CCXXV. STUDIES IN TISSUE METABOLISM.
VII. THE ACTION OF TUMOUR EXTRACTS ON HEXOSEDIPHOSPHATE.

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Warburg [1930], Case [1929] and Barr *et al.* [1928] have found that the active glycolysis of tumour tissue is destroyed by freezing and thawing. In the foregoing paper of this series [Boyland and Boyland, 1935] it was shown that extracts of frozen malignant tissue rapidly destroy adenylpyrophosphate—the coenzyme necessary for lactic acid formation in muscle extracts. We have found that tumour extracts convert hexosediphosphate into lactic acid, if relatively large amounts of adenylpyrophosphate are added in order to compensate for the destruction of this substance.

Meyerhof and Lohmann [1934, 1] have described an enzyme which causes reversible conversion of hexosediphosphate into dihydroxyacetonephosphate which they have named zymohexase. Muscle extract forms a very active preparation of this enzyme which is, according to Meyerhof and Lohmann [1934, 2], 100 times as active as that from a mouse carcinoma (strain not stated). We have estimated the zymohexase activity in extracts of tumours and muscle of mice and rats. In all our experiments the amount of zymohexase in tumours was found to be greater than that found in mouse carcinoma by Meyerhof and Lohmann.

Estimation of zymohexase.

Weighed amounts of muscle and tumour tissue removed from freshly killed mice, rats and rabbits were minced in a well-cooled mortar. The tissue was ground with 2 vols. of water until frozen. After thawing and centrifuging for 15 mins. at 3000 r.p.m. the supernatant fluid was removed and dialysed against running tap water for 2 hours in a cellophane bag. The volume of the dialysed extract was measured, and small portions were incubated with hexosediphosphate in citrate buffer in an atmosphere of N_2 and afterwards precipitated with trichloroacetic acid. For the determinations at zero time the extract was acidified with trichloroacetic acid before mixing with the substrate and buffer solution. The determination of alkali-labile phosphate was carried out as follows. 2 ml. of the solution to be determined were measured into each of two 15 ml. flasks, to the first of which an equal volume of $2N$ NaOH was added. After 15 mins., 1·4 ml. of $10N$ H_2SO_4 were added to each flask and 2 ml. of $2N$ NaOH to the second flask. Molybdate and reducing agent were then added in the usual way. The difference between the amounts of phosphate in the two flasks was equal to the alkali-labile phosphate present.

In order to compare the activities of different tissue extracts the same concentration was always used and in every case approximately equal amounts of hexosediphosphate. Determinations of free and labile phosphate were then made for different times of incubation; the values were plotted against time and the time at which half the equilibrium amount of labile phosphate was

formed was determined graphically. It was found that if oxygen were not excluded from the incubation tubes the triosephosphate was slowly oxidised and low values of labile phosphate were obtained for times greater than 15 mins. If oxygen were excluded the equilibrium value remained constant for an hour. The times required to reach the half equilibrium value are given in Table I; in

Table I. *Zymohexase activity of tissue extracts.*

Tissue	Time required for formation of 50% of maximum triosephosphate (secs.)	Relative activity
Muscle (rabbit)	9	100
(rat)	15	60
(mouse)	20	45
Sarcoma 37 (mouse)	180	5·0
Crocker 180 (mouse)	210	4·3
Mal (mouse)	210	4·3
J.R.S. (rat)	210	4·3
Brain (rat)	210	4·3

all cases the values are averages of 2–6 determinations. Some similar determinations made with rat's brain showed that the rate of triosephosphate formation was identical with that of J.R.S. Zymohexase from tumour tissue and from muscle yielded no alkali-labile phosphorus compound from hexosemonophosphate (kindly supplied by Prof. Robison).

The results show that the zymohexase content of the grafted tumours examined is relatively constant and that it is about one-tenth that of muscle of the same animal. It is interesting to find that the zymohexase activities of rat and mouse muscle are significantly less than that of rabbit muscle.

