68. GLYCOLYSIS IN RETINAL EXTRACTS

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THE relatively high glycolytic power of retinal cells *in vitro* has been recognized since the work of Warburg *et al.* [1924], but it is still not known whether this activity is of any significance in the metabolism of living retina, nor have the intermediary steps of this glycolysis been worked out.

As a preliminary to a series of studies on retinal metabolism we have prepared a cell-free extract of retina [Kerly & Bourne, 1939] which fortunately proved to be very active glycolytically, and we have endeavoured to compare its behaviour with that of cell-free extracts of other tissues described by other workers.

The chief points of interest which emerge from the present work are concerned with the mechanism by which glucose is converted into lactic acid and with the question of the phosphorylation of glucose during the glycolysis. It has been suggested that the *in vitro* conversion of glucose into lactic acid by the cells of retina [Bumm & Fehrenbach, 1931] and of other tissues proceeds by a mechanism which is entirely different from that involved in the conversion of glycogen into lactic acid in tissue extracts. The presence of phosphate is said to be essential for glycogenolysis but unnecessary for glucolysis (see Needham & Lehmann [1937] for a discussion of the literature). The evidence for this hypothesis is unsatisfactory, since it is derived from experiments on intact cells where considerations of permeability and of the composition of the medium inside the cell make it difficult to interpret the results. In those cases where cell-free extracts which will attack glucose have been prepared [Euler *et al.* 1936; Süllman, 1937; Meyerhof, 1938], the evidence does not support the view that there are two separate mechanisms, one for the breakdown of glucose and another for the breakdown of glycogen; it indicates, on the contrary, that glucolysis and glycogenolysis proceed by similar routes. The results of our own experiments reported in this paper show that cell-free extracts of retina convert both glucose and glycogen into lactic acid, but that glucose is more readily attacked. They demonstrate conclusively that the same activators and coenzymes are necessary for both substrates, and that the same enzyme poisons which inhibit glycogenolysis in muscle extracts also prevent glycolysis of both glycogen and glucose in retinal extracts. The intermediary reactions have not been worked out, but the results suggest that phosphorylation and concurrent oxido-reduction of intermediary products are essential reactions in the breakdown of glucose.

After these experiments had been completed Süllman & Vos [1939] reported the preparation of aqueous retinal extracts, and showed that the coenzymes and inhibitors of muscle glycolysis were active. A comparison of our own findings with the results obtained by these authors is included in the discussion.

METHODS

Preparation of extract. Ox eyes were brought from the slaughterhouse on ice from 1 to 6 hr. after the animals had been killed. The retinae were dissected out and frozen solid; they were then ground in a cold mortar with sand and an

equal weight of ice-cold glass-distilled water. The mixture was then frozen solid and centrifuged for 20 min.; the supernatant solution was decanted and again frozen and centrifuged for 20 min. This repeated freezing and thawing was intended to assist in breaking up the cells and in promoting extraction. The topmost layer, which appeared perfectly clear after the second spinning, had no glycolytic activity; therefore the whole of the supernatant solution, except the bottom deeply coloured layer, was used after being decanted and thoroughly mixed. No intact cells could be detected in it on microscopic examination. Extracts which are referred to in the text as "fresh extracts" were not dialysed and were used within 4 hr. of their preparation (unless otherwise stated), being kept ice cold during this time.

Dialysis. Cellophane sacs were used, and the extract was dialysed against cold tap water for 6 hr. followed by distilled water in the cold room for approximately another 15 hr. In the earlier experiments running tap water at about 12–14° was used; in later experiments large volumes of tap water at 5°, changed hourly, were used. During the second period of dialysis the distilled water was changed only if the dialysis was continued for more than 24 hr. The use of glass-distilled in place of copper-distilled water made no difference to the results.

Measurement of glycolysis. The extract, together with substrate and other additions, was incubated at 37° in bicarbonate buffer (pH 7.4) in an atmosphere of N₂ (95 %) and CO₂ (5 %). 1 ml. of the extract, the substrate and other additions were placed in a Warburg flask containing NaHCO₃ (final concentration 0.03 M), coenzymes were placed in the side arm of the flask, and the total volume brought to 2 ml. with distilled water. The amounts of the various additions used in each experiment are given in the tables. The flask was then attached to a manometer and shaken in a water bath at 37°. The gas mixture was passed through the flask for 10 min.; the tap was then closed, and a further 5 min. were allowed for equilibrium to be reached, after which the contents of the side arm were tipped in. As no glycolysis took place without the addition of coenzyme, this procedure allowed the precise time at which glycolysis began to be recorded and small differences in equilibrating time were eliminated.

Manometric measurements were usually made as a guide to the rate of glycolysis, but all results reported are in terms of lactic acid formation measured chemically by a modification of the Friedman *et al.* [1927] method. Glycolysis was allowed to continue for 30 min., then trichloroacetic acid was added and lactic acid estimated on an aliquot of the filtrate after precipitation of the sugar with copper and lime.

Estimation of phosphates. The method of Fiske & Subbarow [1925] was used. In order to obtain sufficient filtrate for the determination of both phosphate and lactic acid, all quantities were doubled for these experiments. For the determination of the various acid-soluble phosphate fractions an equal volume of *N* HCl was added to a portion of the protein-free filtrate, aliquots were used for estimation of inorganic and total phosphate, and for inorganic phosphate after hydrolysis in sealed tubes heated in a boiling water bath for 7, 30 or 180 min.

