LXXXVI. THE ACTIVATION OF LACTIC DEHYDROGENASE AND ITS RELATION TO THE RÔLE OF VITAMIN B₁.

BY THOMAS WILLIAM BIRCH AND
PHILIP JOCELYN GEORGE MANN¹.

From the Nutritional Laboratory, University of Cambridge and Medical Research Council, and the Biochemical Laboratory, University of Cambridge.

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THE necessity of a coenzyme for the oxidation of lactic acid by the lactic dehydrogenase of animal tissues has been shown by Szent-Györgyi [1925]. The preparation of a concentrate of this substance from mammalian heart tissue has been described by Szent-Györgyi and Vargha [1932]. Recently Banga and Szent-Györgyi [1933] have further purified the concentrate by precipitation with phosphotungstic acid and picric acid and have obtained the coenzyme as a semi-crystalline picrate. By investigation of the products of hydrolysis of the coenzyme they concluded that it was a monophosphate of a monoadenylnucleotide not identical with muscle adenylic acid or adenylypyrophosphate, but possibly identical with cozymase.

This coenzyme is of considerable interest owing to the importance of lactic acid as an intermediary in animal metabolism. In certain pathological conditions where accumulation of lactic acid is observed, it appears possible that such an accumulation may be due to a deficiency of the coenzyme. Such an accumulation occurs as a symptom of vitamin B₁ deficiency and in this case there is additional evidence which suggests the possibility of a connection between vitamin B₁ activity and the coenzyme activity.

Kinnersley and Peters [1929; 1930] showed that brain tissue of polyneuritic pigeons had an abnormally high lactic acid content. Fisher [1931] has shown that there is a general defect in lactic acid metabolism in the polyneuritic pigeon as a whole, in addition to the defect in brain described by Kinnersley and Peters. This defect in lactic acid metabolism in vitamin B₁ deficiency is not confined to pigeons. According to Japanese workers, *e.g.* Hayasaka [1930], lactic acid accumulates in the body and reaches abnormally high levels in patients suffering from beri-beri.

Further, a relation between vitamin B₁ and lactic dehydrogenase coenzyme is suggested from the work of Meiklejohn *et al.* [1932] and Passmore *et al.* [1933], who showed that the cerebrum of the brain of polyneuritic pigeons when minced and mixed with lactic acid showed a low oxygen uptake compared with normal brain tissue. The addition of vitamin B₁ concentrate increased the *in vitro* respiration of the polyneuritic brain tissue, and it was assumed that the oxidation affected in this way was that of lactic acid.

Unfortunately, from the recent work of Meiklejohn [1933] it appears that the amount of lactic acid removed during the oxidation is not influenced by the presence of the vitamin. A similar observation has been made by Hahn and Fischbach [1929] using ox muscle. They find that with washed ox muscle, the

¹ Beit Memorial Research Fellow.

renewed oxygen uptake in presence of "Kochsaft" is not due to oxidation of the added lactic acid, for although the amount of respiration may be restored to 90 % of its original value by adding the "Kochsaft," the lactic acid content remains unaltered. On the other hand, while avitaminous brain tissue shows a low oxygen uptake with pyruvic acid similar to that with lactic acid, according to the conclusions of Meiklejohn *et al.* [1933] in this case the addition of vitamin B₁ is without effect. However, on consideration of the results of Peters and his collaborators it appears that this conclusion is somewhat unjustified. The difference in the rate of oxygen uptake between normal and avitaminous brain tissue in presence of lactate is small. Hence the increase obtained on addition of vitamin B₁ to the avitaminous brain is almost sufficient to raise the rate of oxygen uptake to that of normal brain tissue + lactate. On the other hand the difference in the rate of oxygen uptake in the case of normal and avitaminous brain with pyruvate is much greater than in the case of lactate. Hence in this case while addition of vitamin B₁ causes an increase of the same order as in the case of lactate, this addition is insufficient to raise the rate to normal. In the case of lactate and avitaminous brain, using the cerebrum the increase in oxygen uptake produced by addition of vitamin B₁ is 28 %, using the optic lobes 22 %. In the case of pyruvate with the cerebrum the increase in oxygen uptake produced by addition of vitamin B₁ is 20 %, and with the optic lobes 40 %. If the increase in the presence of lactate is to be regarded as significant it appears that the case of pyruvate should not be ignored merely because it does not raise the rate of oxygen uptake to normal. Furthermore since the difference in the rate of oxygen uptake between normal and avitaminous brain with pyruvate is greater than with lactate, it appears that deficiency of vitamin B₁ produces actually a greater effect on the mechanism of pyruvate oxidation than on that of lactate oxidation. Peters explains his conclusion by assuming that the increase obtained with pyruvate is due to the effect of the vitamin on the residual lactic present in the brain tissue.

