CLXXXVIII. STUDIES IN TISSUE METABOLISM.
IV. A COMPARISON OF MUSCLE AND TUMOUR GLYCOLYSIS.

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Since the work of Warburg [1930] on tumour metabolism it has been known that malignant tissue will produce lactic acid at a relatively high rate under either aerobic or anaerobic conditions. Warburg stated that tumour tissues can produce an amount of lactic acid equal to their own weight in ten hours, but this statement has been questioned by Downes [1929]. Chemical estimations indicate that slices of Jensen rat sarcoma will produce about 10 to 15 mg. lactic acid per g. (wet weight) per hour under anaerobic conditions at 37°. Owing to the rapid deterioration of the glycolytic power of minced muscle at this temperature, comparison is difficult, but Boyland [1931] found that minced ox-muscle at 20° formed 3 to 4 mg. lactic acid per g. of tissue per hour.

The mechanism of lactic acid formation in muscle resembles, according to Meyerhof [1930; 1933], the alcoholic fermentation of yeast in the fact that phosphorus compounds form an essential step in the breakdown of the sugar, and he places the glycolysis of erythrocytes in the same class [Meyerhof, 1932]. When materials so dissimilar as yeast, erythrocytes and muscle have similar glycolytic systems, it might be expected that tumour would behave in the same way, but to test this satisfactorily is not an easy matter. Owing to the limited permeability to phosphorus compounds of cell membranes [*cf.* Eggleton, 1933] it is essential that cell-free extracts, or some other non-cellular preparation, should be used.

Barr *et al.* [1928] found that the glycolytic activity of tumour tissue was very easily destroyed by any form of manipulation such as grinding, freezing or extraction. They conclude that there is no evidence for the formation of carbohydrate-phosphate compounds in cancer, and in this opinion they are supported by Harrison and Mellanby [1930]. These authors consider that tumour glycolysis differs fundamentally from that of non-malignant tissues in that it does not involve the reaction of phosphorus compounds.

Downes [1929] found that rat tumours would produce lactic acid from hexose-phosphate at about half the rate of glycolysis of glucose, and an indication that phosphate was at any rate to some extent involved in tumour metabolism was given by Franks [1932], who showed that the increase in free phosphate observed on incubation of mouse tumour C 180 was considerably reduced in the presence of glucose, while the acid-soluble organic phosphate at the same time increased. However, about twenty molecules of lactic acid were formed for each molecule of phosphate esterified, while under similar conditions in muscle three molecules of lactic acid were formed for each molecule of hexose ester produced.

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The presence of hexosephosphate and adenylpyrophosphate in tumour tissue was indicated by the work of Boyland [1932] and Franks [1932], and this suggests that the glycolysis of muscle and tumour are similar. Tumour glycolysis, like that of muscle, is arrested by pancreatic extract, fluoride and iodoacetate. It is known that these substances interfere with the phosphate metabolism.

Glycolytic enzymes from rat muscle.

In order to compare the enzymes of muscle and tumour it was advisable to obtain these tissues from the same animal. Meyerhof [1930] has described the preparation of actively glycolysing cell-free extracts from the muscle of rabbits and frogs (in which experimental tumours were not available), but states that such extracts cannot be prepared from rat-muscle. We found, however, that the following procedure gave active extracts from rat-muscle.

Freshly excised muscle was rapidly chopped with scissors in its own weight of ice-cold water contained in a mortar cooled to about $-12°$. After about five minutes' rapid chopping one-tenth of the muscle's weight of sand was added and the whole thoroughly pounded until, after about ten minutes, it had solidified to a pasty mass. This material was centrifuged until it had completely thawed (usually about 15 minutes) and the clear supernatant liquid poured off through muslin. Occasionally a thixotropic gel was formed at this stage, but such gels were quite as active as the liquid extracts. After an experiment with such an extract the proteins were precipitated with 4 % trichloroacetic acid and lactic acid was estimated by the method described by Lohmann [1928].