Materials. MgCl₂ was used as a source of Mg⁺⁺ throughout. Phosphate was added in the form of the Sørensen mixture (Na₂HPO₄—KH₂PO₄) pH 7.4. In one series of experiments a mixture of Na phosphates was substituted, but no difference in the results was obtained, nor did the addition of KCl produce any effect. The Ca salt of adenosinetriphosphate (abbreviated as Ca-ATP) was prepared from cat muscle by the method of Barrenscheen & Filz [1932]. By analogy with the Ba salt (Barrenscheen's formula), the formula of the Ca salt is C₁₀H₁₂O₁₃N₅P₃Ca₂·6H₂O (theoretical 13.4 % P, found 13.2 %), so that 1 mg.

Ca-ATP is equivalent to 0.73 mg. ATP and very nearly 0.5 mg. adenylic acid (abbreviated AA). For other preparations used we are greatly indebted to a number of biochemists as follows: Dr F. G. Young for glycogen (from mytilus), Dr M. Macfarlane for Ba hexosediphosphate (converted into the Na salt before use), Prof. v. Euler and Dr D. Green for cozymase, Dr Ostern for muscle adenylic acid (used as the Na salt) and Dr Mendel for *dl*-glyceraldehyde.

GLYCOLYSIS

Undialysed extract. Cell-free extract of retina can produce lactic acid from glucose, hexosediphosphate or glycogen provided that either ATP or AA is also added. The amount of lactic acid produced from glucose is greater than that

Table 1. *Effects of duration of incubation and of time at which ATP is added on lactic acid formation*

1 ml. extract, 2 mg. Ca-ATP and 4 mg. glucose (or glycogen). Incubated at 37° in 0.03 *M* NaHCO₃.

No.	Time of adding Ca-ATP min.	Substrate	Time of adding substrate min.	Lactic acid (mg.) formed after	
				30 min.	60 min.
7	0	—	—	0.32	0.40
7	30	—	—	0	0.24
4	0	Glucose	0	1.91	1.97
7	0	"	30	—	0.36
7	30	"	30	—	1.89
4	0	Glycogen	0	0.79	0.96
7	0	"	30	—	0.28
7	30	"	30	—	0.50

obtained from equivalent amounts of the other two substrates (Tables 1 and 4), and the glycolysis proceeds at a more rapid rate. Table 1 shows that lactic acid formation from glucose had almost ceased after 30 min., while that from glycogen was still continuing. This difference in rate was confirmed by manometric readings where it was observed that CO₂ production from glucose fell off continuously during incubation, whilst that from glycogen or hexosediphosphate continued at a nearly uniform rate throughout the experiment. 30 min. was therefore chosen as the standard period for glycolysis. Fig. 1 shows the comparative rates of glycolysis as measured by CO₂ production, using dialysed extract.

Without addition of substrate very little lactic acid was formed (Tables 1 and 3). This small value has not been subtracted from the total lactic acid production, and the figures in the tables represent total lactic acid formed from added substrate and from carbohydrate present in the extract.

Without addition of ATP or AA there was no glycolysis (Table 6). It was found that ATP was destroyed during incubation with extract when no substrate was

Fig. 1. Manometric measurement of glycolysis. 1 ml. extract, phosphate (0.02 *M*; 0.04 *M* for glycogen), MgCl₂ (0.004 *M*), 1 mg. Ca-ATP incubated at 37° in 0.03 *M* NaHCO₃. (a) 4 mg. glucose, (b) 4 mg. hexose equivalent of hexosediphosphate, (c) 4 mg. glycogen.

added (Table 1). This destruction must extend beyond the removal of two phosphate radicles since AA is active under certain conditions (Table 6). In the presence of glucose the amount of ATP remaining after 30 min. incubation varied in different extracts (Table 10). It is therefore important that coenzyme should not be added before substrate.

The activity of the extract remained nearly constant during 44 hr. storage in the refrigerator at 5° (Table 2). The activities of different extracts as measured

Table 2. *Effect on lactic acid formation of keeping retinal extract at 5°*

1 ml. extract, 2 mg. Ca-ATP, 4 mg. glucose (or glycogen). Incubated for 30 min. at 37° in 0.03 *M* NaHCO₃.

No.	Hr. after preparation	Lactic acid (mg.) formed from	
		Glucose	Glycogen
6	0	1.80	0.66
	2.5	1.64	0.47
	6	1.53	0.60
	20	1.77	0.54
	44	1.61	0.65
	92	0.53	0.50
7	0	1.89	—
	20	1.78	—
20	0	3.94*	—
	3	4.04*	—

* Phosphate (0.01 *M*) and MgCl₂ (0.004 *M*) also added.

Table 3. *Lactic acid formation from different extracts*

1 ml. extract, 2 mg. Ca-ATP, incubated for 30 min. at 37° in 0.03 *M* NaHCO₃.

No.	Lactic acid (mg.) formed from		
	No added substrate	4 mg. glucose	4 mg. glycogen
3	—	2.00	0.56
4	0.30	1.91	0.79
5	0.17	1.61	0.49
6	0.30	1.80	0.66
7	0.32	1.78	—
8	0.41	1.73	0.70
10	0.32	1.74	0.67
15	—	1.64	—
17	—	1.62	—

by lactic acid production (Table 3) were sufficiently constant for typical results to be selected in compiling the tables illustrating the behaviour of the extract in the presence of phosphate, Mg and ATP. Each point noted was, of course, confirmed by experiments with a number of different extracts.

