *Biochem. J.* **24**, 51.
Watson and Mitolo (1934). *Biochem. J.* **28**, 811.