CCVIII. THE HEXOSEDIPHOSPHATE METABOLISM OF NORMAL TISSUE EXTRACTS

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THE production of lactic acid from hexosediphosphate and hexosemonophosphate by extracts of muscle and tumour tissue, at an optimum temperature of 50—55°, has been demonstrated by Scharles *et al.* [1935]. Using extracts of various transplantable mouse tumours with hexosediphosphate as substrate Mawson [1936] confirmed these results and found that prolonged dialysis of the extracts considerably reduced their glycolytic activity. The activity was restored, however, by addition of glutathione and the suggestion was made that the lactic acid was formed by means of methylglyoxalase. This hypothesis was supported by the observation that the yield of lactic acid was greatly reduced by the addition of extracts of dried kidney, which are known to contain an antiglyoxalase [Woodward *et al.* 1935]. Since little lactic acid was formed by tumour extracts at 38° even in the presence of glutathione it was concluded that the conversion of hexosediphosphate into methylglyoxal could only take place at higher temperatures, and it was thought unlikely that this part of the reaction was of an enzymic character.

The investigation of the conversion of hexosediphosphate into lactic acid by tissue extracts at high temperatures has been carried out chiefly with malignant tissue and it seemed desirable that information should be obtained on the activity of normal tissue extracts under similar conditions. The present paper records an investigation of the hexosediphosphate metabolism at high temperatures of extracts of blood, brain, kidney, liver, muscle, spleen and testis of mice and rats.

EXPERIMENTAL

Fresh tissues from albino mice and rats have been used throughout. The animals were normal and were fed on the stock laboratory diet. Extracts were made by grinding the minced tissues at about 20° with Ringer solution in the proportion (unless otherwise stated) of 2 g. of tissue to 5 ml. of Ringer. The brei was ground through a fine steel-wire sieve and centrifuged twice for 15 min. at 3500 r.p.m. Dialysis was carried out in thin collodion sacs ($4 \times \frac{1}{2}$ in.) either in Ringer solution at 5° or in running tap water. In the latter case the dialysed extract was brought back to normal concentration by addition of Ringer solution of ten times the normal strength. Corrections for changes of volume were applied in calculating the results, which have been expressed as yield per g. of tissue. Unless otherwise stated incubations have been carried out in stoppered tubes ($3\frac{1}{2} \times \frac{3}{4}$ in.) in the presence of air.

Lactic acid has been estimated by a slight modification of the method of Lohmann [1928] and alkali-labile phosphoric esters by the method of Boyland & Boyland [1935]. The hexosediphosphate used was a pure specimen of the calcium salt (Fraenkel and Landau). Calcium was removed from the HCl solution of this substance by centrifuging after neutralization with Na_2CO_3 and addition of NaOH. The solution was brought back to pH 7.3, usually after addition of phosphate buffer, by addition of HCl.

Normal tissues and glucose

Rat liver extracts. Freshly made rat liver extracts, prepared as above, contain about 0.76 mg. glutathione per ml. (mean of 11 experiments). When incubated with an equal volume of glucose solution containing phosphate buffer there is a very small yield of lactic acid at 38° (Table I) which may be somewhat increased by incubation at 52°. Addition of glutathione, raising the GSH concentration to about 1.2 mg. per ml., sometimes causes a further slight increase in the yield of lactic acid at 52°. The results obtained with fresh liver extract and glucose were variable and inconsistent, but although the amounts of lactic acid obtained were usually small they seem significant when compared with the uniformly negative results obtained with tumour extracts [Mawson, 1936].

When dialysed extracts of mouse and rat liver were used they proved to have practically no glucolytic activity at 38 or 52°, whether glutathione was added or not, and this also applied to extracts kept in glass tubes in the dialysis bath for the same length of time.

Brain extracts. Extracts of rat and mouse brain showed hardly any glucolytic activity under any conditions examined. Most other workers have obtained similar results, although Geiger [1935] and Euler *et al.* [1936] have obtained active extracts under special conditions. It was noticeable that there was nearly always an apparent loss of lactic acid at 38° but as this occurred in dialysed extracts containing no detectable lactic acid the decrease was evidently due to a small change in the blank titre, which has not been further investigated. The results at 52° were variable, but in all cases the increases or decreases of apparent lactic acid were so small as to be hardly significant.