The question of the possible identity of vitamin B₁ and lactic dehydrogenase coenzyme has been investigated by Boyland [1933]. As vitamin B₁ is a sulphur-containing compound and the coenzyme contains no sulphur, it is difficult to imagine how the two can be identical. Further arguments point in the same direction, for example Szent-Györgyi precipitates his coenzyme with mercuric nitrate, followed by precipitation from aqueous solution with acetone. By neither of these reagents would vitamin B₁ be precipitated. Of course it still remains possible that vitamin B₁ might exist in the coenzyme in some such form as the base of a nucleotide which occurred as a small impurity in Szent-Györgyi's preparation, but Boyland's experiments do not allow for this.

Boyland concluded that crude coenzyme preparations possessed only a slight vitamin B₁ activity as determined by rat feeding experiments, and that while crude vitamin B₁ concentrates showed activity as coenzyme for heart-lactic dehydrogenase, purified preparations did not possess this activity.

Table I shows the results obtained by Boyland in testing the activity of his coenzyme preparation by the methylene blue technique.

Table I.

Lactic dehydrogenase coenzyme solution 10 mg. per ml.	M/10 lactate ml.	Reduction time in minutes
0.0	1.0	180
0.1	1.0	47
0.2	1.0	27
0.4	1.0	12½
0.4	0.0	150

Since in the above experiments doubling the concentration of the coenzyme doubles the velocity of the reaction, it appears doubtful whether 0.4 ml. of the coenzyme solution is sufficient to produce maximum activation of the enzyme. Meiklejohn *et al.* [1932] found in their experiments with avitaminous brain tissue and lactate that it was necessary to add only 1/10 of a pigeon dose of vitamin B₁ to each Barcroft cup to obtain the maximum increase in oxygen uptake. Thus in the case of Boyland's results, if it is assumed that 0.4 ml. of the coenzyme solution produces maximum activation of the enzyme then this amount should be equivalent to 1/10 of a pigeon dose. Boyland tested the vitamin B₁ activity of his preparation by rat feeding experiments. He found, as would be expected, that 0.1 ml. and 0.2 ml. showed no activity, while 2 ml. showed some activity, but less than one pigeon dose. From the above calculation, 2 ml. should only be equivalent to half a pigeon dose or less.

Thus Boyland's conclusion that lactic dehydrogenase coenzyme possesses little vitamin B₁ activity appears unjustified and in spite of his work it still seems possible that there may be a close interrelation between vitamin B₁ and the coenzyme activity. The work to be described was started with the view of investigating this possibility.

Preparation of the enzyme.

During the course of this work three different methods have been employed for the preparation of lactic dehydrogenase from heart muscle. The methods so employed may be divided into two sections according to the properties of the enzyme preparation obtained.

Section I. Szent-Györgyi and Vargha's method [1932]. 40 g. pig heart muscle freed from fat and connective tissue are finely chopped and shaken with 800 ml. of distilled water at room temperature for 10 minutes; after squeezing through linen the residue is shaken with another 800 ml. of distilled water for a further 10 minutes. After squeezing through linen, the residue is placed in a mortar and frozen by surrounding it with an ice and salt freezing mixture. The tissue is then ground up and mixed with 120 ml. of *M*/15 phosphate buffer at *p*_H 7.2.

Section II. (a) Boyland's method [1933]. 20 g. of well minced pig's heart are ground up with 250 ml. of ice-cold water and pressed through linen. The residue is washed in this way six times, pressed and then frozen. While frozen it is minced three times to break up the cells and then suspended in 100 ml. *M*/100 phosphate buffer at *p*_H 7.4.