The amounts of lactic acid produced from glucose and from glycogen by fresh extract in the presence of ATP were increased by addition of phosphate (Table 4). The yield of lactic acid from glucose was maximal when sufficient phosphate was added to give a concentration between 0.01 *M* and 0.02 *M*. The final concentration in the mixture was slightly higher than that produced by the added phosphate owing to the presence of a small amount of phosphate in the extract. With higher concentrations of phosphate (0.08 *M*), glycolysis was inhibited to some extent. With glycogen maximal glycolysis occurred with a higher phosphate concentration (0.05 *M*) and inhibition at a concentration of 0.08 *M* was slight.

Table 4. *Effect of alteration of phosphate concentration on lactic acid formation*

1 ml. extract, 4 mg. glucose, glycogen or hexose equivalent of hexosediphosphate, incubated for 30 min. at 37° in 0.03 *M* NaHCO₃.

No.	Substrate	Additions		Lactic acid (mg.) formed with phosphate concentrations (<i>M</i>) of								
		Ca-ATP mg.	MgCl ₂ <i>M</i>	0	0.001	0.002	0.01	0.02	0.04	0.05	0.06	0.08
Fresh extract												
8	—	2	0	0.41	—	—	0.56	—	—	0.67	—	0.57
8	Glucose	2	0	1.73	—	—	2.86	2.80	—	2.52	—	2.28
8	Glycogen	2	0	0.70	—	—	—	1.08	—	1.15	—	1.04
15	Hexosediphosphate	2	0	1.09	—	—	1.04	—	—	—	—	—
Dialysed extract												
18	—	2	0.004	0.17	—	—	—	0.19	—	—	—	—
13	Glucose	1	0.004	0.23	—	1.23	2.16	2.62	2.52	—	2.64	—
17	"	2	0.004	0.86	1.24	—	2.80	3.06	3.20	—	2.96	—
13	Glycogen	1	0.004	0.04	—	0.23	0.31	0.53	0.65	—	0.67	—
12	"	2	0.02	0.10	—	0.23	0.64	0.91	1.08	—	—	—
14	Hexosediphosphate	1	0.004	1.22	—	1.33	1.37	1.48	1.36	1.35	—	—

Note. The concentrations stated are the final concentrations which would be produced by the amounts of phosphate or MgCl₂ added in each case. In the fresh extract, the actual concentrations are slightly higher, owing to the presence of small amounts of these substances in the extract.

Table 5. *Effect of alteration of the concentration of MgCl₂ on lactic acid formation*

1 ml. extract, 4 mg. glucose, glycogen or hexose equivalent of hexosediphosphate, incubated for 30 min. at 37° in 0.03 *M* NaHCO₃.

No.	Substrate	Additions		Lactic acid (mg.) formed with MgCl ₂ concentrations (<i>M</i>) of						
		Ca-ATP mg.	Phos- phate <i>M</i>	0	0.0004	0.001	0.004	0.01	0.02	0.04
		Fresh extract								
17	Glucose	2	0.01	2.66	—	—	—	—	3.49	3.02
15	Hexosedi- phosphate	2	0.01	1.04	—	—	—	—	2.17	—
Dialysed extract										
17	Glucose	2	0.02	0.55	1.61	2.43	3.09	2.89	—	2.15
14	Glycogen	1	0.04	0.60	0.76	0.80	0.79	0.71	0.43	—
14	Hexosedi- phosphate	1	0.02	0.91	1.15	1.44	1.48	1.43	1.30	—

See note to Table 4.

In the presence of optimal concentrations of phosphate, the addition of MgCl₂ caused a further increase in lactic acid production from glucose. With hexosediphosphate as substrate the addition of phosphate caused no increase in lactic acid production, but the addition of MgCl₂ caused a considerable increase (Tables 4 and 5).

In fresh extracts AA could replace ATP as coenzyme (Table 6). In the absence of added phosphate the amount of lactic acid formed from glucose was less with AA than with ATP, but in the presence of optimal concentrations of phosphate the amount of lactic acid formed was greater if AA were used. With hexosediphosphate AA was always more effective, the presence or absence of added phosphate making little difference. With glycogen it was shown that AA could replace ATP, but the relative amounts of lactic acid formed and the influence of phosphate were not thoroughly investigated.

Table 6. *Effect of alteration of quantity of Ca-ATP (or AA) on lactic acid formation*

1 ml. extract, 4 mg. glucose, glycogen or hexose equivalent of hexosediphosphate, incubated for 30 min. at 37° in 0.03 *M* NaHCO₃.

