LXIII. STUDIES IN TISSUE METABOLISM

X. THE EFFECT OF COZYMASE ON GLYCOLYSIS IN TUMOUR EXTRACTS

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It has been shown that tumour extracts contain nucleosidases, adenine deaminase and xanthine dehydrogenase and destroy adenylypyrophosphate, a coenzyme of muscle glycolysis [Boyland & Boyland, 1935, 1]. By adding large amounts of adenylypyrophosphate to compensate for its breakdown some glycolysis of hexosediphosphate but not of glucose was obtained in extracts of the Crocker 180 mouse sarcoma [Boyland & Boyland, 1935, 2]. It was also shown that tumour extracts rapidly converted hexosediphosphate into triosephosphate. During the last year further work on the intermediate stages of glycolysis has suggested that some factor similar to cozymase is essential for glycolysis. This culminated in the publication by Meyerhof & Ohlmeyer [1936] which appeared after the essential facts of the present paper had been obtained, stating that cozymase was essential for enzymic lactic acid formation in well-dialyzed muscle extract; this was in agreement with the results which we have found with tumour extract. Two facts suggested that cozymase might be required: (1) tumour extracts alone converted hexosediphosphate into triosephosphate and the next step in carbohydrate breakdown by yeast, an oxido-reduction, requires cozymase; (2) Euler & Myrbäck [1928] showed that cozymase was destroyed by nucleosidase, and Euler [1936] and Warburg & Christian [1936] have shown cozymase to be a nucleotide containing adenylic acid and nicotinamide, so that it would be rapidly destroyed in tumour extract which attacks other adenine compounds.

Jensen rat sarcoma extracts destroy adenylic acid much more rapidly than do extracts of the Crocker 180 mouse sarcoma and for this reason extracts of the latter were mainly used in the following experiments.

EXPERIMENTAL

Extracts of frozen Crocker 180 mouse sarcomata were prepared as previously described and incubated with substrates and coenzymes in 0.2% NaHCO_3 solution in the Warburg apparatus at 38° in the presence of N_2 containing 5% CO_2 . Coenzymes were added from the side vessel after shaking for 10 min. in the thermostat. Manometric estimations of the CO_2 displaced by lactic acid were made every 5 or 10 min. and at the end of the incubation CCl_3COOH was added and lactic acid estimated in the protein-free filtrate as described by Lohmann [1928]. The manometric and chemical estimations of lactic acid were in approximate agreement with each other in experiments with glucose, and it was assumed

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that the rate of CO_2 evolution was proportional to the lactic acid formation. In order to construct the curves shown in Fig. 1, the same assumption was made in the glycolysis of phosphoric esters, although the CO_2 evolved from them was less than equivalent to the lactic acid formation. In some cases triosephosphate (dihydroxyacetonephosphate and glyceraldehydephosphate) was estimated as the difference between the free inorganic phosphate and phosphate present after treatment with N NaOH in the cold.

Fig. 1. Lactic acid production in extract of frozen Crocker sarcoma 180. 1 ml. extract (corresponding to 0.5 g. tissue) incubated with 2.0 mg. NaHCO_3 + 0.1 mg. MgCl_2 at 38° for 1 hour.

Additions:

- A. Hexosediphosphate (15 mg. hexose).
- B. As A + 0.5 mg. cozymase.
- C. As A + 1.0 mg. cozymase.
- D. 10 mg. glucose + hexosediphosphate (0.05 mg. hexose) + 0.5 mg. cozymase.
- E. As D but with 1.0 mg. cozymase.
- F. Hexosemonophosphate (18 mg. hexose) + 1.0 mg. cozymase.

Cozymase was prepared from D.C.L. yeast (a baker's yeast which is rich in cozymase) as described by Euler *et al.* [1936]. The preparation was controlled at every stage by activity determinations using apozymase standardized against apozymase and cozymase (ACo 500,000) kindly given by Prof. v. Euler to Prof. E. C. Dodds. The cozymase used in these experiments had passed through the cuprous salt stage but had not been fractionated with alcohol. It was of high activity, ACo 600,000 as confirmed by Prof. v. Euler, for whose help we are most grateful. Lactic dehydrogenase coenzyme was prepared as described by Banga *et al.* [1932] and yeast adenylic acid from Roche Products Ltd. was used.

