CIV. STUDIES IN TISSUE METABOLISM.

I. VITAMIN B₁ AND THE COENZYME OF LACTIC DEHYDROGENASE.

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(Received May 1st, 1933.)

THE suggestion that the antiberiberi factor was concerned with carbohydrate metabolism arose early in vitamin work [Braddon and Cooper, 1914; Funk and Schönborn, 1914] and has received much attention since. It is only within the last few years, however, that work carried out by Peters and his collaborators has defined the rôle of alkali-labile vitamin B₁. Kinnersley and Peters [1929; 1930] showed that the brains of polyneuritic pigeons had a relatively high lactic acid content, and Gavrilescu and Peters [1931, 1] found that the brain tissue of avitaminous polyneuritic pigeons had a sub-normal oxygen uptake *in vitro*. Soon afterwards [1931, 2] they showed that the addition of vitamin B₁ concentrates increased the *in vitro* respiration of this polyneuritic brain tissue. Meiklejohn, Passmore and Peters [1932, 1] have shown that the oxidation affected in this way is that of lactic acid, indophenol oxidase and succinic dehydrogenase being unaffected. They have also [1932, 2] been able to show that when polyneuritis of avitaminosis was cured by vitamin B₁ concentrates, the ability of the brain to increase its oxygen uptake in the presence of lactate returned. The action of vitamin B₁ concentrates containing a heat-stable substance, which stimulates oxidation of lactate, is like the action of a coenzyme added to an enzyme deficient in its coenzyme.

Recently Banga and Szent-Györgyi [1932] have described a coenzyme of lactic acid dehydrogenase. When washed heart muscle alone is incubated with lactate and methylene blue, the latter is not reduced. Methylene blue is not reduced by coenzyme alone or by lactate alone in the presence of washed heart muscle. If, however, heart muscle extract or the purified coenzyme obtained from such an extract is added to the washed muscle, it is then able to oxidise lactate in the presence of methylene blue. Anaerobically methylene blue is decolorised, aerobically oxygen is taken up [Banga, Szent-Györgyi and Vargha, 1932].

In the experiments of Gavrilescu *et al.* [1932] it was found that addition of lactate doubled the respiration of normal brain, but that the respiration of avitaminous brain was only increased by a quarter by addition of lactate. Addition of vitamin B₁ concentrates to normal brain in presence of lactate increased respiration by 4 % on the average, but with avitaminous brain the respiration was increased 20–32 %. The deficient respiration of avitaminous brain is peculiar to lactate metabolism. In these experiments the avitaminous brain appears as an enzyme deficient in coenzyme and the vitamin concentrates act as coenzyme.

Szent-Györgyi's coenzyme and vitamin B₁ are similar to some extent in chemical properties. The coenzyme is described as an adenine-like derivative containing sulphur; it is not adenylyl pyrophosphate. Vitamin B₁ is stated to be a nitrogenous base containing sulphur.

An obvious difference between the lactic oxidase coenzyme described by Szent-Györgyi and the purest vitamin B₁ preparation is that the former is described as being yellow-coloured while vitamin B₁ is colourless. Preparations of coenzyme, however, have been made, both by the original technique and by modified methods, which are colourless and yet active. Banga, Szent-Györgyi and Vargha [1932] suggested that the activity ran parallel with colour in different preparations. The fact that the active coenzyme could be obtained colourless suggested that it might be identical with the antineuritic factor of yeast.

In order to test this hypothesis, crude and purified preparations of vitamin B₁ were tested for coenzyme activity in the presence of washed heart muscle, lactate and methylene blue by means of the Thunberg technique. Crude vitamin B₁ concentrates activated the lactic dehydrogenase of washed heart muscle, but purified preparations did not. A purified preparation of heart muscle coenzyme was then tested for vitamin B₁ activity by means of the rat curative test and was found to have no vitamin activity.

EXPERIMENTAL.

Lactic dehydrogenase coenzyme.