No.	Substrate	Additions		Lactic acid (mg.) formed with added											
		Phos- phate <i>M</i>	MgCl ₂ <i>M</i>	Ca-ATP (mg.)								AA (mg.)			
				0	0.01	0.1	0.5	1.0	2.0	5.0	0.5	1.0	2.0	2.5	
Fresh extract															
5	—	0	0	0	—	—	—	—	0.17	—	—	0.12	0.25	—	
12	—	0.01	0	—	—	—	—	0.46	0.37	0.26	0.19	0.49	—	0.46	
5	Glucose	0	0	0	—	—	—	—	1.61	—	—	1.21	1.44	—	
12	„	0.01	0	—	—	—	—	2.74	2.69	1.86	3.14	3.14	—	3.22	
5	Glycogen	0	0	—	—	—	—	—	0.49	—	—	0.28	0.80	—	
15	Hexosediphosphate	0	0	—	—	—	—	—	1.09	—	—	1.46	—	—	
15	„	0.01	0	—	—	—	—	—	1.04	—	—	1.47	—	—	
Dialysed extract															
18	—	0.01	0.004	0	—	—	—	—	0.19	—	—	—	—	—	
8	Glucose	0.02	0.02	—	—	—	—	—	2.60	—	—	0.63	1.17	—	
13	„	0.02	0.004	—	—	—	—	2.62	—	—	0.05	—	—	—	
16	„	0.02	0.004	0	0	0.12	2.52	2.94	3.34	3.22	—	—	—	—	
13	Glycogen	0.04	0.004	—	—	—	—	0.65	—	—	0.30	—	—	—	
16	„	0.04	0.004	0	0	—	0.31	0.54	0.72	0.85	—	—	—	—	
16	Hexosediphosphate	0.02	0.004	0.23	0.20	0.53	1.32	1.69	2.06	2.48	—	—	—	—	

Note. 2 mg. Ca-ATP ≡ 1 mg. AA. See note to Table 4.

Table 7. *Effect of adding cozymase on lactic acid formation*

1 ml. extract, 4 mg. glucose, glycogen or hexose equivalent of hexosediphosphate, incubated for 30 min. at 37° in 0.03 *M* NaHCO₃. For dialysed extract 0.004 *M* MgCl₂ and 0.02 *M* phosphate (0.04 *M* for glycogen) also added.

Additions			Lactic acid (mg.) formed from			
Ca-ATP mg.	Cozymase mg.	Dialysis hr.	No added substrate	Glucose	Glycogen	Hexosediphosphate
Fresh extract						
2	0	—	—	1.77	—	—
0	0.2	—	—	0.37	—	—
2	0.2	—	—	1.74	—	—
Dialysed extract						
2	0	20	—	2.86	0.82	1.64
0	2	20	—	0	0	0.26
2	2	20	0.19	2.97	0.86	1.56
2	0	48	—	2.25	0.49	1.34
2	2	48	—	2.86	0.51	1.41

ATP could not be replaced by cozymase for inducing glycolysis in fresh extracts, nor did the addition of cozymase in the presence of ATP increase the yield of lactic acid (Table 7).

The effect of dialysis. After the extract had been dialysed against cold water for 20 hr. addition of coenzyme (ATP) only was not sufficient to produce glycolysis. In order that glucose might be converted into lactic acid it was necessary to add both phosphate and MgCl₂. The concentrations of these substances required for producing maximal glycolysis have been determined and are recorded in Tables 4 and 5.

When hexosediphosphate was used as substrate the addition of phosphate produced only a small increase in glycolysis, whilst the addition of MgCl_2 greatly increased lactic acid production. With glycogen, on the other hand, MgCl_2 had little effect, but it was essential to add phosphate; increasing concentrations gave greater yields of lactic acid and no inhibitory effect was observed with the highest concentration (0.06 M) used.

Without the addition of ATP or AA there was no glycolysis of either glucose or glycogen, but a trace of lactic acid was produced when hexosediphosphate was incubated with extract (Table 6). The effect of substituting AA for ATP was tried in only two experiments with dialysed extract. In one case, extract no. 8, AA produced some activity when added together with phosphate and MgCl_2 , but extract no. 13, which showed normal activity with ATP, was quite inactive when an equivalent amount of AA was substituted. The only difference in the preparation of these two extracts recorded was in the temperature of the tap water during the preliminary dialysis. In the case of extract no. 8 the temperature did not rise above 12° , but with extract no. 13 it had risen to 18° at the end of 6 hr. The possible bearing of this on the observed result is discussed later.

Cozymase could not replace ATP when added to extract dialysed for 20 hr. (Table 7). When it was added together with ATP it caused no increase in lactic acid formation. But, in the case of extract which had been dialysed for 48 hr. and which showed a diminution of glycolytic activity in the presence of ATP, the addition of cozymase caused a definite increase in lactic acid production from glucose. The increases obtained in the case of hexosediphosphate and glycogen were not significant. It is possible that cozymase is removed from retinal extract only after prolonged dialysis. Sym *et al.* [1930] have shown that retina contains large amounts of cozymase and Green *et al.* [1937] state that cozymase associated with triosephosphate mutase is not completely removed by dialysis.

Effect of inhibitors. Glycolysis of all substrates was inhibited by addition of iodoacetate (IAA), NaF or glyceraldehyde (Table 8). A concentration of $M/20,000$ IAA inhibited lactic acid production from glucose almost completely, from glycogen by about 50 %, and from hexosediphosphate hardly at all, while a tenfold increase in concentration ($M/2000$) effectively inhibited glycolysis

Table 8. *Inhibition of glycolysis*

1 ml. extract, 2 mg. Ca-ATP, 4 mg. glucose, glycogen or hexose equivalent of hexosediphosphate, incubated for 30 min. at 37° in 0.03 M NaHCO_3 . For extracts nos. 20 and 21 phosphate, to give a concentration of 0.01 M , and MgCl_2 , to give a concentration of 0.004 M , were also added.