(b) Birch and Mann's method. In this method the enzyme is extracted from the tissue. The method is identical in its initial stages with that of Szent-Györgyi. It is essential that the frozen tissue should be thoroughly ground up so as to break up the cells as far as possible. The pounded tissue is suspended in phosphate buffer and allowed to stand for 2–3 hours at room temperature. The suspension is then squeezed through linen, and the liquid obtained is fully saturated with ammonium sulphate. The precipitate is filtered off and dried in a vacuum-desiccator. The yield from 100 g. of tissue is about 7 g. This enzyme preparation shows little loss of activity after several weeks if it is left in the dry form in a vacuum-desiccator. It may be further purified by dissolving in phosphate buffer, centrifuging off the insoluble residue and then reprecipitating with ammonium sulphate.

The differentiation of the methods of preparation is based on the amount of washing which the tissue undergoes. Szent-Györgyi's enzyme preparation consists of comparatively unwashed tissue, while by Boyland's method the tissue is

very thoroughly washed. In our own method the enzyme is further purified by extraction from the tissue and by precipitation with ammonium sulphate. This difference in the degree of washing of the tissue is accompanied by a fundamental difference in the properties of the resultant enzyme preparations.

Preparation of the coenzyme.

At the beginning of this work the method of Szent-Györgyi and Vargha [1932] was used for the preparation of the coenzyme from bullock's heart and Boyland's enzyme was used as a means of testing the activity of the coenzyme. The yield of the coenzyme is small—about 0.7 g. of the crude concentrate from 5 kg. of heart, and in the subsequent purification by phosphotungstic acid most of the activity was lost. In place of the phosphotungstic precipitation other methods were used for the purification of the crude coenzyme, and in some cases it was apparently separated into two fractions, either of which separately showed little coenzyme activity but together gave good activation of the enzyme. An example of this, where precipitation with silver nitrate was used as the means of fractionation is shown in Table II.

Solution I. The crude coenzyme solution is precipitated at p_H 1.5 with $AgNO_3$. The precipitate is decomposed with H_2S , evaporated to a small volume and precipitated by the addition of 10 volumes of acetone. This precipitate dissolved in water forms solution I.

Solution II. The filtrate from the above acid silver precipitate is brought to p_H 6 with ammonia and again precipitated with $AgNO_3$. The filtrate is treated with H_2S , evaporated and precipitated with acetone. The acetone filtrate is evaporated until all the acetone is removed.

Using these two solutions with Boyland's enzyme the following results were obtained by the Thunberg technique of methylene blue reduction. The total volume in each tube was 3 ml. In general 1 ml. of enzyme was used and 1 ml. of 1 in 5000 methylene blue, together with phosphate buffer at p_H 7.4. In some cases when the enzyme was found to be weak the methylene blue was cut down to 0.5 or 0.25 ml.

Table II.

	Reduction time (mins.)
0.25 ml. of (I) + enzyme + lactate + MB	80
1.0 ml. of (II) + enzyme + lactate + MB	120
0.25 ml. of (I) + 1.0 ml. of (II) + enzyme + lactate + MB	10
0.5 ml. of (I) + 0.5 ml. of (II) + enzyme + MB (no lactate)	68

Attempts to separate these two factors from heart completely were unsuccessful, though results such as those shown in the above table were sometimes obtained. One of the causes of this failure was the minute amounts of the substances with which we were dealing. It was found that yeast contained considerable amounts of the coenzyme. Since yeast is more readily obtainable and easier to work up on a large scale than heart tissue, it formed the source of most of our subsequent preparations of coenzyme. Even using yeast it has so far proved impossible to effect a complete separation of the two factors. One factor, which is probably identical with Szent-Györgyi's coenzyme is readily obtained more or less free from the other. The method of obtaining this is shown in the following scheme.

Preparation A.

Attempts to obtain the second factor free from the first have up to the present been unsuccessful. The best preparations so far consist of a mixture of the two factors. The method of preparation of this mixture is shown in the following scheme.

Preparation B.

Most of the activity is found in the silver ppt. at p_H 6 while some activity is also found in the p_H 1.5 ppt. These ppts. are again fractionated with silver nitrate. The silver fractionation does not give clear cut results. The majority of the activity usually comes down at p_H 6, but often some comes down at p_H 1.5. These solutions obtained after refractionation with silver are called "coenzyme preparation B" in the subsequent experiments.

