CCXXVI. HEXOSEPHOSPHATE METABOLISM OF TUMOUR EXTRACTS

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The investigation of the glycolytic mechanism of tumour tissue would be simplified if it were possible to obtain cell-free tissue extracts with properties of glycolysis analogous to those of the muscle extracts first described by Meyerhof. Barr *et al.* [1928], Warburg [1930], Boyland & Mawson [1934] and others all found, however, that injury to malignant tissue was accompanied by reduction of its power of glycolysis and that cell-free extracts were practically inactive. The substrate used in these experiments has generally been glucose, but equal lack of success has attended most attempts to obtain lactic acid from hexosephosphates by the use of tumour slices or of preparations subjected to various degrees of cell destruction. Downes [1929] observed that rat tumours would produce lactic acid from hexosephosphate at about half the rate at which they produced it from glucose, but Boyland & Mawson [1934], using hexosemonophosphate and Jensen rat sarcoma slices, obtained lactic acid at only about 1/6 the rate of formation from glucose. Results of this kind have led to a belief that the series of events in tumour glycolysis is entirely different from that observed in muscle, and doubt exists that phosphorus compounds play any part in tumour metabolism.

Boyland & Boyland [1935, 1] obtained extracts from mouse sarcomas which, after dialysis for one hour, would produce small amounts of lactic acid from hexosediphosphate, but on addition of adenylpyrophosphate the yield was greatly increased until it was about 1/4 of the amount produced by tissue slices in glucose. They suggested that previous failures to demonstrate glycolytic activity in tumour extracts might be due to rapid destruction of the coenzyme by the powerful nucleosidase known to occur in tumour tissue [Boyland & Boyland, 1935, 2]. It is noteworthy that none of the extracts used by these authors would produce lactic acid from glucose.

Scharles *et al.* [1935, 1, 2] have also described an extract of mouse sarcoma which would produce lactic acid from hexose-diphosphate and -monophosphate, but not from glycogen or glucose, but it differed from that of Boyland & Boyland in the fact that its activity did not depend on the presence of adenylpyrophosphate. The maximum rate of production of lactic acid occurred at a temperature of 50–55° and its activity was not affected by dialysis at 10° for 36 hours. The present paper is concerned with an investigation of the properties of extracts similar to those described by Scharles *et al.*

Experimental.

Transplantable mouse tumours have been used which have been recorded in the tables as follows:

C = carcinoma C 63. E = Ehrlich carcinoma. L = sarcoma L.M. 154. S = Vienna sarcoma.

The sarcoma L.M. 154 was originally produced by Barry & Cook [1934] by injection of 5:6-cyclopenteno-1:2-benzanthracene.

Extracts and suspensions have been made with non-necrotic material in sterile Ringer solution, and incubations have been carried out in presence of $M/30$ phosphate, the pH after additions being 7·0–7·5. Incubations were done in presence of toluene in stoppered $3\frac{1}{2} \times \frac{3}{4}$ in. tubes, and protein was precipitated with trichloroacetic acid.

Lactic acid was estimated by the method of Lohmann [1928] and alkaline precipitation of carbohydrate was avoided [Boyland & Boyland, 1935, 1]. Solutions were always distilled for 10 min. before the condensers were fitted in order to drive off volatile products. In order to prevent errors due to the fading which results from the presence of carbohydrate, the end-point used was persistence of blue colour for 30 sec. instead of 60 sec., and excess of $N/10$ iodine was added during the preliminary titration and removed with a drop of bisulphite. Yields of lactic acid have been expressed throughout as mg. lactic acid produced in one hour by an amount of extract or suspension derived from 1 g. of tissue.

Lactic acid from tumour cell suspensions.

Preliminary experiments with minced tumour tissue and with suspensions of tumour cells made by grinding the minced tissue with Ringer solution and filtering through a fine wire mesh, showed that lactic acid was produced from hexosediphosphate in considerable amount at an optimum temperature of 50–55°. A series of experiments was then carried out with cell suspensions to see whether the addition of glutathione would influence the yield of lactic acid at 38° or 52°, using glucose and hexosediphosphate as substrates. The results are given in Table I. (It should be noted that the mean values appearing in all

Table I. *Influence of glutathione on lactic acid production of tumour cell suspensions.*

1·0 ml. suspension, 1·2 ml. additions, 6·0 mg. glucose or hexosediphosphate, 1 mg. glutathione. Incubated 1 hr.

mg. lactic acid per g. tissue per hr.