No.	Inhibitor	Lactic acid (mg.) formed from		
		Glucose	Glycogen	Hexosediphosphate
Fresh extract				
10	—	1.74	0.67	1.25
10	<i>M</i> /20,000 IAA	0.15	0.29	1.15
10	<i>M</i> /2000 IAA	0.02	0.01	0.19
10	<i>M</i> /20 NaF	0.01	0	0.25
20	—	4.04	—	—
20	<i>M</i> /50 NaF	0.04	—	—
Dialysed extract				
21	—	3.78	1.38	2.20
21	<i>M</i> /500 <i>dl</i> -glyceraldehyde	3.59	1.38	2.12
21	<i>M</i> /5 <i>dl</i> -glyceraldehyde	0.63	0.22	0.50

from all substrates. NaF was effective in a concentration of $M/20$ for all substrates and also at $M/50$ for glucose; the effect of the smaller concentration was not determined with the other substrates.

Dialysed extracts were used for testing the effect of glyceraldehyde, since Boyland & Boyland [1938] have shown that undialysed muscle extract contains a substance which will neutralize its inhibitory effect. It was found necessary to use concentrations as high as $M/10$ *l*-glyceraldehyde (added as $M/5$ *dl*-glyceraldehyde) before appreciable inhibition was induced. With this inhibitor retinal extract behaves in a manner similar to muscle extract and is not analogous to the tumour tissue studied by Mendel [Mendel, 1929; Mendel *et al.* 1938] where glycolysis could be inhibited by very much lower concentrations of *l*-glyceraldehyde. Süllman & Vos [1939] found glyceraldehyde to be inhibitory in rather lower concentrations than those which were effective in our experiments, but in neither case was inhibition quite complete.

FORMATION OF PHOSPHORIC ESTERS

The foregoing results prove that the presence of phosphate is necessary for the production of lactic acid from glucose or from glycogen, but they give no indication of the part played by the phosphate ion. In the case of muscle extract it has been shown that a series of carbohydrate-phosphate esters are formed, and, if the same enzyme system were present in retinal extract, the formation of the same esters might be expected to occur during glycolysis. We have attempted to obtain evidence of the formation of phosphoric esters by determining the inorganic phosphate and the organic phosphate fractions before and after incubation, and also after incubation of the mixture in the presence of fluoride.

By observing the rate of hydrolysis in $N/2$ HCl at 100° , although it is not possible to characterize each ester in the mixture, evidence of an accumulation of esterified phosphate or of the transfer of phosphate from the easily hydrolysable ATP to the more difficultly hydrolysable carbohydrate-phosphate esters can be obtained.

For all these experiments $MgCl_2$, phosphate and coenzyme (either ATP or AA) were added to fresh extract.

Initial values. Each of the two extracts studied contained inorganic phosphate and some difficultly hydrolysable ester. A little ATP (i.e. phosphoric ester hydrolysed in 7 min.) was present in extract no. 20, but none in extract no. 19 (Table 9).

Table 9. *Acid-soluble phosphate esters of retinal extract*

Values represent mg. inorganic P in 2 ml. extract before and after hydrolysis in $N/2$ HCl. Suffixes denote time of hydrolysis in min.; P_t , total phosphate.

No.	P_0	P_7-P_0	$P_{30}-P_7$	$P_{180}-P_7$	P_t-P_{180}
19	0.29	0	0.08	0.06	0.19
20	0.17	0.10	-0.04	0.03	0.21

Glucose as substrate. After incubation of glucose with ATP as coenzyme there was no decrease in inorganic phosphate. In one experiment (Table 10, [1]) it hardly changed in amount, in another (Table 10, [2]) it increased. In most experiments in which AA was used instead of ATP there was some decrease in inorganic phosphate, but this decrease was accounted for by gain in ATP, that is in the fraction hydrolysed in 7 min. (Table 10, [6] and [7]). In no case was any accumulation of phosphoric ester with a longer hydrolysis time observed.

Table 10. *Rate of hydrolysis of acid-soluble phosphate esters before and after incubation*

2 ml. fresh extract, substrate, phosphate (0.01 *M*), MgCl_2 (0.004 *M*) and other additions as stated, incubated for 30 min. at 37° in 0.03 *M* NaHCO_3 , final volume 4 ml. Concentration of NaF, 0.02 *M*; of IAA, *M*/2000. Values represent mg. inorganic P before and after hydrolysis in *N*/2 HCl for 2 ml. extract plus additions. Suffixes denote time of hydrolysis, P_t = total phosphate.