Using preparation "A" in the Thunberg technique of methylene blue reduction only small amounts are necessary to produce maximum activation (0.5 to 2 mg.; the actual amount seems to vary with the enzyme used); the activation produced is small. With preparation "B" relatively large amounts are often necessary but the activation produced is 5–8 times as great when using

the pure enzyme. It is seen from the schemes of preparation shown that the first factor "A" is precipitated by HgSO_4 and by acetone from aqueous solution. The second factor does not seem to follow these reactions, but if the HgSO_4 filtrate or the acetone filtrate is examined for coenzyme activity it is usually found to possess only slight activity. In some cases by mixing the two solutions reactivation has been obtained as is shown in Table II, but more often than not the mixture is found to be inactive. Thus it appears that the second factor is unstable except when in the presence of the first, and the best preparation obtainable is a mixture of the two, containing a greater percentage of the second factor.

Further evidence for the existence of two factors.

In addition to the results shown in Table I other evidence has been found which suggests that two factors are necessary. This has been obtained by comparing the maximum activation produced by coenzyme preparations "A" and "B" with each type of enzyme.

Fig. 1.

Fig. 2.

Fig. 1. Comparison of the two coenzyme preparations "A" and "B" using the unpurified enzyme at p_H 7.2. Abscissa = relative amounts of coenzyme added; ordinate = $1/T$.

In this experiment 1 ml. of Szent-Györgyi's enzyme was used with 1 ml. of 1 in 5000 methylene blue, the lactate concentration being $M/10$. The minimum reduction time with coenzyme preparation "B" was 3 minutes. The minimum reduction time with coenzyme preparation "A" was 5 minutes. The blanks were enzyme + lactate + MB = 135 minutes, enzyme + maximum amount of coenzyme "B" + MB = 43 minutes, enzyme + coenzyme "A" + MB = 150 minutes.

Fig. 2. Comparison of coenzyme preparations "A" and "B" with the purified enzyme at p_H 7.2. This enzyme was twice precipitated with ammonium sulphate. Starting with 1 g. of the original precipitate the final volume was made up to 20 ml., and $\frac{1}{2}$ ml. of the solution was used. The minimum reduction time with coenzyme preparation "B" was $4\frac{1}{2}$ minutes. The minimum with coenzyme preparation "A" $31\frac{1}{2}$ minutes. The blanks were enzyme + lactate + MB = 6 hours + ; enzyme + coenzyme "A" + MB = 6 hours; enzyme + coenzyme "B" + MB = 51 minutes.

Fig. 1 shows curves obtained using Szent-Györgyi's enzyme; curve A with increasing amounts of coenzyme "A," curve B with coenzyme "B." With this unpurified enzyme coenzyme "A" gave an activation which was almost two-thirds of that obtained with "B."

When a more purified enzyme was used with increasing amounts of the coenzyme results were obtained which are shown in Fig. 2. Here it is seen that

maximum activation with coenzyme "A" was only one-seventh of that given by coenzyme "B."

Thus using an enzyme consisting of practically unwashed tissue both co-enzymes show similar activity. Using a purified enzyme there is a marked difference in the activation produced by the coenzyme preparations "A" and

Fig. 3.

Fig. 4.

Fig. 3. Rate of oxygen uptake using Szent-Györgyi's enzyme at p_H 7.2.

- ▲, { Right-hand cup contained enzyme + lactate + coenzyme "A"
Left-hand cup contained enzyme + lactate
- , { Right-hand cup contained enzyme + lactate + coenzyme "A"
Left-hand cup contained enzyme + coenzyme "A"
- , { Right-hand cup contained enzyme + lactate + coenzyme "B"
Left-hand cup contained enzyme + lactate
- , { Right-hand cup contained enzyme + lactate + coenzyme "B"
Left-hand cup contained enzyme + coenzyme "B"

2 ml. of enzyme were used, the lactate concentration being $M/20$. The amount of coenzyme used was that which was found necessary to give maximum activation in 3 ml. when using the methylene blue technique.