Extracts from rat-muscle formed lactic acid from glycogen, hexosephosphate and α-glycerophosphate + pyruvate. (We are indebted to Dr F. L. Pyman for the pure sodium α-glycerophosphate.) Dialysis destroyed their glycolytic activity, which was restored on addition of adenylpyrophosphate and magnesium. No lactic acid was obtained from β-glycerophosphate in any circumstances. Results of experiments using rat-muscle extracts are given in Table I. The optimum concentrations of both phosphate and hydrogen ions for the formation of lactic

Table I. *Lactic acid formation in rat-muscle extract.*

(1) Dialysed extract.

Substrate	Glycogen	Glycogen + adenylpyro-phosphate + $MgCl_2$	Hexose-phosphate	Hexose-phosphate + adenylpyro-phosphate + $MgCl_2$
mg. lactic acid formed in 2 hours by 2 ml. extract	0·16	3·46	0·19	1·35

(2) Undialysed extract.

Substrate	Glycogen	Pyruvate	α-Glycero-phosphate	Pyruvate + α-glycero-phosphate
mg. lactic acid formed in 2 hours by 2 ml. extract	4·59	0·68	1·19	4·13

Table II. *Optimum* p_H *of lactic acid formation in rat-muscle extract.*

1·5 ml. extract, incubated with glycogen, adenylpyrophosphate + Mg and phosphate buffer.

Exp. 1.							
p_H of solution	6·0	6·8	6·9	7·2	7·5	8·0	
mg. lactic acid formed	2·34	2·88	2·92	3·32	3·72	2·26	
Exp. 2.							
p_H of solution	5·7	6·1	6·8	7·3	8·0	8·9	9·3
mg. lactic acid formed	0·37	0·46	1·23	1·77	1·76	0·96	0·23

acid from either glycogen or α-glycerophosphate + pyruvate were determined. The optimum p_H was found to be about 7·5 (Table II) while at this p_H the optimum concentration of phosphate was approximately 0·03 *M* (Table III). The hydrogen ion concentration was determined colorimetrically immediately after all additions had been made.

Table III. *Optimum phosphate concentration for lactic acid formation in rat-muscle extract at* p_H *7·5.*

Exp. 1.					
PO_4 concentration	*M*/3000	*M*/1000	*M*/300	*M*/100	*M*/30
mg. lactic acid formed	0·12	0·11	0·46	2·66	3·69
Exp. 2.					
PO_4 concentration	*M*/64	*M*/32	*M*/16		
mg. lactic acid formed	2·40	3·40	3·13		

Comparison of muscle and tumour glycolysis.

A study was made of the effect of changing p_H and phosphate concentration on tumour glycolysis. The presence or absence of phosphate in the medium had no effect on the glycolysis even if the tissue had been soaked in a large volume of oxygenated Ringer solution overnight and thoroughly washed next morning. This may be due to the fact that the tumour cell is impermeable to phosphate or contains large stores of available phosphate in the form of nucleic acids and phospholipins.

No sharply defined optimum p_H could be determined for tumour tissue, but it appeared to be about p_H 8 (Table IV).

Table IV. *Optimum* p_H *for glycolysis of J.R.S. slices in phosphate and bicarbonate.*

With bicarbonate buffer (*M*/50).					
p_H	8·9	8·4	7·6	7·1	6·5
mg. lactic acid formed	2·57	2·82	2·51	2·66	1·81
With phosphate buffer (*M*/30).					
p_H	8·7	7·3	6·8	6·3	5·3
mg. lactic acid formed	2·38	2·38	1·78	0·92	0·49

Tumour slices formed lactic acid from hexosemonophosphate and from α-glycerophosphate + pyruvate, but to a smaller extent than from glucose (Table V). Addition of pyruvate did not appreciably increase the yield of lactic

Table V. *Glycolysis of J.R.S. slices with glucose, hexosemonophosphate and pyruvate + α-glycerophosphate, shaken in Warburg apparatus in presence of* $N_2 + 5\ \%\ CO_2$ *at 37°.*