No.		P_0	P_7-P_0	$P_{30}-P_7$	$P_{180}-P_{30}$	P_t-P_{180}	Lactic acid mg.
Extract 19 + 4 mg. Ca-ATP							
	Before incubation	1.59	0.29	0.09	0.17	0.21	—
1	After incubation + 8 mg. glucose	1.58	0.24	0.11	0.13	0.22	7.01
Extract 20 + 4 mg. Ca-ATP							
	Before incubation	1.46	0.42	-0.01	0.11	0.30	—
2	After incubation + 8 mg. glucose	1.82	0.15	0	0.15	0.31	7.87
3	After incubation + 8 mg. glucose + NaF	1.41	0.10	0.03	0.01	0.94	0.08
4	After incubation + 8 mg. pyruvate + NaF	1.44	0.15	-0.02	0.06	0.79	1.75
5	After incubation + 8 mg. pyruvate + 8 mg. glucose + NaF	0.39	0.18	0.07	0.18	1.58	3.96
Extract 19 + 2 mg. AA							
	Before incubation	1.50	0	0.12	0.11	0.25	—
6	After incubation + 8 mg. glucose	1.32	0.22	0.02	0.26	0.05	7.67
Extract 20 + 2 mg. AA							
	Before incubation	1.45	0.10	-0.03	0.10	0.30	—
7	After incubation + 8 mg. glucose	1.53	0.19	0	0.30	-0.05	8.01
8	After incubation + 8 mg. glucose + NaF	1.51	0.08	0.11	-0.11	0.45	0.03
9	After incubation + 8 mg. pyruvate + NaF	1.10	0.14	-0.06	0.11	0.71	1.62
10	After incubation + 8 mg. pyruvate + 8 mg. glucose + NaF	0.43	0.13	-0.01	0.13	1.29	3.62
Extract 11 + 4 mg. Ca-ATP							
	Before incubation	2.00	0.28	0.50	0.40	0	—
11	After incubation + 8 mg. glucose + IAA	2.32	0.04	0.30	0.34	0	0.06
Extract 20 + 4 mg. Ca-ATP + hexosediphosphate ($\equiv$ 6.3 mg. hexose)							
	Before incubation	1.52	1.20	0.31	0.95	0.36	—
12	After incubation	3.38	0.36	0.06	0.04	0.74	5.12
13	After incubation + 8 mg. glucose	2.04	0.86	0.40	0.30	0.76	8.86
14	After incubation + 8 mg. pyruvate + NaF	1.24	0.62	0.32	0.35	2.00	4.95
15	After incubation + 8 mg. pyruvate + 8 mg. glucose + NaF	0.50	0.83	0.48	0.59	2.02	5.22
Extract 19 + 2 mg. AA + hexosediphosphate ($\equiv$ 6.8 mg. hexose)							
	Before incubation	1.64	0.91	0.58	0.98	0.21	—
16	After incubation	2.83	0.51	0.23	0.10	0.48	4.80

See note to Table 4.

Hexosediphosphate as substrate. After incubation of the extract with hexosediphosphate there was a large increase in inorganic phosphate and an increase in difficultly hydrolysable ester, i.e. ester not hydrolysed in 180 min. (Table 10, [12] and [16]). The amount of lactic acid formed was approximately that calculated as equivalent to the increase in inorganic phosphate.

Hexosediphosphate plus glucose as substrate. When a mixture of approximately equivalent amounts of glucose and hexosediphosphate was incubated with extract (Table 10, [13]), the yield of lactic acid (8.86 mg.) was a little greater than that from the same amount of glucose alone (7.87 mg.), but not nearly so great as the sum of the amounts formed when the two substrates were incubated separately (7.87 + 5.12 mg., Table 10). About one-third of the hexosediphosphate remained unchanged after incubation and some ester largely hydrolysed in 30 min. was formed. Inorganic phosphate increased and it was not possible to

decide whether the added glucose was esterified and the lactic acid derived entirely from hexosediphosphate or whether the glucose was converted into lactic acid directly. However, the results indicate that the same enzymes are concerned in the conversion of both glucose and hexosediphosphate into lactic acid, for if there were two entirely different mechanisms operating one might expect that the total lactic acid formed from the mixture would be equal to the sum of the amounts formed from each substrate separately.

Glucose as substrate in the presence of NaF. When glucose was incubated with NaF-poisoned extract no lactic acid was formed, added ATP disappeared and there was a considerable formation of difficultly hydrolysable ester (Table 10, [3] and [8]).

Incubation of glucose with pyruvate in the presence of NaF. It has been suggested that in yeast extract and in blood corpuscles glucose is phosphorylated concurrently with oxidation of triosephosphate ester. In a system poisoned with NaF, any phosphoglycerate which may be formed cannot be converted into pyruvic acid, which acts as acceptor for hydrogen during oxidation of triosephosphate; therefore, the formation of lactic acid is inhibited. If a similar oxidation-reduction system is present in retinal extract, a fluoride-poisoned extract should be able to form lactic acid from glucose provided that pyruvate is also added, and at the same time there should be an increase in phosphoric ester. This was found to be the case (Table 10, [5] and [10]). The esterified phosphate was found in the fraction not hydrolysed in 180 min. The increase in phosphate ester was balanced by a decrease in inorganic phosphate and ATP phosphate, if ATP were used as coenzyme; if AA were used all of the esterified phosphate was derived from inorganic phosphate.

Incubation of pyruvate alone in the presence of NaF. When pyruvate without glucose was incubated with fluoride-poisoned extract there was a considerable formation of lactic acid and some increase in difficultly hydrolysable phosphate ester (Table 10, [4] and [9]). As in the presence of glucose, inorganic phosphate was esterified if AA was used as coenzyme, but phosphate was transferred from ATP if it was present. The extracts used for these experiments were not dialysed and therefore probably contained some carbohydrate which could be esterified and possibly some other substrate which could be oxidized concurrently with reduction of pyruvate to lactate.

Incubation of hexosediphosphate with pyruvate in the presence of NaF. When hexosediphosphate was incubated with pyruvate in fluoride-poisoned extract

Table 11

Comparison of increase in phosphate ester unhydrolysed in 180 min. with that calculated from the lactic acid formation (on the assumption that 1 mol. of lactic acid is equivalent to 1 mol. of phosphate), in extract incubated with NaF and pyruvate. For experimental details see Table 10.