The diminished labile phosphate formation in air suggests that the "Pasteur reaction" may possibly be concerned with this stage of glycolysis. The dialysed extracts could not convert the hexosediphosphate into lactic acid, yet in the presence of oxygen some of the triosephosphate formed from hexosediphosphate disappeared. The nature of the oxidation of the triosephosphate has not been further investigated, but the results suggest that oxidation in tumour extracts can remove triosephosphate, the first product of the decomposition of hexosephosphate, and thus prevent its conversion into lactic acid.

Occurrence of dihydroxyacetonephosphate in tissues.

Dihydroxyacetonephosphate and glyceraldehydephosphate are the only compounds known which yield inorganic phosphate rapidly in cold *N* NaOH solutions. Of these compounds only dihydroxyacetonephosphate has been isolated from tissues. If cold trichloroacetic acid extracts of tumour or brain tissue from which some free phosphate has been precipitated with calcium acetate or barium carbonate are examined it is found that an increase in the inorganic phosphate content occurs on standing 10 mins. in *N* NaOH. Estimations of this alkali-labile phosphorus in three different batches of J.R.S. showed that 0·02, 0·03, 0·04 mg. of P as alkali-labile phosphate per g. was present. The average of these figures would correspond to 0·15 mg. of dihydroxyacetonephosphate per g. of tumour. Similar estimations on two separate ox brain extracts showed 0·12 and 0·15 mg. of dihydroxyacetonephosphate per g. Until

the compound is isolated however this labile phosphate cannot be definitely attributed to a triosephosphate.

That tumour extracts form dihydroxyacetonephosphate from hexosediphosphate was confirmed by the isolation of the triosephosphate after incubation of tumour extract with hexosediphosphate and sodium sulphite. Sodium sulphite combines with dihydroxyacetonephosphate and hence all the hexosediphosphate in solution is converted into triosephosphate. The hydrolysis of the triosephosphate in N HCl at 100° was followed and the hydrolysis constant $k = 33{\cdot}2 \times 10^{-3}$ agrees with that found for pure dihydroxyacetonephosphate by Kiesling [1934].

Lactic acid formation from hexosediphosphate.

Extracts of rat and mouse tumours were prepared in a similar way to that described for the preparation of zymohexase but with only 1 vol. of water in order to keep the extracts as concentrated as possible. The extract was dialysed at 0° for 1 hour. Measured volumes of the dialysed extract were mixed in Thunberg tubes with various reagents in $KHCO_3$ buffer; samples were removed for estimation of the original lactic acid content. The tubes were then repeatedly evacuated and refilled with N_2-CO_2 mixture and incubated at 38°. At the end of incubation the samples were all precipitated with trichloroacetic acid solution (final concentration about 3%) and the estimations of lactic acid carried out by the method of Lohmann [1928].

Precipitation with copper sulphate and lime before estimation of the lactic acid was avoided, for in acid solution methylglyoxal has no effect on the estimation but if the solution is made alkaline methylglyoxal is partially converted into lactic acid and dihydroxyacetonephosphate completely so. Thus if the solution is never allowed to become alkaline the long extraction of methylglyoxal recommended by Stewart *et al.* [1934] is unnecessary. Some results shown in Table II demonstrate that the small lactic acid formation from hexosediphosphate is greatly increased by addition of adenylpyrophosphate.

No extracts have been found that will produce lactic acid from glucose. Lactic acid formation from hexosephosphate by active extracts of different tumours is shown in Table III.

Table II. *Effect of varying the adenylpyrophosphate concentration on lactic acid formation from magnesium hexosediphosphate by extract of Crocker 180 sarcoma.*

Exp.	Concentration of adenyl-pyrophosphate*	mg. lactic acid formed by extract from 1 g. original tissue	
		In 30 mins.	In 60 mins.
A	0·10	0·80	0·86
	0·40	0·64	1·54
B	Nil	—	0·71
	0·07	0·84	0·75
	0·13	0·74	1·02
	0·26	—	2·95

* Expressed as pyrophosphate-P in mg. per ml.

These results confirm the suggestion that tumour extracts do not readily show lactic acid formation because of the extreme rapidity with which they decompose adenylpyrophosphate. Not all extracts which were made under similar conditions formed lactic acid from hexosediphosphate, although all the extracts tested for zymohexase contained this enzyme.