Normal tissues and hexosediphosphate

Fresh liver extracts. Small yields of lactic acid were obtained when fresh rat liver extract was incubated with hexosediphosphate at 38° (Table I). A considerable increase was obtained at 52°, but addition of glutathione at this

Table I. *Fresh rat liver extract. Glycolysis with hexosediphosphate and glucose at 38 and 52°*

1 ml. extract. 1 ml. Hdp (6 mg. free acid) or 1 ml. glucose (6 mg.) containing phosphate pH 7.3 (M/15). 1 mg. glutathione (GSH). Incubated 1 hr.

mg. lactic acid per g. tissue per hr.					
Hexosediphosphate			Glucose		
38°	52°	52° + GSH	38°	52°	52° + GSH
0.07	0.83	0.55	0.16	0.01	0.15
0.08	1.08	0.08	0.12	0.31	0.97
0.68	1.31	0.89	-0.28	0.07	0.39
0.81	1.35	1.29	-0.18	1.14	0.88

temperature gave a smaller yield than that obtained in its absence. The presence of excessive amounts of glutathione seems to inhibit glycolysis, and this is in accordance with a similar effect noticeable in the case of fresh tumour extracts. In this connexion Geiger [1935] mentions that a reduction in the rate of glycolysis occurs in presence of oxidized glutathione.

Dialysed liver extracts. When liver extracts are thoroughly dialysed the glutathione content is reduced to a very low level. Under these conditions

Table II. *Addition of glutathione to dialysed and undialysed liver extracts with hexosediphosphate as substrate*1 ml. extract. 1 ml. Hdp, containing phosphate pH 7.3 (*M*/15). Incubated 1 hr.

Hours dialysed	Temp. 0° C.	Dialysed					Undialysed				
		GSH mg. per ml.	mg. lactic acid per g. tissue per hr.			GSH mg. per ml.	mg. lactic acid per g. tissue per hr.				
			38°	52°	52° + GSH 1 mg.		38°	52°	52° + GSH 1 mg.		
Rat:											
20	5	0.037	0.18	0.90	1.45	0.063	0.45	1.66	1.30		
18	5	0.005	0.12	1.07	2.33	0.148	0.00	2.32	2.22		
18	5	0.012	0.64	1.45	1.93	0.210	0.26	1.82	1.72		
23	15	0.003	0.00	0.56	2.16	0.077	0.04	1.22	2.75		
21	16	0.000	0.16	0.22	2.07	0.161	0.02	1.34	1.71		
Mouse:											
22	16	0.000	0.00	1.00	1.74	0.039	0.28	0.91	1.07		
17	12	0.010	—	1.64	1.95	0.276	0.29	1.20	0.98		
17	11	0.000	0.00	1.27	1.38	0.082	0.14	0.50	0.41

Fig. 1. Rate of formation of lactic acid from hexosediphosphate by rat liver extract at 52° in presence and absence of glutathione. A, with added glutathione; GSH at 0 hr. = 0.68 mg. per ml., GSH at 5.5 hr. = 0.14 mg. per ml. B, without added glutathione.

(Table II) the amount of lactic acid produced by rat liver extracts from hexose-diphosphate at 38° is small. A larger yield is obtained at 52°, which is greatly increased by addition of glutathione. Extracts kept in the dialysis bath in glass tubes lose a proportion of their glutathione but sufficient remains to permit the formation of a considerable amount of lactic acid without addition of coenzyme; when additional glutathione is introduced a marked increase in lactic acid yields occurs.

These points are clear in the case of rat liver extracts, but Table II suggests that the glutathione effect is not so significant in mouse liver extracts. The course of lactic acid production over an extended period was therefore compared, using rat and mouse liver extracts. Fig. 1 shows that in the case of dialysed rat liver extracts with added glutathione, incubated at 52°, glycolysis follows a linear path for about 4 hr. Thereafter the rate gradually falls off until after 6 hr. it has become very small. If no glutathione is added glycolysis occurs at a lower rate from the beginning and ceases after 2 hr.

Fig. 2. Lactic acid production of mouse liver and hexosediphosphate in presence and absence of glutathione. Temperature 52°. A, added glutathione; GSH at 0 hr. = 0.68 mg. per ml., GSH at 6 hr. = 0.27 mg. per ml. B, without added glutathione.