Two bullocks' hearts were obtained from the slaughter-house and stored in ice until used. The hearts were treated within 1½ hours after death. The heart muscle separated from fat and connective tissue was minced and the minced tissue (3 kg.) stirred into 3 litres boiling 1 % trichloroacetic acid. After 10 minutes' stirring the material was pressed through muslin and the extract filtered. The filtrate (which had coenzyme activity) was treated with excess of mercuric nitrate dissolved in nitric acid. The precipitate so formed was filtered off, washed with acetone and then extracted with HCl containing HgCl₂. The acid extract and washings (50 cc.) were then poured into 500 cc. of dried acetone, which precipitated the coenzyme. It was centrifuged and collected, dissolved in water and treated with H₂S to remove mercury. The mercury sulphide formed a colloidal suspension which was precipitated by addition of one-third volume of alcohol. The colourless filtrate (60 cc.) was then aerated to remove H₂S, and the coenzyme in the colourless solution was precipitated with dried acetone (500 cc.), removed by centrifuging and dried in a vacuum desiccator.

Vitamin B₁ preparations.

The first crude vitamin B₁ concentrate was prepared in the Lister Institute (preparation A in Table II) from acetic acid extracts of brewer's yeast. The extract was treated with lead acetate, baryta, mercuric sulphate and then H₂S. The vitamin was adsorbed on norite at p_H 7.0, eluted with *N*/10 HCl and the extract neutralised. The second crude preparation (B) was made from baker's yeast in the Biochemical Laboratory, Oxford, as described by Kinnersley, O'Brien, Peters and Reader [1933]. Both preparations A and B contained 3 pigeon doses per cc. These crude concentrates were free from vitamin B₂. The purified vitamin preparations were made in the Biochemical Laboratory, Oxford. The preparation purified by phosphotungstate contained 3 pigeon doses per cc. and the crystalline preparation 2.5 doses per cc. (in 5 γ solids). The crystals were prepared by the method described by Kinnersley, O'Brien and Peters [1933].

Comparison of vitamin B₁ and heart muscle extract as coenzyme.

20 g. of well-minced bullock's heart muscle were ground with 250 cc. ice-cold water and pressed through muslin. The residue was washed in this way 6 times, pressed and then frozen solid and suspended in 100 cc. *M*/10 phosphate buffer, *p*_H 7.4. While frozen it was minced 3 times to break up the tissue.

2 cc. lots of this suspension with 1 cc. 0.01 % methylene blue were placed in Thunberg tubes with additions shown in Table I, evacuated and incubated in a glass-sided thermostat at 37°. The reduction times are shown in Table I.

Table I. *The reduction of methylene blue by washed heart muscle in the presence of lactate and lactic dehydrogenase coenzyme or vitamin B₁ preparations.*

<i>M</i> /20 lactate cc.	Lactic dehydrogenase coenzyme solution 10 mg. per cc. cc.	Vitamin solution		Vitamin preparation	Reduction time in minutes
		cc.	Pigeon doses		
—	—	—	—	—	>200
1	—	—	—	—	180
—	0.1	—	—	—	200
—	0.2	—	—	—	180
—	0.4	—	—	—	150
1	0.1	—	—	—	47
1	0.2	—	—	—	27
1	0.4	—	—	—	12½
—	—	0.5	1.5	Crude A	70
—	—	1.0	3.0	"	60
1	—	0.1	0.3	"	47
1	—	0.5	1.5	"	40
1	—	1.0	3.0	"	27
—	—	0.1	0.3	Crude B	115
—	—	0.2	0.6	"	50
—	—	0.4	1.2	"	36
1	—	0.1	0.3	"	42
1	—	0.2	0.6	"	33
1	—	0.4	1.2	"	25
—	—	0.2	0.6	Purified by phosphotungstate	>200
—	—	0.4	1.2	"	200
1	—	0.2	0.6	"	180 (approx.)
1	—	0.4	1.2	"	180 "
—	—	0.2	0.5	Crystalline	>200 "
—	—	0.4	1.0	"	>200 "
1	—	0.2	0.5	"	200 "
1	—	0.4	1.0	"	180 "

Washed heart muscle, either alone or in the presence of lactate alone, coenzyme alone or any of the vitamin preparations alone hardly reduced methylene blue. The addition of crude vitamin B₁ preparations, however, in the presence of lactate caused the reduction of methylene blue in the same way as did the coenzyme prepared from heart muscle. The activity of 0.4 cc. crude vitamin concentrate was equivalent to 0.2 cc. lactic dehydrogenase coenzyme solution. The vitamin preparations which had been further purified by phosphotungstate fractionation had no coenzyme-like activity, which shows that the coenzyme and vitamin cannot be identical.

Test of heart muscle extract for vitamin activity.