The difference between the two curves obtained with coenzyme "B" is presumably due to some blank in the coenzyme preparation, which is accounted for in ○ where it is balanced against enzyme + coenzyme.

Fig. 4. Oxygen uptake using ammonium sulphate enzyme at p_H 7.2.

- ×, { Right-hand cup contained enzyme + lactate + coenzyme preparation "B"
Left-hand cup contained enzyme + lactate
- , { Right-hand cup contained enzyme + lactate + coenzyme preparation "B"
Left-hand cup contained enzyme + coenzyme preparation "B"
- ▲, { Right-hand cup contained enzyme + lactate + coenzyme preparation "A"
Left-hand cup contained enzyme + lactate
- , { Right-hand cup contained enzyme + lactate + coenzyme preparation "A"
Left-hand cup contained enzyme + coenzyme preparation "A"

Ammonium sulphate coenzyme 1 g. made up to 18 ml. in phosphate buffer, then 2 ml. used. The lactate and coenzyme concentrations were the same as in the previous experiment.

"B." With a purified enzyme preparation "A" is practically inactive. Thus preparation "B" contains an additional factor necessary for the activation of lactic dehydrogenase. This factor is present in Szent-Györgyi's crude enzyme, and thus coenzyme preparation "A" shows activity. On purification of the enzyme

however this factor is removed and preparation "A" becomes inactive with this enzyme, while preparation "B" still remains active.

This explanation is again supported by similar results obtained by using the Barcroft technique for the measurement of the rate of oxygen uptake. The results using Szent-Györgyi's enzyme are shown in Fig. 3. The amount of coenzyme added was the same as that which was required to give maximum activation in 3 ml. when using the Thunberg technique. It is seen that coenzyme "A" gives a rate of oxygen uptake which is about half that given by using coenzyme "B."

When using the purified enzyme curves were obtained which are shown in Fig. 4. In this experiment the rate of oxygen uptake with coenzyme "A" is only one-fifth of the rate obtained by using coenzyme "B."

Thus the results obtained by the Barcroft technique support those obtained by the Thunberg technique and indicate that two factors are necessary for the activation of lactic dehydrogenase. In the Barcroft experiment just described with the ammonium sulphate enzyme the partially purified enzyme was used, not that purified by a second precipitation with $(\text{NH}_4)_2\text{SO}_4$. This was because by further purification of the enzyme in this manner we appear to remove a factor necessary for oxygen uptake by the system. This is shown in Fig. 5 where we have used the twice precipitated enzyme with coenzyme preparation "B" with and without methylene blue.

It is seen that the addition of methylene blue enables the system to take up oxygen about twice as fast as when methylene blue is absent. The system has not yet been obtained in such a state that it gave no oxygen uptake without methylene blue but these results seem to indicate that lactic dehydrogenase with its coenzymes is unable to take up oxygen by itself. Apparently it needs some system such as the cytochrome system to enable it to take up oxygen. The addition of methylene blue to systems containing the cruder enzyme has no effect on the oxygen uptake.

The possible identity of these coenzymes with cozymase.

In his latest paper Szent-Györgyi suggests that his factor is possibly identical with cozymase. During the course of this work two preparations of cozymase have been made according to the method of Euler and Myrbach [1928] and it was found that both were completely inactive as coenzyme for lactic dehydrogenase, while they showed a high activity as coenzyme for glucose dehydrogenase [Mann, 1932]. The estimation of the preparations as cozymase is at present being carried out and until this is done it cannot be claimed definitely that cozymase is inactive as the coenzyme. But from the results with one cozymase preparation which are shown in Table III and from the fact that the method of preparation of cozymase differs markedly from the preparation of lactic coenzyme the conclusion that they are not identical is fairly safe.

Table III. *Activity of cozymase as lactic coenzyme. Cozymase prepared according to Euler and Myrbach [1928]. Szent-Györgyi's crude enzyme used.*

	R.T.
0.1 mg. cozymase + enzyme + lactate + MB	4 hrs. +
0.5 mg. " "	4 hrs. +
2.5 mg. " "	4 hrs.
4.0 mg. " "	4 hrs.
Blank with lactate + enzyme + MB	4 hrs. +
1.6 mg. P.T.A. heart coenzyme + enzyme + lactate + MB	4½ mins.