	Glucose		Hexose-monophosphate		Pyruvate + α-glycero-phosphate	
Substrate						
Time (mins.)	45	90	45	90	45	90
mg. lactic acid formed	0·85	2·16	0·14	0·37	0·17	0·30

acid from hexosemonophosphate with either muscle extract or tumour slices. In order to investigate muscle and tumour under approximately similar conditions, comparisons of glycolytic power were made using the minced tissues. Results are given in Table VI, which show that an appreciable yield of lactic acid can be obtained from α-glycerophosphate + pyruvate with minced tumour, but this yield is not very much greater than that from pyruvate alone unless adenyl-

Table VI. *Glycolysis of minced tumour and rat-muscle, shaken in Warburg apparatus in presence of $N_2+5\%$ CO_2.*

mg. lactic acid from 0·5 g. tissue + 3 ml. additions in two hours at 37°.

	Substrate				
Tissue	Pyruvate	Pyruvate + α-glycero-phosphate	Pyruvate + α-glycero-phosphate + adenylpyro-phosphate + Mg	Glucose (or glycogen with muscle)	Glucose + adenylpyro-phosphate + Mg
1. J.R.S.	0·33	0·53	0·63	2·45	—
2. J.R.S.	0·40	0·68	0·97	3·19	3·04
3. J.R.S.	0·65	0·99	1·09	2·79	—
4. L.R. 10	0·69	0·86	1·33	2·78	3·09
5. L.R. 10	0·34	0·62	0·99	2·82	3·06
6. Muscle	0·47	1·62	—	3·35	—
7. Muscle	0·47	2·15	—	5·16	—

The tumour L.R. 10 (no. 5 above) gave with hexosemonophosphate 0·30 mg. lactic acid, and with hexosemonophosphate + adenylpyrophosphate + Mg, 0·49 mg. L.R. 10 is a rat sarcoma, produced originally by injection of 1:2:5:6-dibenzanthracene [Burrows *et al.*, 1932].

pyrophosphate is added. If we consider the figures as a percentage of the maximum glycolysis (*i.e.* with glucose in the case of tumour and with glycogen in the case of muscle) it will be seen that the yield of lactic acid from tumour with α-glycerophosphate and pyruvate is comparable with the yield from muscle with the same compounds (Table VII).

Table VII. *Glycolysis of minced tumour and minced rat-muscle, shaken in Warburg apparatus in presence of $N_2+5\%$ CO_2.*

Mean glycolysis as % of glycolysis with glucose (or glycogen for muscle).

	Substrate		
Tissue	Pyruvate	Pyruvate + α-glycerophosphate	Pyruvate + α-glycerophosphate + adenylpyro-phosphate + Mg
J.R.S.	16	28	32
L.R. 10	18	26	41
Muscle	11	44	—

Smythe and Gerischer [1933] have shown that the glyceraldehydephosphate which was synthesised by Fischer and Baer [1932] is rapidly fermented by yeast preparations. Through the generosity of Prof. H. Fischer of Basel and Dr Feldmann of Berlin, who gave us some calcium glyceraldehydephosphate, we have been able to observe the lactic acid formation from this substance by tumour slices and rat erythrocytes (Table VII). The amount of lactic acid formed in this way by tumour tissue is less than half that formed from glucose; it is therefore attacked at about the same rate as hexosephosphate or glycerophosphate + pyruvate. Rat erythrocytes also formed some lactic acid from glyceraldehydephosphate under the same conditions.

Attempts to prepare cell-free extracts from tumours.

Warburg [1930] and Barr *et al.* [1928] have attempted, without success, to obtain a glycolytic enzyme from tumour tissue, but as these experiments were done before the nature of the muscle coenzyme was known it was thought that

the addition of adenylpyrophosphate + Mg to tumour press juice might give an active cell-free extract: no success was attained by this method. While it was possible repeatedly to freeze rat-muscle extracts without loss of activity, tumour tissue suffered severe reduction in its glycolytic power if frozen once. Inactive extracts were obtained by grinding minced tumour or tumour slices with water, saline, boiled muscle extract, various antiprotease preparations, glucose solution, fluoride and alkaline phosphate solutions.