Mouse liver extracts (Fig. 2) give curves of a slightly different character. The rate of glycolysis of the dialysed extracts with hexosediphosphate at 52° is about the same in presence or absence of glutathione for nearly 1 hr., and this accounts for the very small increase in the glycolytic activity of these extracts on addition of coenzyme when, as in Table II, the incubation period is only 1 hr. If the curve is followed further, however, the rate of glycolysis of the extracts containing glutathione is maintained, but that of the untreated extracts rapidly falls to a very low level. If the curves are taken as a whole, therefore, mouse and rat liver extracts are found to behave in the same way.

Brain extracts. The production of lactic acid from hexosediphosphate by rat and mouse brain extracts after various periods of dialysis is shown in Table III. After short periods of dialysis there is a small glycolytic activity at 38 and 52° which disappears after prolonged dialysis. Although not shown in the table,

Table III. *Brain extracts and hexosediphosphate*

1 ml. extract. 1 ml. Hdp (6 mg. free acid) containing phosphate ($M/15$, pH 7.3). 1 mg. GSH. Incubated 1 hr.

Hours dialysed	Temp. 0° C.	GSH mg. per ml.	mg. lactic acid per g. tissue		
			38°	52°	52° + GSH
Rat:					
1½	16.0	0.021	0.41	1.37	3.80
2½	15.4	0.007	0.16	0.11	3.77
19½	11.5	0.000	-0.04	0.02	2.00
22	14.0	—	0.16	-0.13	2.02
24	10.5	—	0.08	-0.07	2.79
Mouse:					
2½	15.0	0.000	0.61	0.60	3.31
17	11.5	0.003	-0.04	-0.09	2.44

addition of glutathione has no influence on lactic acid production at 38° either in brain extracts or in those of other tissues examined. At 52° addition of the coenzyme raises the glycolytic activity to a point which is usually higher than that of liver extracts. Except in the case of rat brain dialysed for 1½ hr., where there was a residual glutathione concentration of 0.021 mg. per ml., the amount of lactic acid obtained at 52° without glutathione was very small as compared with that obtained with liver extracts. There is some evidence in Table III that the activity of brain extracts decreases during dialysis independently of the glutathione content.

Kidney extracts. Since addition of extracts of dried kidney inhibits the hexosediphosphate metabolism of tumour, it was of interest to investigate the glycolytic activity of fresh kidney. Table IV shows the results obtained with fresh and

Table IV. *Kidney extract and hexosediphosphate*

1 ml. extract. 1 ml. Hdp (6 mg.). 1 ml. phosphate ($M/10$, pH 7.3). 1 mg. GSH.

Treatment of extract	Strength of extract	Incubation time hr.	mg. lactic acid per g. tissue per hr.			
			38°	38° + GSH	52°	52° + GSH
Mouse:						
Fresh	2 : 7	2	0.16	0.06	- 0.04	0.33
Rat:						
Fresh	2 : 12	1.33	- 0.09	0.12	- 0.07	0.12
Dialysed 18 hr. at 10.5°	2 : 12	2	0.00	0.26	0.21	0.34
Dialysed 18 hr. at 10.5°	2 : 7	2	0.10	0.11	0.06	0.25
Dialysed 19 hr. at 11.5°	2 : 7	1	0.13	—	0.31	0.48

dialysed extracts of mouse and rat kidney. At 38°, both in presence and absence of added glutathione, the yields of lactic acid were small and irregular, the results being very similar to those obtained with brain. Addition of glutathione at 52° caused an increase of glycolytic activity, but the level reached was only about 1/5 of that observed with liver extracts. It seems very likely, therefore, that although the enzyme system under consideration is present in extracts of kidney, its activity is largely masked by the presence of the antglyoxalase described by Woodward *et al.* [1935].

Other normal tissue extracts. Similar experiments have been performed with blood, muscle, spleen and testis, and the results are reported in Table V. The extract of blood was made by using freshly clotted blood and extracting the

Table V. *Dialysed extracts of various tissues with hexosediphosphate*

1 ml. extract. 1 ml. Hdp (6 mg. free acid) containing phosphate ($M/15$ pH 7.3). 1 mg. GSH. Incubated 2 hr.