	38°		52°		38°		52°	
Tumour	Glucose	Glucose + GSH	Glucose	Glucose + GSH	Hdp	Hdp + GSH	Hdp	Hdp + GSH
E	0·00	0·01	0·00	0·19	0·42	0·75	1·94	2·43
S*	0·02	0·00	0·00	0·00	0·30	0·06	0·27	2·07
S	0·11	0·00	0·00	0·01	0·12	0·07	0·31	0·85
S	0·13	0·04	0·00	0·00	0·15	0·13	0·60	0·71
S	0·00	0·04	0·00	0·15	0·00	0·13	0·41	0·64
Mean	0·05	0·02	0·00	0·07	0·20	0·23	0·71	1·34

* 2·5 g. tumour. 11·5 ml. Ringer. Incubated 2 hours. GSH = glutathione. Hdp = hexosediphosphate.

the tables are given only for convenience in comparison of results and are not necessarily significant in themselves.) Very little lactic acid arises from glucose at 38° or 52°, and addition of glutathione does not increase this yield. The small yield of lactic acid from hexosediphosphate at 38° is not increased by addition of glutathione, but at 52° there is a considerably greater yield of lactic acid which is definitely increased by addition of glutathione. The variation in activity of the cell suspensions made the results statistically unsatisfactory, so the work was repeated using cell-free extracts and hexosediphosphate. It was not considered necessary to repeat the experiments using glucose as substrate.

Lactic acid from tumour extracts.

Extracts were prepared at 20–25° by mincing 4 g. tumour, grinding into a fine brei and adding 10 ml. of Ringer solution. The mixture was very thoroughly ground and centrifuged twice for 10 min. at 3500 r.p.m. Such extracts usually

contained about 0·07 mg. glutathione per ml. Table II shows that at 38° addition of glutathione caused an apparent decrease in the yield of lactic acid, but the amounts determined were too small for confidence to be placed in the significance of this observation. At 52°, however, the amount of lactic acid produced after addition of glutathione was always greater than with hexosediphosphate and extract alone. These results suggested that glutathione was acting as coenzyme in the reaction, and the hypothesis was adopted that a glyoxalase was involved in the system.

Table II. *Influence of glutathione on lactic acid production of tumour extracts.*

1·0 ml. extract. 1·2 ml. additions. 6·0 mg. hexosediphosphate. 1·0 mg. glutathione. Incubated 1 hour.

mg. lactic acid per g. tissue per hr.

	38°		52°	
Tumour	Hexosediphosphate	Hexosediphosphate + GSH	Hexosediphosphate	Hexosediphosphate + GSH
S	0·36	0·11	0·78	0·99
E	0·24	0·03	1·03	1·51
E	0·61	0·50	1·52	2·36
E	0·00	0·00	0·88	1·78
E	0·27	0·00	0·55	1·17
Mean	0·26	0·13	0·95	1·56

Effects of "antiglyoxalase".

Woodward *et al.* [1935] have shown that rat kidney contains a substance which can act as a powerful antiglyoxalase, and experiments were therefore carried out to determine whether such a preparation would prevent the formation of lactic acid from hexosediphosphate at 52°. Mouse kidneys were cut into small pieces and dropped into 20 volumes of acetone, which was replaced after 10 min. by fresh acetone. After a further 10 min. the kidney was washed with ether and left to dry in the refrigerator overnight. It was then ground into a fine powder. Extracts of this material were made by grinding 100 mg. of powder with 1·0 ml. water and centrifuging twice. It was found (Table III) that addition

Table III. *Influence of kidney antiglyoxalase on lactic acid production of tumour extracts.*

1·0 ml. extract. 1·2 ml. additions. 6·0 mg. hexosediphosphate. 0·2 ml. antiglyoxalase. Incubated 1 hr. at 52°.

mg. lactic acid per g. tissue per hr.