No. in Table 10	Substrate	Coenzyme	Decrease in inorganic phosphate mg.	Increase in ester- P (mg.)		Lactic acid formed mg.
				Found	Calc.	
4	—	Ca-ATP	0.02	0.49	0.60	1.75
9	—	AA	0.35	0.41	0.56	1.62
5	Glucose	Ca-ATP	1.07	1.28	1.36	3.96
10	„	AA	1.02	0.99	1.25	3.62
14	Hexosediphos- phate	Ca-ATP	0.28	1.64	1.70	4.95
15	Glucose + hexose- diphosphate	Ca-ATP	1.02	1.66	1.80	5.22

(Table 10, [14]) it was not dephosphorylated but was converted into an ester not hydrolysed in 180 min. The increase in this fraction was approximately equivalent to the lactic acid formed (Table 11). At the same time some inorganic phosphate was esterified.

Incubation of glucose plus hexosediphosphate with pyruvate in the presence of NaF. If glucose, hexosediphosphate and pyruvate were incubated together in fluoride-poisoned extract (Table 10, [15]) there was a decrease in inorganic phosphate, but the increase in difficultly hydrolysable ester greatly exceeded the decrease in phosphate; the amount formed was almost the same as when hexosediphosphate and pyruvate were incubated alone under the same conditions.

Incubation of extract poisoned with IAA. When glucose was incubated with extract poisoned with IAA there was no esterification of phosphate or transference of phosphate from ATP (Table 10, [11]).

DISCUSSION

According to the present conception of the mechanism of anaerobic carbohydrate breakdown the three most important steps are: (1) phosphorylation, leading to formation of triosephosphate; (2) oxidation of triosephosphate to phosphoglycerate with concurrent reduction of either a molecule of pyruvate or another molecule of triosephosphate; (3) dehydration and dephosphorylation of phosphoglycerate to form pyruvate, which, after the induction period, supplies the main reductant in the second step.

The results reported in this paper support the hypothesis that this system is present in retinal extract. Not only are the same activators and coenzymes essential but fluoride inhibition of glycolysis can be reversed by addition of pyruvate, lactic acid formation then being accompanied by formation of a difficultly hydrolysable phosphoric ester.

The initial step in the process, phosphorylation of carbohydrate, may occur in three ways, by direct phosphorolysis, by phosphorylation coupled with either oxidoreduction or with dephosphorylation of phosphopyruvate. So far as is known the first method is applicable only to polysaccharides, when phosphate is added directly to the molecule as the glucosidic linkages are opened. The second and third methods may result in phosphorylation of glucose, glycogen, hexosemonophosphate or creatine, and the necessary enzyme systems have been found in various tissue extracts.

The inability to detect phosphoric esters during glucose breakdown does not necessarily mean that such esters are not formed; for only if the rate of formation is greater than the rate of breakdown will a measurable quantity of phosphoric esters accumulate. When the extract was poisoned with NaF, although there was no decrease in inorganic phosphate, there was an increase in difficultly hydrolysable phosphoric ester at the expense of ATP phosphate. If pyruvate was added together with glucose in fluoride-poisoned extract there was a large decrease in inorganic phosphate and a corresponding increase in difficultly hydrolysable ester. This can be explained on the assumption that pyruvate is reduced to lactate while phosphoric ester is oxidized to the difficultly hydrolysable phosphoglycerate and at the same time inorganic phosphate is esterified. If the esterified phosphate remained as hexosemonophosphate one would expect that for every molecule of lactic acid formed two molecules of phosphate would appear as difficultly hydrolysable ester. In fact not quite one molecule accumulated per molecule of lactic acid formed (Table 11); therefore, we conclude that the difficultly hydrolysable ester is phosphoglycerate, formed by the oxidation

of the phosphoric ester first formed. Further evidence that phosphorylation of glucose is accompanied by oxido-reduction is provided by the experiment on an extract poisoned with IAA (known to inhibit the oxido-reduction system), in which no phosphorylation at all occurred.

In the presence of fluoride and pyruvate lactic acid formation from glucose was only about half as great as in unpoisoned extract, suggesting that normally glucose esterification is accomplished by an additional route, namely, by transference of phosphate from phosphopyruvate. Increase in phosphate concentration may be expected to aid phosphorylation until a point is reached at which further increase inhibits dephosphorylation of phosphopyruvate [see Lennerstrand, 1936], so that the finding of an optimum phosphate concentration is explained.

Since glycolysis took place in dialysed extract on addition of coenzyme, $MgCl_2$ and phosphate, some oxidizable or phosphorylated substrate must still have been present. Our extracts were not shaken or stirred during dialysis, so that it is quite possible that traces of substrate, especially if they were associated with protein, would not be removed.

In fresh extract addition of both glucose and hexosediphosphate resulted in a slightly larger formation of lactic acid than addition of glucose alone, probably because glycolysis proceeded at the maximal rate from the beginning of incubation. This experiment affords strong evidence for supposing that the same system is concerned in breakdown of both glucose and hexosediphosphate, for if the two substrates are attacked by entirely different enzymes the amount of lactic acid formed when both are incubated together should approximate to the sum of that formed from the two incubated separately.

The results reported here do not settle the question of which form of nucleotide is active in retinal extract. For oxido-reduction in brain extract Meyerhof [1938] found that only adenosinediphosphate was active; in retinal extract adenosinediphosphate may well be formed from either ATP or AA. ATP was more active than AA in promoting glycolysis in low phosphate concentrations, but when the phosphate concentration was optimal, AA gave better results. If phosphate were split off from ATP the actual phosphate concentration of the extract would be increased, so that if the initial concentration were low the glycolytic rate would be increased, but at higher concentrations the optimum might be exceeded.