Tissue	Dilution of extract	mg. lactic acid per g. tissue per hr.			
		38°	38° + GSH	52°	52° + GSH
Rat blood	1 : 5.6	0.01	0.17	0.00	0.17
Rat blood with glucose	1 : 5.6	0.03	0.06	0.20	0.06
Rat muscle	1 : 3.5	0.05	0.19	0.06	1.37
Rat muscle with glycogen	1 : 3.5	0.01	0.03	-0.03	-0.01
Mouse spleen	1 : 3.5	0.29	0.26	0.34	1.28
Rat testis	1 : 6	—	—	0.09	2.76

minced clot in a manner analogous to that used for other tissues. No appreciable glycolytic activity was found in the blood extracts with hexosediphosphate or glucose as substrate after 24 hr. dialysis, even at 52° and after addition of glutathione. This is of interest as it shows that the activity of other tissues is not influenced by their blood content and it also emphasizes the fact that the activity of the other tissue extracts must be due to a true enzyme system.

An experiment with dialysed mouse spleen extract showed slight but definite glycolysis with hexosediphosphate at 38°, unaffected by glutathione. At 52° the activity was about the same and was considerably increased by addition of glutathione, which also raised the activity of testis extract at 52° to a level higher than that of liver.

In the case of dialysed muscle extracts no lactic acid was produced from hexosediphosphate at 38 or 52° in absence of glutathione. Slight glycolysis occurred at 38° on addition of glutathione but at 52° addition of the coenzyme caused a considerable production of lactic acid. With glycogen as substrate, muscle extract gave no lactic acid under any conditions. It should be mentioned in this connexion that the method of preparation of all the extracts described in this paper is not that which is best calculated to produce active glycolytic enzyme solutions in the usual sense of that term. The technique adopted was designed to make the results comparable with those described by Mawson [1936] using tumour extracts. This point must be borne in mind when comparing the glycolytic activity at 38° with that observed by other workers using extracts prepared under different conditions.

Notes on mechanism of reaction

Optimum temperature. Experiments with normal tissue extracts have shown that the optimum temperature for production of lactic acid from hexosediphosphate is about 55°. In an experiment with rat liver slices and hexosediphosphate a similar optimum temperature was observed. The slices were washed three times with Ringer solution and glutathione was added.

Optimum glutathione concentration. It will be recalled that some lactic acid is produced by liver extracts and hexosediphosphate at 52° even when the extracts have been thoroughly dialysed and no glutathione has been added (Table II, Figs. 1 and 2). This glycolytic activity might be due to traces of residual glutathione, or to a second enzyme system quite independent of this substance. In the former case the yield of lactic acid would rise steeply at once

with progressive small additions of glutathione. In the latter case, however, the glutathione concentration of the system could be increased up to a certain point before any increase in glycolytic activity occurred. Thereafter further additions of the coenzyme should cause a sharp rise in the lactic acid production. Fig. 3 shows that up to a concentration of 0.027 mg. glutathione per ml. addition

Fig. 3. Rate of production of lactic acid by rat liver extract and hexosediphosphate in presence of varying amounts of glutathione. Temperature 52°.

of the coenzyme has no effect on the glycolytic activity and therefore the lactic acid production of extracts containing less than 0.027 mg. glutathione per ml. cannot be dependent upon glyoxalase activity.

Changes in alkali-labile phosphate. Although it seems likely that methylglyoxal is the immediate precursor of lactic acid in the reaction at 52° in presence of glutathione, nothing is known of the mechanism of production of the methylglyoxal. Boyland & Boyland [1935] have shown that tumour tissue is an excellent source of zymohexase, which catalyses the formation of alkali-labile phosphoric esters (probably mainly dihydroxyacetonephosphate) from hexosediphosphate, and it was of interest to find whether such compounds were the source of methylglyoxal in the reaction under investigation. The results of an experiment to show the changes in alkali-labile phosphate concentration in presence and absence of glutathione in citrate buffer solution are shown in Table VI (1). The phosphate changes were identical whether glutathione was present or not. The production of alkali-labile phosphate from hexosediphosphate was complete

Table VI. *Phosphate changes with rat liver extract at 52°.*

(1) *Citrate buffer.* Extract dialysed 16 hr. at 10·5° in running tap water. 1·5 ml. extract. 1·5 ml. *M*/10 sodium citrate pH 7·3. 1·5 ml. Hdp (4·5 mg.). 0·1 ml. GSH (1 mg.) or 0·1 ml. Ringer. Incubated in air.