Tumour	Hexosediphosphate	Hexosediphosphate + antiglyoxalase
C	1·03	0·16
E	1·07	0·40
L	1·60	0·49
C	0·36	0·00
C	0·98	0·43
E	1·12	0·23
Mean	1·03	0·29

of this antiglyoxalase preparation caused a considerable decrease in the amount of lactic acid produced from hexosediphosphate and tumour extract at 52°. The yield obtained was, in fact, about what would have been expected at 38° in absence of antiglyoxalase (Table II).

Dialysed extracts.

Scharles *et al.* [1935, 1] stated that the glycolytic activity of their extracts was not reduced by dialysis in cellophane sacs for 36 hours at 10°. The results just described, however, pointed so definitely to the significance of glutathione that a series of dialysis experiments was carried out. Thin sacs were made of B.D.H. collodion "Special for preparing permeable membranes" in $4 \times \frac{1}{2}$ in. tubes. Dialysis was carried out in Ringer solution at 7°, 1·2 litres of Ringer being used for each ml. of extract. During dialysis a parallel sample of extract was kept at 7° in a glass tube and in each experiment dialysed and undialysed extracts were investigated simultaneously.

Contrary to the observation of Scharles *et al.* it was found (Table IV) that the glycolytic activity of the extract was very much reduced by dialysis. The undialysed extract, kept at the same temperature for the same length of time,

Table IV. *Influence of dialysis and addition of glutathione on lactic acid production of tumour extracts.*

1·0 ml. extract. 1·2 ml. additions. 6·0 mg. hexosediphosphate. Incubated 1 hr. at 52°.

mg. lactic acid per g. tissue per hr.

	Undialysed		Dialysed		
Tumour	Without GSH	With GSH	Without GSH	With GSH	Time of dialysis hr.
E	0·37	0·75 (1·3)*	0·09	0·65 (1·3)*	26
L	0·82	1·30 (1·5)	0·05	0·70 (1·5)	20
L	0·73	1·17 (2·0)	0·01	1·22 (2·0)	68
L	1·26	1·44 (3·0)	0·43	1·66 (3·0)	24
E	1·12	1·42 (3·0)	0·11	1·08 (3·0)	48
Mean	0·86	1·22	0·14	1·06	

* Figures in brackets represent mg. glutathione added.

retained much of its original glycolytic power. In one experiment, for example, the fresh extract produced 1·60 mg. lactic acid per g. tissue per hour and after keeping for 24 hr. at 7° the yield was 1·26 mg./g./hr. in spite of the fact that the extract had been diluted 14% to compensate for the liquid taken up by the dialysed extract. The latter only produced 0·43 mg./g./hr. The addition of glutathione to dialysed extracts always caused a large increase in their lactic acid production, and although this did not always reach the level attained by the undialysed extract plus glutathione there seems to be little doubt that the loss of glycolytic activity observed after dialysis is due to the disappearance of glutathione.

Discussion.

The normal source of lactic acid in the tumour cell is probably glucose. Tumour slices will produce lactic acid from glucose at a very high rate, but damage to the tissue cells destroys their glycolytic power to such an extent that suspensions of cells are quite inactive. If the loss of activity were strictly proportional to the degree of breakdown of cell structure this result would hardly be anticipated, for the suspensions used in this work (Table I) contained about 10^6 apparently undamaged cells per ml., and though they often gave no lactic acid at all with glucose they were capable of producing tumours. Boyland & Boyland [1935, 1] were able to obtain lactic acid from hexosediphosphate and tumour extracts on addition of adenylpyrophosphate, but no lactic acid could be obtained with glucose. If we assume that hexosediphosphate is a normal metabolic product of tumour, destruction of cells must damage the enzyme system

at some point before the production of hexosediphosphate as well as causing decomposition of adenylpyrophosphate, and in tumour suspensions this damage extends to cells which remain histologically intact. It has, however, been shown that adenylpyrophosphate is not necessary for the conversion of hexosediphosphate into lactic acid by tumour extracts, but for this reaction to take place in the absence of the muscle coenzyme it is necessary for the temperature to be comparatively high and glutathione must be present. Even under these conditions no lactic acid is produced from glucose.