Effect of haemolytic reagents.

It seems probable that tumour does not attack glycogen (a large molecule), hexosephosphate and α-glycerophosphate (by analogy with sodium phosphate) because these substances do not penetrate the cell membrane, and so do not come into contact with the cell enzymes. In an attempt to increase the amount of diffusion into the cells various substances were added to tumour slices which should increase their permeability. Neither saponin, digitonin, taurocholate, oleate, ricinoleate nor choline appreciably increased the yield of lactic acid from α-glycerophosphate + pyruvate with tumour slices, though in some cases taurocholate and choline slightly increased the yield from glucose.

Experiments with acetone slices.

Harden and Young [Harden, 1932] were able to show the effect of phosphate on fermentation not only in yeast juice, which corresponds to muscle extract, but also in dried yeast and zymin. Meyerhof [1930] has described the preparation of an acetone precipitate from muscle extract having 40 % of the activity of the original extract. Using a similar precipitate from muscle extract, we obtained 1·91 mg. of lactic acid from glycogen and 1·74 mg. from α-glycerophosphate + pyruvate, whereas an equivalent amount of the original extract had given 5·63 mg. of lactic acid from glycogen. Analogous preparations were therefore made from tumour material.

Slices of good Jensen rat sarcoma (J.R.S.) tissue (0·25 mm. thick) were dried over P_2O_5, but had very little glycolytic activity even with the muscle coenzyme. Preparations with acetone, however, gave better results. Thin slices of J.R.S. or mouse sarcoma L.M. 154 were soaked in alkaline Ringer-bicarbonate solution (p_H 8·7) for 15 minutes, the Ringer drained away and the slices dropped into about 100 volumes of acetone at 37°. The acetone was rapidly stirred and decanted after two minutes. A.R. acetone at 37° (dried over anhydrous Na_2SO_4) was then added and left at this temperature for 15 minutes. After pouring off the acetone, the slices were rapidly drained and dried overnight *in vacuo* over $CaCl_2$. The dried material (9–12 % of the original wet weight) was white and became swollen in water so as to resemble the original tissue. Although such preparations were sometimes inactive, acetone slices have been obtained having up to about one-tenth of the glycolytic activity of the original tissue.

0·3 g. of dried acetone J.R.S. slices was soaked in 3 ml. rat Ringer solution and finely minced. 0·3 ml. of the emulsion so formed was injected into each of ten rats. In no case was there any growth of tumour tissue even after 3 months.

The acetone slices contained some lactic acid precursor, for on incubation alone some lactic acid was usually obtained, but an increased amount was produced on incubation with glucose or pyruvate + α-glycerophosphate. If the preparation was washed with $M/100$ phosphate buffer (p_H 7·4) containing toluene, and then repeatedly with toluene water, the lactic acid precursor could be removed.

Table VIII. *Lactic acid formation from glyceraldehydephosphate by tumour and erythrocytes.*

Lactic acid formed in 2 hours while shaking at 37° in Warburg apparatus.

	Substrate	mg. lactic acid
100 mg. J.R.S.	None	0·10
	Glucose (4 mg.)	1·25
	Calcium glyceraldehydephosphate (4 mg.)	0·53
20 mg. Rat erythrocytes	None	0·02
	Calcium glyceraldehydephosphate (4 mg.)	0·36
	Theoretical lactic acid formation from 4 mg. calcium glyceraldehydephosphate	1·48

Table IX. *Lactic acid formation by acetone-treated tumour slices incubated in N_2+5 % CO_2 at 37°.*

mg. lactic acid per 100 mg. dry weight per hour.