Table III. *Lactic acid formation from magnesium hexosediphosphate by tumour extracts at 38°.*

Exp.	Tumour	Concentration of adenyl-pyrophosphate*	mg. lactic acid formed by extract from 1 g. original tissue in 30 mins.	60 mins.
1	Crocker mouse sarcoma 180	0	0·58	0·76
		0·27	1·04	2·40
2	" "	0	—	0·70
		0·22	—	2·50
3	" "	0	—	0·34
		0·31	0·94	1·20
4	" "	0·14	0·32	0·54
		0·56	0·31	0·72
5	Mouse sarcoma 37	0·15	0·76	—
6	" "	0	—	0·6
		0·6	2·2	3·2
7	Mouse sarcoma Mal	0	0·28	0·31
		0·15	0·91	1·15
8	" "	0	—	0·10
		0·22	—	2·21

* Expressed as pyrophosphate-P in mg. per ml.

Two extracts were made from J.R.S. but neither of these showed any activity at all. This can be explained by the extremely rapid destruction of adenylpyrophosphate by J.R.S. extracts [Boyland and Boyland, 1935]. With our best conditions the extract corresponding to 1 g. of mouse tumour forms 2–3 mg. of lactic acid per hour from hexosediphosphate. Tumour slices under optimum anaerobic conditions (with $Q_M^{N_2}=25$) form 10 mg. lactic acid per g. per hour from glucose but they will not form lactic acid from hexosediphosphate. It has been pointed out [Boyland and Mawson, 1934] that the glycolysis of hexosephosphate could not occur with whole cells because the phosphoric ester could not pass into the cells. But mouse tumour extracts can convert hexosediphosphate into triosephosphate more rapidly than the original cells can convert glucose into lactic acid, and they can convert hexosephosphate into lactic acid at one-quarter to one-third the rate of normal glycolysis. Moreover the conversion of hexosediphosphate into lactic acid necessitates the presence of the coenzyme adenylpyrophosphate which is rapidly broken down by the tissue extracts.

It is improbable that in extraction of the enzyme from tumour with an equal volume of water more than one-half of the activity would be found in solution. This loss, combined with the destruction of coenzyme which occurs in the extracts, shows that in the living cell hexosediphosphate would be converted into lactic acid as rapidly as glucose is transformed. It is thus possible that hexosediphosphate is an intermediate in tumour glycolysis.

It has been shown [Boyland, 1932] that whole tumour tissue contains adenylpyrophosphate. It is conceivable that the nucleosidases which attack adenylpyrophosphate in extracts are segregated in the living cell. One possibility is that the nucleosidases are in the nucleus, where the nucleic acid metabolism may occur, whilst the adenylpyrophosphate is in the cytoplasm where lactic acid formation takes place. These results explain why freezing and thawing of tumour tissue destroys the glycolytic power, whilst freezing and thawing of muscle does not greatly decrease the lactic acid formation. The rapid breakdown

of hexosediphosphate by tumour extracts along the same paths as occur in muscle extracts gives support to the idea that the mechanisms of lactic acid formation in tumour and muscle are similar.

The effect of freezing and thawing on the glycolytic process is possibly allied to the viability of the tumour under these conditions. Cramer [1930] has found the S 37 tumour to be more resistant to freezing than the J.R.S. The Mal. sarcoma (used in this work) is also fairly resistant to freezing and like the S 37 has given extracts after freezing which will convert hexosephosphate into lactic acid. The less viable J.R.S. has failed to give any glycolytic extract.

SUMMARY.

1. Dialysed tumour extracts contain zymohexase which converts hexosediphosphate into dihydroxyacetonephosphate. The zymohexase activity of tumour is about one-tenth that of the muscle of the same animal.

2. In the presence of oxygen, dihydroxyacetonephosphate is oxidised by dialysed tissue extracts to some more stable phosphorus compound. It is possible that the Pasteur effect might involve oxidation of triosephosphate.

3. Dialysed extracts of mouse tumours convert hexosediphosphate into lactic acid if sufficient adenylpyrophosphate is added to compensate for the rapid decomposition of this latter substance which occurs in tumour extracts.

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