When AA was used as the coenzyme there was always a decrease in inorganic phosphate and formation of ATP (or adenosinediphosphate). If this phosphorylation is independent of glycolysis, then the difference in behaviour of extracts nos. 8 and 13 (Table 6) may be explained by supposing that the phosphatase was destroyed by the higher temperature (18°) reached during dialysis of extract no. 13, so that the active form of the nucleotide could not be formed from added AA. Süllman & Vos [1939] found that AA could no longer activate their extracts after dialysis for 24 hr.

Lactic acid formation from hexosediphosphate was less than from glucose, and was accompanied by formation of difficultly hydrolysable ester; the reason for this may be that dephosphorylation is disturbed by lack of a phosphate acceptor. ATP can activate the breakdown of hexosediphosphate in dialysed extract; therefore, either it is dephosphorylated to provide an acceptor, or oxido-reduction can proceed at a reduced rate without concurrent phosphorylation. Furthermore, when hexosediphosphate was incubated with pyruvate in fluoride-poisoned extract both lactic acid and difficultly hydrolysable ester formation greatly exceeded esterification of inorganic phosphate (Table 11). This suggests that, either oxido-reduction takes place without concurrent phosphorylation or

the phosphorylated product is immediately dephosphorylated. The activating effect of Mg^{++} on hexosediphosphate breakdown is to be expected as Lohmann [1933] has shown that Mg is necessary for its dephosphorylation.

The slow rate of glycogenolysis may be due to lack of the enzymes necessary for the initial phosphorolysis, since Süllman & Vos [1939] found that glucose-1-phosphate is attacked nearly as readily as glucose by retinal extract. Increase in phosphate concentration would be expected to aid phosphorolysis, and the small activating effect of Mg^{++} may be due to the fact that it is not essential for this first step in breakdown of glycogen [Cori *et al.* 1938].

The results of Süllman & Vos [1939] on glycolysis in retinal extract are in general agreement with those reported here, and they also support the hypothesis that carbohydrate breakdown proceeds according to the Meyerhof-Embden scheme. These authors used AA as coenzyme in all their experiments but found that it was necessary to add phosphate, some of which was esterified during incubation. They report that many of their extracts were active without addition of coenzyme; none of our preparations which were tested in this way showed any activity unless ATP or AA were added. Probably, in our preparation, nucleotide originally present in the cells was destroyed by a nucleotidase extracted along with the other enzymes; a similar explanation would account for the destruction of added ATP (Table 1). Reis [1934] and Gulland & Jackson [1938] have found a very active nucleotidase in retina. Süllman & Vos did not freeze the retinae during preparation of their extracts, so that extraction of cell contents may have been less complete and in some cases insufficient nucleotidase extracted to destroy all the nucleotide before incubation. The same explanation, namely, incomplete extraction, would account for their finding that addition of cozymase and AA increased glycolytic activity more than addition of AA alone.

It is difficult to make a direct comparison between the activities of our extracts and those of Süllman & Vos, because in their work the conditions, particularly of time of incubation, were not standard throughout. If lactic acid production had almost ceased after 30 min. incubation, as in our experiments, then their extracts were, with one exception, rather less active than ours; on the other hand, if lactic acid production continued during the whole of their incubation periods, then their extracts were very much less active.

SUMMARY

1. An aqueous extract of ox retina which on addition of ATP or AA converted added glucose, glycogen or hexosediphosphate into lactic acid was prepared.

2. The amount of lactic acid formed from glucose was increased by the addition of phosphate and $MgCl_2$; the yield from glycogen was increased by phosphate, but not by $MgCl_2$; and the yield from hexosediphosphate was increased by $MgCl_2$, but not by phosphate.

3. After the extract had been dialysed for 20 hr. no lactic acid was formed from glucose unless phosphate and $MgCl_2$, as well as ATP, were added; with glycogen as substrate it was necessary to add phosphate; and with hexosediphosphate $MgCl_2$ without phosphate produced activity.

4. Adenylic acid could replace ATP as coenzyme, but with glucose as substrate the amount of lactic acid formed was less; if, however, phosphate was also added, a higher yield of lactic acid was obtained with AA. With hexosediphosphate AA was always more effective.

5. Cozymase could not take the place of ATP or AA as coenzyme. When added in addition to ATP it did not increase glycolysis in fresh or in 20 hr.-dialysed extract, but with extracts which had been dialysed for 48 hr. the addition of cozymase in the presence of ATP increased the yield of lactic acid from glucose.

6. Glycolysis of all three substrates was inhibited by addition of IAA, NaF or glyceraldehyde. In fluoride-poisoned extract, addition of pyruvate caused formation of lactic acid.

7. In unpoisoned extract incubated with glucose no evidence of the formation of carbohydrate-phosphoric esters could be obtained. When AA was used as coenzyme there was a transference of inorganic phosphate to an easily hydrolysed ester, probably ATP. When hexosediphosphate was used as substrate it was almost all broken down, there was a large increase in inorganic phosphate and some formation of a difficultly hydrolysable ester.

8. In fluoride-poisoned extract there was some formation of difficultly hydrolysable ester when glucose was used as substrate; when pyruvate was added in addition to glucose this formation of difficultly hydrolysable ester was greatly increased and was approximately equivalent to the amount of lactic acid formed, and to the decrease in inorganic phosphate.

9. Incubation of glucose and hexosediphosphate together yielded only a slightly larger amount of lactic acid than incubation of glucose alone.

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