Fig. 1 and Table I show results from two typical experiments on the effect of cozymase. With the extract alone very little lactic acid was produced from any substrate, but when cozymase was added lactic acid was formed from glucose containing a small amount of hexosediphosphate, from hexosemonophosphate and from hexosediphosphate in amount varying with the concentration of cozymase present.

Table I and Fig. 2 show that the glycolysis of glucose was further increased by addition of adenylic acid, which is analogous to the findings of Meyerhof & Ohlmeyer [1936] with muscle extract dialyzed for 36–48 hours.

Table I. *Glycolysis in extract of frozen Crocker sarcoma 180 at 38°*1 ml. extract (0.5 g. tissue) + 1 mg. cozymase + 0.1 mg. MgCl_2 + 2.0 mg. NaHCO_3

Substrates			Mg. lactic acid formed in extract equivalent to 1 g. tissue in 1 hour	
Glucose mg.	Hexose-diphosphate as mg. hexose	Hexose-monophosphate as mg. hexose	Without adenylic acid	With 2 mg. adenylic acid
10	—	—	1.7	3.6
10	15	—	5.4	6.4
10	0.015	—	2.7	6.3
—	15	—	5.4	6.0
—	—	18	6.0	6.2

Fig. 2. Lactic acid production in extract of frozen Crocker sarcoma 180. 1 ml. extract (equivalent to 0.5 g. tissue) incubated with 10 mg. glucose + hexosediphosphate (0.05 mg. hexose) + 2.0 mg. NaHCO_3 + 0.1 mg. MgCl_2 at 38° for 1 hour.

Additions:

- A. With 2 mg. adenylic acid and 0.5 mg. cozymase after 1 hour.
- B. With 1 mg. cozymase.
- C. With 2 mg. adenylic acid + 0.5 mg. cozymase and 0.5 mg. cozymase after 1 hour.
- D. With 2 mg. adenylic acid + 1.0 mg. cozymase.
- E. With 2 mg. lactic dehydrogenase coenzyme.

The effect of hexosediphosphate in increasing the glycolysis of glucose is shown in Table I. It is remarkable that addition of hexosediphosphate should be necessary for glucose breakdown as CCl_3COOH solution extracts of tumour

tissue contain traces of triosephosphate, as measured by the alkali-labile phosphate, and therefore probably contain hexosediphosphate. The rate of glycolysis with excess of both glucose and hexosediphosphate is the same as with either of them alone and therefore it is improbable that there is a different glycolytic system present for each of these substrates.

As the experiments shown in Figs. 1 and 2 indicate, the effect of cozymase in increasing the glycolysis is of short duration, but addition of more cozymase increases the rate of glycolysis a second time (Fig. 2). Lactic dehydrogenase coenzyme, which is probably similar in structure to cozymase, has some activating power in tumour glycolysis but never gave full activation of an extract (Fig. 2).

That the cozymase is concerned with the further change of triosephosphate was shown by estimations of triosephosphate in extracts with and without cozymase (Table II). Without cozymase the normal equilibrium amount of

Table II. *Changes in lactic acid, free phosphate and triosephosphate (expressed as mg. formed in extract equivalent to 1 g. tissue) in Crocker 180 sarcoma extract after 1 hour's incubation*

Substrate	Cozymase mg.	Products formed		
		Lactic acid mg.	Free phosphate as mg. P	Triose- phosphate as mg. P
Glucose + hexosediphosphate (0.01 mg. P)	—	0.1	0.106	0.002
„ „	0.5	1.2	0.205	0.000
Hexosediphosphate (1.2 mg. P)	—	0.1	0.112	0.275
„ „	0.5	2.5	0.810	0.018
Hexosemonophosphate (1.1 mg. P)	—	0.1	0.080	0.265
„ „	0.5	2.2	0.017	0.018

triosephosphate formed from hexosediphosphate was found. With cozymase present the triosephosphate was broken down and there was a large increase in free phosphate.

The tumour extract with cozymase, adenylic acid and a trace of hexosediphosphate was not only able to produce lactic acid from glucose but also from fructose and glycogen (Table III).