Inhibition by pyruvic acid.

It is difficult to understand why for a simple reaction such as the conversion of lactic acid into pyruvic acid two coenzymes should be necessary. Is the second factor a coenzyme of this reaction or of some subsequent one? Using the Thunberg technique with purified enzyme and coenzyme preparation "A" or, as we

Fig. 5.

Fig. 6.

Fig. 5. The difference between the rate of oxygen uptake when methylene blue is added to the purified lactic dehydrogenase system.

- , { Right-hand cup contained enzyme + lactate + coenzyme preparation "B"
 Left-hand cup contained 3 ml. phosphate buffer.
 ×, { Right-hand cup contained enzyme + lactate + coenzyme preparation "B" + MB
 Left-hand cup contained 3 ml. phosphate buffer.

The enzyme was prepared by dissolving the 1st ammonium sulphate precipitate in phosphate buffer, centrifuging off the insoluble matter and then reprecipitating with ammonium sulphate. 0.5 g. of this precipitate was made up to 8 ml. and 1 ml. was used in each cup.

Fig. 6. Inhibition of the activity of lactic dehydrogenase by small amounts of pyruvic acid.

- , Coenzyme preparation "B"
 ×, Coenzyme preparation "A"

Ammonium sulphate-precipitated enzyme used; 1 g. made up to 28 ml. 0.5 ml. of this solution was used with coenzyme preparation "B." The minimum reduction time (*i.e.* without pyruvate) was $2\frac{3}{4}$ minutes and the maximum when pyruvate was added 28 minutes. The blank on coenzyme + enzyme alone was 68 minutes. 1 ml. of the enzyme solution was used with coenzyme preparation "A" and a minimum reduction time of $6\frac{3}{4}$ minutes was obtained. The maximum when pyruvate was added was 98 minutes.

The concentration of lactate was $M/10$, and 1 ml. of 1 in 5000 methylene blue was used throughout. The pyruvic acid was purified according to the method of Case [1932], and was added as the sodium salt.

believe, Szent-Györgyi's coenzyme, it was often found that for a short period reduction of methylene blue proceeded quite rapidly, after which further reduction was very slow. It therefore appeared that some substance which was being formed was inhibiting the reaction and it appeared probable that this substance might be pyruvic acid. This view was supported by results obtained from the effect of added pyruvic acid on the velocity of reduction of methylene blue in presence of $M/10$ lactate. The results are shown in Fig. 6.

It is seen from Fig. 6 that a minute concentration of pyruvate inhibited the reaction, using coenzyme "A" with the ammonium sulphate enzyme. This may

afford an explanation of the slowing off in the speed of reduction of M.B. when this coenzyme was used, as immediately a trace of pyruvic acid was formed it inhibited the reaction.

Using coenzyme "B" a much greater concentration of pyruvate was necessary to give the same percentage inhibition. It is suggested as an explanation of this that in the case of coenzyme "B" some factor was present which enabled the removal of pyruvic acid. In the case of coenzyme "A" this factor was missing.

The second factor present in the coenzyme "B" would therefore be a coenzyme for removal of pyruvic acid.

One would naturally suppose that this removal would be carried out by oxidation, but if this is so it seems that the oxidation is not accomplished by pyruvic dehydrogenase since pyruvate without lactate in the presence of enzyme and coenzyme hardly reduced methylene blue at all.

Fig. 6 shows that an apparent equilibrium or maximum inhibition with pyruvate was obtained with a pyruvate concentration between 0.002 and 0.004 *M*. This differs from the results of Bernheim [1928] who found that this "apparent equilibrium" was obtained between 0.033 and 0.066 *M* concentration of pyruvate. This was presumably due to the presence of our second factor in his enzyme. It must be borne in mind however that it has not yet been shown that the soluble lactic dehydrogenase from yeast needs a coenzyme but, as the coenzyme for heart muscle-lactic dehydrogenase occurs in relatively large amounts in yeast, it is conceivable that it does. This matter is at present under investigation.

Activity of the coenzyme preparations "A" and "B" as vitamin B₁.