Time incubated (min.) ...	No GSH				0·217 mg. GSH per ml.			
	10	20	30	60	10	20	30	60
	mg. per g. tissue							
Alkali-labile P	0·66	0·62	0·66	0·61	0·65	0·62	0·63	0·59
Inorganic P	0·09	0·12	0·19	0·25	0·07	0·12	0·14	0·26
Lactic acid	—	—	—	0·37	—	—	—	0·71

(2) *Bicarbonate buffer.* Extract dialysed 18 hr. at 10·5° in running tap water. 2·0 ml. extract. 2·0 ml. *M*/5 sodium bicarbonate pH 7·3. 2·0 ml. Hdp (6 mg.). 0·2 ml. GSH (2 mg.). Incubated in 94·5% N₂, 5·5% CO₂.

Time incubated (min.) ...	0	5	10	30	60
	mg. per g. tissue				
Alkali-labile P	0·01	1·27	1·30	1·24	1·04
Inorganic P	0·03	0·11	0·11	0·32	0·54
Lactic acid	—	—	—	—	1·42

within 10 min. and in both cases the amount remained fairly constant during 1 hr. Lactic acid production in presence of glutathione was 0·71 mg. per g. tissue, which is approximately equivalent to the increase found in the inorganic phosphate concentration. In absence of glutathione, although the lactic acid yield was only half that obtained in presence of the coenzyme, the production of inorganic phosphate was the same. The significance of this observation will be referred to in the discussion.

In the experiments with citrate buffer very large blanks were obtained in the lactic acid estimations. Control experiments showed that the estimations were nevertheless quite reliable, but in order to ensure that the citrate had no specific influence on the results an experiment is recorded in Table VI (2) in which bicarbonate buffer (pH 7·3) was used in presence of N₂ (94·5%) and CO₂ (5·5%). Similar results were obtained, and it was again found that the rapid production of alkali-labile phosphate was not followed by the sharp fall which would have been expected had these compounds been the source of the lactic acid produced.

Nature of intermediate compounds. It has just been shown that when dialysed liver extract is incubated at 52° with hexosediphosphate an amount of inorganic phosphate is liberated which is equivalent to the lactic acid which would have been produced if glutathione had been present. Scharles *et al.* [1935] have stated that under such conditions no methylglyoxal is produced by tumour tissue extracts and it was thought desirable to confirm that none was produced by liver extracts in absence of glutathione. The methylglyoxalase of liver is very active [Jowett & Quastel, 1933] and heat-stable [Lohmann, 1932] and in a control experiment it was found that in the presence of glutathione the rate of lactic acid production from methylglyoxal was exceedingly high and was practically the same at 20, 38, 51 and 67°. Only a slight maximum could be detected at about 50°, which was, however, much more sharply defined at about 55° in dialysed extract containing only traces of the coenzyme. If dialysed liver extract and hexosediphosphate were heated together at 52° and complete formation of methylglyoxal took place, addition of glutathione and continuation of incubation at 38° should give the same amount of lactic acid as that obtained by incubation of extract + substrate + coenzyme at 52°.

The results of an experiment of this nature are set out in Table VII. Extract, substrate, and extract+substrate were each heated for 30 min. at 50·5° in absence of glutathione. The tubes were then cooled and glutathione added to

Table VII. *Enzyme and substrate heated before addition of glutathione*

Preliminary heating at 50·5° for 30 min. Then 1 ml. extract, 1 ml. Hdp, 1 ml. phosphate (*M*/10, pH 7·3), 0·1 ml. GSH (1 mg.), incubated 2 hr.

	Hdp preheated alone	Extract preheated alone	Extract + Hdp preheated	No pre-heating		
	38°	38°	38°	38°	50·5°	50·5°
Incubated at GSH added	+	+	+	-	-	+
mg. lactic acid per g. tissue per hr.	0·04	0·06	0·19	0·08	0·39	0·96

each. Incubation was then resumed, this time at 38°, and continued for 2 hr. During this time the whole of any preformed methylglyoxal would have been converted into lactic acid. At the same time extract+substrate which had not been "preheated", were incubated at 50·5 and 38°, and extract+substrate with glutathione at 50·5°, for 2 hr. In the latter case a yield of 0·96 mg. lactic acid per g. liver per hr. was obtained. The yield at 50·5° without glutathione was 0·39 mg. per g. per hr. No significant amount of lactic acid was produced by extract or substrate alone, but incubation of "preheated" extract+substrate+glutathione at 38° gave a yield of 0·19 mg. lactic acid per g. per hr. It is evident from Table VII that this 0·19 mg. is the amount of lactic acid which was formed during the period of "preheating" at 50·5°, so we can say that no lactic acid whatever was produced during the 2 hr. incubation at 38°. It can therefore be concluded that no methylglyoxal is produced by incubation of liver extract and hexosediphosphate in absence of glutathione.