Table III. *Glycolysis in extract of frozen Crocker sarcoma 180 at 38°*

1 ml. extract with 0.5 mg. cozymase and hexosediphosphate (0.05 mg. hexose)

Substrates	mg. lactic acid formed in extract equivalent to 1 g. tissue in 1 hour	
	Without adenylic acid	With adenylic acid
10 mg. glucose	5.0	5.8
10 mg. fructose	3.9	9.0
10 mg. glycogen	—	6.0

If the mechanisms for glycolysis are the same in tumour tissue and extract, then the tissue should contain cozymase. Boiled tumour extracts were made from Jensen rat sarcoma and Crocker 180 sarcoma by grinding with an equal volume of boiling water and centrifuging. The effects of these extracts in increasing the rate of fermentation of washed dried yeast were determined, the cozymase content being estimated by the method of Myrbäck [1933]. The apozymase was made from Messrs Barclay Perkins' brewer's bottom yeast: the

initial pH was 6.7 and the phosphate concentration 0.2 *M* (optimum). In order to reduce the induction period it was necessary to add acetaldehyde (2 mg.) as well as hexosediphosphate. The results indicate that the Crocker 180 extracts contained up to 65 Co-units per ml., the J.R.S. up to 110 Co-units per ml. Three samples of yeast gave values between 150 and 300 Co-units per ml. The boiled tumour extracts also caused some glycolysis in frozen Crocker sarcoma 180 extract (Table IV).

Table IV. *Lactic acid produced by an extract of frozen Crocker sarcoma 180 activated by boiled extracts*

Expressed as mg. lactic acid formed by extract equivalent to 1 g. tissue per hour, from 10 mg. glucose + 2 mg. hexosediphosphate + 2 mg. adenylic acid

ml. of boiled extract	Type of boiled extract added to frozen Crocker extract		
	Boiled yeast	Boiled J.R.S.	Boiled Crocker 180
0.1	2.7	—	—
0.2	3.1	—	—
0.5	—	0.2	1.5
1.0	—	0.5	2.5

The action of tumour extract on cozymase was determined by estimating the cozymase activity (as shown by the ability to increase the fermentation of washed dried yeast) of a known amount of cozymase incubated with frozen

Table V. *Lactic acid production and inactivation of added yeast cozymase by 2 ml. frozen Crocker sarcoma 180 extract in the presence of glucose (10 mg.) and hexosediphosphate (0.01 mg. P)*

0.5 mg. ($\equiv$ 300 Co-units) added					
Exp. 1			Exp. 2		
Time	Lactic acid formed in previous 10 min. mg.	Cozymase (Co-units) present	Time	Lactic acid formed in previous 10 min. mg.	Cozymase (Co-units) present
0	—	340	0	—	290
15	1.3	215	10	0.52	80
30	0.8	170	25	0.45	10
45	0.7	130	40	0.14	10
60	0.3	80	40*	—	260

* Extract heated to 80° before incubation.

tumour extract for different times. The results shown in Table V show how rapidly the tumour extract destroys the cozymase isolated from yeast, and that the rate of lactic acid production falls as the cozymase disappears.

DISCUSSION

In the experiments with Crocker 180 tumour extract to which cozymase and adenylic acid are added lactic acid is produced at the rate of 8–10 mg. an hour in an extract equivalent to 1 g. of tissue. Assuming $Q_{\text{Lactic acid}}^{N_2} = 20\text{--}22$ and a water content of 88%, which are the values we have found for the tumours used, normal Crocker tumour slices produce about 11 mg. lactic acid per g. wet weight. Thus in the tumour extract for the period when sufficient cozymase and adenylic

acid are present, glycolysis is nearly as rapid as it would be in the equivalent amount of whole tissue.

The absence of glycolytic activity from a tumour extract can be explained as a result of the nucleosidase activity of the extract destroying the necessary coenzymes, cozymase and adenylic acid. In the living tissue the enzymes attacking nucleosides may be mainly concentrated in the nucleus but in breaking the cell walls to obtain the extract the nuclei are also broken down. In the whole tumour tissue a substance with cozymase activity is present as it is in muscle and it is presumably essential for glycolysis in both tissues. No essential differences have been found in the intermediate stages of carbohydrate breakdown in tumour tissue and muscle.

SUMMARY

1. The addition of yeast cozymase and adenylic acid to a cell-free extract of the Crocker 180 tumour increases the glycolytic power to that of the tissue from which the extract was made. In the presence of added cozymase and adenylic acid, glucose, hexosemonophosphate and hexosediphosphate are converted into lactic acid in the extract as rapidly as is glucose by the original tissue. Cozymase and adenylic acid are however rapidly destroyed by the extract.

2. Extracts of boiled tumour tissue increase the rate of fermentation by washed dried yeast and therefore contain a cozymase.

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