Meyerhof [1933] has dismissed methylglyoxal as of no importance in muscle glycolysis. It has no place in the Embden-Meyerhof scheme and Lohmann [1932] has shown that lactic acid production by muscle extract can take place in absence of glutathione, the coenzyme of glyoxalase. However Gaddie & Stewart [1935] have reopened this question with the observation that addition of glutathione to dialysed muscle extract containing magnesium ions does lead to considerable lactic acid production from glycogen, and add the interesting observation that glutathione appears to stimulate the production of methylglyoxal as well as to aid in its conversion into lactic acid. It seems, in fact, that production of methylglyoxal from glycogen or glucose may be conditioned by the possibility of its rapid disappearance, for Toenniessen & Fischer [1926] found that methylglyoxal was produced from hexosediphosphate, but not from glucose or glycogen, in presence of muscle brei and pancreatic antiglyoxalase. In the case of tumour tissue no analogy can be found at 38° with the observations of Gaddie & Stewart, but at 52°, using hexosediphosphate as substrate, the resemblance is very close. It is known [Lohmann, 1932] that glyoxalase is very stable to heat and it may be that some change of a non-enzymic nature takes place at 52° as a stage between hexosediphosphate and methylglyoxal which is a necessary step in tumour metabolism but which can be achieved by an enzymic process at 38° with muscle extracts. It is difficult to imagine an enzyme of mammalian tissue which would be active at 52° and inactive at 38°.

The observation of Scharles *et al.* [1935, 1] that no methylglyoxal is detectable among the end-products of the action of tumour extracts on hexosediphosphate at 52° is doubtless due to the fact that it is decomposed as soon as it is formed, but it is more difficult to explain their failure to observe any decrease of activity in their extracts after dialysis. In the present work every dialysed extract has shown greatly decreased glycolytic power, which has invariably been increased on addition of glutathione. This observation, together with the fact that kidney antiglyoxalase reduces the production of lactic acid from hexosediphosphate at 52° to about the level observed at 38° in absence of added glutathione, leaves no doubt that the extra glycolysis observed at the higher temperature is due to a system, involving glutathione as coenzyme, which is probably a glyoxalase.

In a comparison of the glycolytic properties of muscle and tumour extracts the following points are of importance:

(1) Muscle extract will form lactic acid from carbohydrate or hexosephosphate in presence of adenylpyrophosphate at 38°, but tumour extract will only utilize hexosephosphate for this purpose.

(2) Muscle extract will form lactic acid from carbohydrate in the presence of glutathione and magnesium at 38°, but tumour will not utilize either carbohydrate or hexosediphosphate at this temperature in the absence of adenylpyrophosphate.

(3) Tumour extract will form lactic acid from hexosediphosphate in the absence of adenylpyrophosphate at 52°, but only in the presence of glutathione.

Two distinct differences can now be detected in the glycolytic system of tumour extract as compared with that of muscle. Firstly, hexosediphosphate cannot be produced from carbohydrate. If it could, lactic acid could be produced from glucose at 38° on addition of adenylpyrophosphate and also at 52° on addition of glutathione. Secondly, lactic acid cannot be produced by tumour extract at 38° from hexosediphosphate even on addition of glutathione. The latter defect can be remedied by addition of adenylpyrophosphate. It is unlikely that the destruction of this compound by nucleosidase is the whole reason for the difference because presumably muscle extract can form lactic acid from hexosediphosphate as well as from glycogen on addition of glutathione and magnesium. An enzyme capable of carrying out the reaction is certainly present in tumour extract because lactic acid is produced from hexosephosphate at 52° if glutathione is present, and the link in the chain which is absent seems to be the capacity to convert hexosediphosphate into methylglyoxal at 38°.

Summary.

1. Lactic acid is not produced from glucose by mouse tumour cell suspensions at 38° or 52°, with or without addition of glutathione.

2. Only small amounts of lactic acid are produced by tumour cell suspensions or extracts from hexosediphosphate at 38°, and these are not increased by addition of glutathione.

3. At 52° tumour cell suspensions and extracts produce considerable amounts of lactic acid from hexosediphosphate, and these yields are greatly increased by addition of glutathione.

4. Dialysed tumour extracts produce very little lactic acid from hexosediphosphate at 52°, but their glycolytic activity is restored by addition of glutathione.

5. The lactic acid production of tumour extracts at 52°, with hexosediphosphate as substrate, is greatly reduced by addition of kidney antiglyoxalase.

6. The contrast between the glycolytic systems of extracts of muscle and tumour is discussed.

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