Heat-precipitation of extracts. When tissue extracts are heated at 50–55° for 15 min. or more a considerable amount of coagulation occurs. The experiments described in this paper have been done with the whole extract and it was always observed that after incubation the tubes contained a sediment of somewhat gelatinous appearance and a clear supernatant which, when the extracts contained a visible amount of haemoglobin, was red in the presence of glutathione and pale buff in its absence. The incubation tubes were not shaken in the bath and it was important to determine whether the activity of the enzyme remained in the supernatant or was wholly or partially associated with the unevenly distributed sediment. The results of an experiment designed to investigate this point are given in Table VIII. Rat liver extract was heated for 30 min. at 51° and the coagulated material separated on the centrifuge. The deposit was

Table VIII. *Activity of coagulated fraction of heated rat liver extract*

Extract heated 30 min. at 51° and centrifuged. Deposit washed twice with Ringer. 1 ml. enzyme. 1 ml. Hdp (6 mg.). 1 ml. phosphate (*M*/10 pH 7·3). 0·1 ml. GSH (1 mg.). Incubated 2 hr. at 51°.

	Unheated extract	Unheated extract + GSH	Suspen- sion of deposit	Suspen- sion of deposit + GSH	Super- natant	Super- natant + GSH
mg. lactic acid per g. tissue per hr.	0·08	1·30	0·00	0·07	0·09	1·01
Mean wt. of protein pre- cipitate, mg.	32·1	—	17·5	—	9·8	—

washed twice with Ringer solution and made up to the original volume with Ringer. Supernatant and deposit were then put up with hexosediphosphate and glutathione, together with controls containing unheated extract. The supernatant had about 80 % of the activity of the unheated extract, but the deposit retained only about 5 % of that activity. During the course of an ordinary incubation, therefore, practically the whole of the glycolytic activity must remain in solution. Table VIII also shows that although a certain amount of protein was lost during the washing of the deposit, roughly 2/3 of the protein content of the extract was removed as inactive material by heating for 30 min. at 51°. No further attempt has been made to purify the enzyme, but removal of heat-coagulable protein should be a useful first step in this direction.

Effect of dilution on activity of enzyme. Kendal & Stickland [1937] have observed that the yield of lactic acid from starch in muscle extracts in the presence of boiled muscle extract, stated as mg. per ml. extract used, increases with increasing dilution of the extract. A similar phenomenon occurs with liver extracts and hexosediphosphate at high temperatures, and the same thing has also been found with tumour extracts [Mawson, unpublished]. Two experiments are illustrated in Fig. 4 which show a very marked rise of activity on dilution of

Fig. 4. Effect of dilution on activity of enzyme. Rat liver extract with hexosediphosphate and GSH at 52°.

the extracts. It is worth stating that in the highest dilution used (1 ml. extract + 15 ml. Ringer solution—1 ml. mixture used as enzyme) the titre after subtracting the appropriate blank was 1.75 ml. *N*/500 iodine, representing 0.175 mg. lactic acid. The experimental error of the titration is about ± 0.05 ml. *N*/500 iodine.

Specificity of glutathione. In order to test the specificity of glutathione as coenzyme, an experiment was done in which it was replaced by cysteine. It will

Table IX. *Coenzyme effect of cysteine*

Rat liver extract dialysed 40 hr. at 10-5° in running tap water. 1 ml. extract. 1 ml. Hdp. 1 ml. phosphate (*M*/10, *pH* 7-3). 0-1 ml. GSH or cysteine hydrochloride (cyst.). Incubated 2 hr.

mg. lactic acid per g. tissue per hr.							
No addition		0-33 mg. GSH per ml.	0-33 mg. cyst per ml.	0-33 mg. GSH per ml.	0-33 mg. cyst per ml.	0-11 mg. cyst per ml.	0-04 mg. cyst per ml.
38°	55°	38°	38°	55°	55°	55°	55°
0-08	0-38	0-03	0-13	1-53	0-13	0-08	0-10

be seen from Table IX that cysteine had no coenzyme activity even in so high a concentration as 0-33 mg. per ml. although the effect of glutathione has been shown to be visible at all concentrations above 0-027 mg. per ml. The figures given in Table VII for lactic acid yields at 55° would even seem to suggest that cysteine has an inhibitory effect upon that part of the enzyme system which at this temperature does not depend upon the coenzyme effect of glutathione.