The lactic dehydrogenase prepared from heart muscle was tested for its vitamin B₁ content by estimation of its power to promote growth and prevent or cure polyneuritis in rats [Chick and Roscoe, 1929]. These tests were carried

out at the Lister Institute, London, through the kindness of Miss M. H. Roscoe. No vitamin B₁ could be detected in doses of 0.1 cc. or 0.2 cc. of the extract, these being insufficient to prevent loss of weight or the development of polyneuritis. Traces of the vitamin were detectable in doses of 2.0 cc. Not sufficient material was available for an accurate test; it could only be stated that 2.0 cc. contained less than one vitamin B₁ unit, or that one pigeon dose was more than 3.0 cc. This material was therefore less than one-tenth as rich in vitamin B₁ as the crude yeast concentrate.

Meiklejohn, Passmore and Peters [1932, 2] have found that the oxygen uptake of avitaminous brain with added vitamin B₁ concentrates was not increased by the addition of pyruvate, while the respiration of normal brain was increased by pyruvate. The system of avitaminous brain *plus* vitamin B₁ concentrate differs from the heart muscle system which oxidises pyruvate readily. The oxidation of pyruvate and of succinate by washed heart muscle occurs without any coenzyme, as is shown by the results in Table II. Pyruvate and

Table II. *Reduction of methylene blue by washed heart muscle in presence of different substrates.*

2 cc. washed heart muscle suspension in phosphate buffer p_H 7.4 + 1 cc. 0.01 % methylene blue. Substrate concentrations $M/100$.

Substrate	Reduction time in minutes
None	> 200
Lactate	> 200
Pyruvate	39
Succinate	33
Fumarate	> 200
Malate	> 200
Malonate	> 200
Lactate + succinate	36
Pyruvate + succinate	15

succinate are oxidised readily by the preparation, which indicates that pyruvic dehydrogenase and succinic dehydrogenase require no coenzyme or that the coenzyme is not removed by extensive washing.

Effect of coenzyme on tissue respiration.

The brain tissue of avitaminous pigeons resembles tumour tissue in respect of being deficient in respiratory power. As the respiration of avitaminous brain

Table III. *Oxygen uptake of thin slices of Jensen rat sarcomata in Ringer lactate solution.*

mm.³ O₂ per g. tissue per hour.

	Conditions	First hour	Second hour
<i>Experiment I</i>			
	Tissue alone	2200	2200
	+ 0.1 cc. coenzyme solution	2130	2300
	+ 0.2 cc. coenzyme solution	2200	1850
	+ 0.1 cc. vitamin B ₁ (purified by phosphotungstate)		
	0.3 pigeon dose	1950	1850
	+ 0.2 cc. vitamin B ₁ (purified by phosphotungstate)		
	0.6 pigeon dose	1860	1920
<i>Experiment II</i>			
	Tissue alone	1950	1900
	+ 0.1 cc. coenzyme	1970	2000
	+ 0.2 cc. coenzyme	2180	1850

in vitro is raised by vitamin B₁, the effect of the vitamin on tumour respiration was investigated. Thin slices of small non-necrotic Jensen rat sarcomata were suspended in 1 cc. Ringer solution containing *M*/100 lactate in the vessels of the Warburg apparatus and the vessels filled with oxygen. The oxygen uptake at 37·5° was measured over 2 hours as shown in Table III.

The experiments show a slightly decreased respiration in the presence of vitamin. In the coenzyme experiments the respiration did not differ significantly from that of the controls. The deficient respiration of the Jensen rat sarcoma is therefore not likely to be due to lack of vitamin B₁ or of lactic dehydrogenase coenzyme.

SUMMARY.

1. Crude vitamin B₁ concentrates resemble Szent-Györgyi's lactic dehydrogenase preparation in enabling washed heart muscle to oxidise lactate. Purified preparations of vitamin B₁ do not behave in this way.

2. Lactic dehydrogenase coenzyme preparations from heart muscle have no vitamin B₁ activity.

3. Washed heart muscle, which oxidises lactate only on the addition of lactic dehydrogenase coenzyme, oxidises pyruvate and succinate without the addition of any coenzyme.

4. The respiration of slices of Jensen rat sarcoma in the presence of lactate was not increased by the addition of lactic dehydrogenase coenzyme or vitamin B₁ solution.

I am deeply indebted to Prof. R. A. Peters and Miss M. H. Roscoe for their valuable help in this work.

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