The difference between the coenzymes "A" and "B" as activators of lactic dehydrogenase is accompanied by a difference in their action on vitamin B₁-deficient rats. Rats as the experimental animals have been used and the heart rate method as the method of estimating vitamin B₁ activity. Normal rats (heart rate 500–550); vitamin B₁-deficient rats (heart rate 300–350). Injection of vitamin B₁ intraperitoneally causes the heart rate of vitamin B₁-deficient rats to return to normal, usually in about 6 hours.

Injection of coenzyme "A" into vitamin B₁-deficient rats caused an almost instantaneous drop in heart rate. This supports Szent-Györgyi's opinion that his coenzyme is an adenylic acid, as Drury and Szent-Györgyi [1929] have shown that certain adenine compounds, notably adenosine and adenylic acid, caused bradycardia in normal rats when injected. Using coenzyme "A" this drop in the heart rate lasted for 1–2 hours depending on the amount injected and then the rate returned to nearly its original value. It showed no rise above its original value and therefore no vitamin B activity.

In these experiments that amount of coenzyme which produced maximum activation in a total volume of 3 ml. in a M.B. tube was called one dose.

Using the coenzyme "B," if 9 doses were injected a similar drop in heart rate was produced to that obtained with coenzyme "A," showing that the adenine compound was again present. The heart rate returned to its original value after 1–2 hours, and tended to rise slightly above the original value, but this slight rise was only maintained for a few hours. Using half the amount the drop was less, and the subsequent rise was quicker, the heart rate returning to its original value in about half an hour and continuing to rise above its original value during the next 2 or 3 hours. This seemed to show some definite curative effect.

Using 2½ doses, while the initial drop in heart rate was smaller and recovery to original rate more rapid, the subsequent rise in heart rate was much smaller than with 4½ doses. These results are shown in Fig. 7.

The curative effect of the coenzyme "B" was limited to a few hours whereas injection of vitamin B₁ with or without the coenzyme gave a curative effect lasting for several days.

This was shown in Fig. 8 where 9 doses of coenzyme "B" and vitamin B₁ were injected simultaneously.

Fig. 7.

Fig. 8.

Fig. 7. Effect of coenzyme preparation "B" on the heart rate of vitamin B₁-deficient rats. One dose is the amount of coenzyme necessary to give maximum activation of the enzyme in 3 ml.

- , 9 doses injected intraperitoneally
- ×, 4½ doses injected intraperitoneally
- , 2½ doses injected intraperitoneally

The coenzyme preparation used in this experiment was prepared by a slight modification of scheme B. The original yeast extract was first of all treated with acid clay at p_H 4.5 in order to adsorb as much vitamin B₁ as possible. The acid clay was then filtered off and the filtrate treated according to scheme B. The coenzyme is apparently not adsorbed by the acid clay.

Fig. 8. Injection of 9 doses of coenzyme preparation "B" + vitamin B₁ concentrate. Approximately two international units of vitamin B₁ were used.

It seems from this evidence therefore that if vitamin B₁ occurred in the coenzyme it only occurred in a minute amount, and it appears doubtful whether the rise in heart rate produced was due to vitamin B₁. It is therefore unlikely that vitamin B₁ or any compound containing it participates in the oxidation of lactic acid by the systems described in this paper. Nevertheless from Peters's evidence, and from the fact that a temporary cure of the bradycardia is produced by coenzyme "B" it is possible that it is this second factor, which is missing in vitamin B₁ deficiency; but it is obviously useless to form any rigid theory until more evidence has been obtained.

SUMMARY.

A soluble cell-free lactic dehydrogenase from heart muscle has been prepared in a less impure form than hitherto obtained. By using this enzyme it has been shown that two factors are necessary for its activation. Methods for the preparation from yeast of the first factor, which seems to be identical with Szent-

Györgyi's coenzyme, and for a mixture of the two factors have been worked out. Neither of the factors appeared to be identical with cozymase.

Using the system enzyme + lactate + mixture of two coenzymes, addition of methylene blue was found to be necessary before the system could take up oxygen.

Some evidence has been obtained which tends to show that the second coenzyme may be concerned with the removal of pyruvic acid.

The coenzymes have been obtained free from vitamin B₁ but the second factor seems to show some temporary curative effect on the bradycardia produced in vitamin B₁-deficient rats.

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