DISCUSSION

Many workers have found that tissue extracts do not produce lactic acid from glucose even when the phosphate concentration is suitable and large amounts of adenylypyrophosphoric acid or glutathione are added. In the present work, little glucolytic activity was observed with any of the extracts used, and addition of glutathione did not increase their activity. Tissue extracts for work on glycolysis are usually prepared at very low temperatures and the final extracts are obtained in the shortest possible time, but it has already been pointed out that these precautions were not necessary in this investigation. If, however, extracts had been prepared under more rigorous conditions it is unlikely that the results with glucose would have been very different. It has been clearly shown by Boyland *et al.* [1937] that the reason for the inactivity of tumour extracts is to be found in the very rapid destruction of cozymase and adenylypyrophosphate which takes place immediately damage occurs to the cells. Similar results have been obtained by Euler *et al.* [1936] using extracts of an acetone preparation of brain. It is possible that the same explanation might in some cases account for the failure of normal tissue extracts to form lactic acid from glucose. The production of lactic acid from hexosediphosphate at 52° does not appear to depend upon the presence of cozymase or adenylypyrophosphate. Extracts lose little of their activity during very prolonged dialysis providing that glutathione is added. They can be kept for a long time at room temperature without appreciable inactivation and during prolonged incubation at 50-55° their capacity to convert hexosediphosphate into lactic acid decreases remarkably slowly. The absolute yields of lactic acid obtained with the extracts was considerable, though no cozymase or adenylypyrophosphate was added in any of the experiments described in this paper.

Addition of glutathione did not increase the glycolytic activity at 38° of any of the tissue extracts used, though Geiger [1935] found that it would increase the activity of his brain and liver extracts. When a temperature of 50-55° was used with hexosediphosphate as substrate the amount of lactic acid obtained was considerable. Very little difference was found between the activity of extracts of the various tissues used except for those of kidney. In all cases the lactic acid production of extracts which had been subjected to prolonged dialysis

was comparatively low and was greatly increased by addition of glutathione. It has been shown that glutathione may be added to dialysed liver extract until a concentration of 0.027 mg. GSH per ml. is reached before a coenzyme effect begins to appear, and this indicates that in liver extract an enzyme system must exist which will produce lactic acid from hexosediphosphate at 50–55°, but which is unrelated to glutathione. It is considerably less thermostable than the glutathione-sensitive enzyme and it does not appear to exist in brain extract. It will be seen from Table III that except in the first experiment with brain extract the lactic acid yield at 52° without glutathione was never greater than that at 38°. The exception was due to the fact that in that case the dialysis was incomplete. The increase of glycolytic activity obtained on addition of glutathione at 50–55° is so striking that there can be no doubt of the reality of the phenomenon in all the tissues examined, with the exception of blood, which gave no significant amount of lactic acid under the conditions of the experiment.

Although the results were so definite with hexosediphosphate, it is noteworthy that even at 55° no lactic acid could be obtained from glucose. The glyoxalase activity of the extracts was so high at all temperatures examined that no satisfactory optimum temperature could be found when methylglyoxal was used as substrate, but results with dialysed extracts suggested that it was at about 55°. This would suggest that no hexosediphosphate was formed from glucose by the extracts used in these experiments.