			Substrate			
Exp.	Tumour	Incubation time (mins.)	Glucose	Glucose + adenylpyrophosphate + $MgCl_2$	Sodium glycerophosphate + pyruvate	Sodium glycerophosphate + pyruvate + adenylpyrophosphate + $MgCl_2$
1	L.M. 154*	90	0·47	0·62	0·42	—
2	J.R.S.	150	0·35	0·34	—	—
3	J.R.S.	180	0·49	0·61	0·10	0·12
4	J.R.S.	180	0·53	0·55	0·15	0·64
5	J.R.S.	180	0·05	0·32	0·03	0·02
6	J.R.S.	180	—	0·13	—	0·09
7	L.M. 154	180	—	0·53	—	0·28
8	J.R.S.	180	0·18	0·36	0·36	0·42
9	J.R.S.	180	0·07	0·13	0·33	0·36
10	J.R.S.	15	1·05	—	0·43	—
		30	0·86	—	0·28	—
		45	0·62	—	0·23	—
11	J.R.S.	15	—	0·64	—	0·48
		30	—	0·58	—	0·56
		60	0·29	0·50	0·38	0·44
12	J.R.S.	15	—	1·08	—	0·20
		33	—	0·68	—	0·22
		56	0·85	0·73	0·19	0·23
13	J.R.S.†	30	0·11	—	0·08	0·13
	washed	70	0·10	0·07	0·10	0·13
14	J.R.S.	45	—	0·30	—	0·36
		90	—	0·23	—	0·23
15	J.R.S.	45	—	0·85	—	0·90
16	J.R.S.	45	—	0·56	—	0·40
		90	—	0·36	—	0·32
17	J.R.S.‡	60	—	0·20	—	0·19
	washed	120	0·09	0·52	—	0·34

* L.M. 154 refers to a grafted tumour produced in mice by the injection of 5:6-*cyclo*-penteno-1:2-benzanthracene [Barry and Cook, 1934].

† The media used for this experiment contained toluene and gave a vigorous growth of *B. coli* on incubation with broth containing toluene.

‡ Samples of the media used were taken at the end of incubation and found to be sterile.

The lactic acid formation of this non-living preparation of tumour tissue was small compared with that of the original tissue. In order to show that this glycolysis was not due to bacterial contamination, experiments under sterile conditions were required. Even after taking great precautions to ensure sterility by aseptic methods alone, incubation of the media at the end of the experiments showed that bacteria were present. The bacteria did not appear to be affected by saturation with thymol or with toluene. Eventually sterility was attained by working under aseptic conditions and adding 0·1 % phenol to the media. The sterile experiment (17 of Table IX) which was carried out on washed material gave a larger yield of lactic acid from glucose in the presence of coenzyme than did many experiments which showed contamination with either *Staphylococcus albus* or *B. coli*.

The foregoing experiments indicate the difficulty of obtaining a cell-free glycolysing extract which could be compared with muscle extract. The most active of the acetone slice preparations had less than one-tenth of the original activity. This remaining activity involves the same chemical mechanism as that of muscle. It may be dangerous to argue from this to the total glycolysis, since Ashford [1933] has suggested that glucose breakdown in brain may follow two paths, one involving phosphorylation and the other not. However, no glycolysis without phosphorylation has yet been clearly demonstrated. It is also possible that some of the residual activity remaining after treatment with acetone is due to red blood corpuscles which remain in the tissue.

Summary.

1. Actively glycolysing extracts have been prepared from rat-muscle which give lactic acid with glycogen, hexosemonophosphate and α-glycerophosphate + pyruvate.

2. The optimum p_H for glycolysis in rat-muscle extract is about 7·5, and the optimum phosphate concentration about $M/30$.

3. The glycolysis of tumour tissue is unaffected by the presence or absence of phosphate. The optimum p_H is about 8·0.

4. A little lactic acid was formed from hexosemonophosphate and from α-glycerophosphate + pyruvate by tumour slices, but the amount is very much smaller than that formed by muscle extract from the same substances.

5. It was not found possible to obtain glycolytically active extracts from tumour tissue or to increase its metabolism of phosphorus compounds by the addition of haemolytic reagents.

6. A preparation made from tumour slices with acetone is described which possesses glycolytic activity; the yield of lactic acid from α-glycerophosphate + pyruvate is of the same order as that from glucose.

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