Jowett & Quastel [1934] and Crabtree [1936] have stated that the glyoxalase system in tissue slices decreases in activity very rapidly at 38°, although Lohmann [1932] claimed that glyoxalase is very stable to heat. It has been shown that lactic acid production from hexosediphosphate at high temperatures is effected by a very stable system and the question therefore arose as to whether methylglyoxal was really produced at any stage in the reaction. Inoue [1931], using dialysed extracts of rabbit liver freed from glyoxalase by alcohol, obtained almost complete conversion of hexosediphosphate into methylglyoxal, and a 54% yield was observed by Neuberg *et al.* [1930] with extracts of an acetone preparation of rat sarcoma. In the present work no direct evidence has been found of methylglyoxal formation. This would not be surprising in the complete system where active glycolysis was taking place, because of the extraordinarily high activity of tissue glyoxalase [cf. Jowett & Quastel, 1933]. In the absence of glutathione, however, methylglyoxal might be expected to accumulate, but the fact that this does not occur is shown by the observation that addition of glutathione to a dialysed extract containing hexosediphosphate which has been incubated at 52°, and continuation of the incubation at 38°, does not lead to any production of lactic acid. Had methylglyoxal been formed during the preliminary treatment at 52° it would have been converted quantitatively into lactic acid during the subsequent incubation in presence of the coenzyme.

If methylglyoxal is formed during the reaction it must arise from a precursor whose breakdown is dependent upon the presence of glutathione at 50–55°. There is much indirect evidence in favour of this view. The antiglyoxalase of kidney prevents conversion of hexosediphosphate into lactic acid at high temperatures, and under these conditions the glycolytic activity of fresh extracts of whole kidneys is strikingly less than that of other tissues. In this connexion it is difficult to explain the observation of Jowett & Quastel [1934], who found that slices of rat and guinea-pig kidney had a higher glyoxalase activity than any other tissues examined. It has been noticed in the case of all tissues examined that addition of glutathione to fresh tissue extracts containing large amounts of natural glutathione often causes a reduction of their glycolytic activity at 50–55°.

and a parallel to this is to be found in the observation of Giršavičius & Cheifetz [1936], who state that beyond a certain optimum concentration, increase in glutathione content depresses the activity of glyoxalase preparations.

If the hypothesis of methylglyoxal formation is not accepted it must be assumed that glutathione can act as coenzyme in the conversion of some other substance, free from phosphate, into lactic acid. The effect must be relatively specific since cysteine has been shown to have no coenzyme activity. The precursor must be free from phosphate since the same amount of inorganic phosphate is formed whether glutathione is present or not, and the inorganic phosphate produced is equivalent to the amount of lactic acid formed in presence of coenzyme. It has been shown that alkali-labile phosphate is not directly concerned in the reaction and can therefore be excluded in this connexion. It has not yet been possible to identify the precursor, but it is interesting that Lawson [1936] has postulated a non-phosphate precursor in the glycolysis of kidney at 38°. It may be possible, by the use of cozymase, to establish a connexion between the non-phosphate glycolytic systems of Ashford [1934] and Lawson and the precursor which, at 50–55°, is the intermediate compound between hexosediphosphate and methylglyoxal.

SUMMARY

1. Dialysed normal tissue extracts prepared at 20° do not convert glucose into lactic acid either at 38 or 50–55° in the presence or absence of glutathione.

2. With the exception of blood, which has no glycolytic activity under these conditions, all the dialysed normal tissue extracts examined form small amounts of lactic acid from hexosediphosphate at 50–55°, and addition of glutathione at this temperature causes a large and sustained increase of glycolytic activity. Little lactic acid is formed from hexosediphosphate at 38° and the yield is not increased by addition of glutathione.

3. The coenzyme effect of glutathione does not commence until a concentration of 0.027 mg. per ml. is reached. Below this concentration glycolysis is dependent upon a comparatively thermo-labile system which is not influenced by glutathione.

4. The activity of tissue extracts which convert hexosediphosphate into lactic acid at 50–55° in presence of glutathione increases greatly on dilution of the enzyme.

5. Formation of alkali-labile phosphoric esters from hexosediphosphate is very rapid at 52° in liver extract containing citrate or bicarbonate buffer. Addition of glutathione causes no change in the alkali-labile phosphoric ester concentration.

6. The same amount of inorganic phosphate is formed from hexosediphosphate by liver extract at 52° in presence or absence of glutathione.

7. No methylglyoxal is formed by liver extract from hexosediphosphate at 50.5° in absence of glutathione.

8. Almost the whole of the enzymic activity remains in the uncoagulated portion of tissue extracts heated for 30 min. at 51°.

9. Cysteine cannot replace glutathione as coenzyme for the conversion of hexosediphosphate into lactic acid at 50–55°.

10. The significance of these results is discussed.

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