CCX. THE LACTIC DEHYDROGENASE OF ANIMAL TISSUES.

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Meyerhof [1919] first observed the oxidation of lactic acid in presence of muscle and demonstrated the participation of a coenzyme in this oxidation. Szent-Györgyi and his co-workers [Szent-Györgyi, 1925, 1, 2; 1930; Banga *et al.* 1931; 1932; 1933] then studied in great detail the properties of the lactic coenzyme prepared from heart muscle and went a long way towards elucidating its chemical nature. Since the first description of the lactic dehydrogenase of animal tissues, some hundreds of papers have been written on the subject—most of which are discussed in Euler's monograph [1934]. But despite the vast accumulation of literature, definite information is lacking concerning the most fundamental characteristics of the lactic dehydrogenase, e.g. the nature of the oxidation product, the mechanism of the reaction with molecular oxygen, the quantitative distribution in various tissues, the role of the coenzyme etc. The difficulty which has impeded progress in the systematic investigation of this enzyme has been the inability of the various investigators to prepare an extract which could oxidize lactic acid aerobically with an appreciable velocity. In consequence, the study has been carried out mainly with minced tissue which allows of but a limited analysis; and in those cases where active extracts were prepared, the oxidation of lactic acid was measured only anaerobically in Thunberg tubes.

The purpose of the present investigation was to analyse the conditions necessary for the aerobic oxidation of lactic acid by the dehydrogenase system of animal tissues and to study in detail the properties and characteristics of the different components of the catalytic system. One of the significant conclusions which may be drawn from our study is that short-duration methylene blue experiments may present an unreliable account of the oxidation process, and that no study of dehydrogenases based entirely on simple methylene blue tube experiments can throw much light on the question of mechanism of reaction.

I. *Preparation of the components.*

Enzyme. The heart of the pig offers the most convenient source of the lactic dehydrogenase. The method of preparation however is generally applicable to the various tissues of any animal. Four hearts are divested of fat and connective tissue, passed through a Latapie mincer and washed exhaustively with tap water until the washings are haemoglobin-free. The washed mince is mixed with sand and 500 ml. $M/50$ phosphate buffer of pH 7 and ground to a fine paste in a mechanical mortar. The sand and insoluble debris are filtered off through muslin. The filtrate is centrifuged hard for 20 min. and the sediment resuspended in 200 ml. $M/5$ phosphate buffer of pH 7·2 (solution A) whilst the supernatant fluid is treated with 50 ml. $M/2$ acetate buffer of pH 4·6, and the flocculent precipitate centrifuged. The supernatant is discarded and the precipitate resuspended in 200 ml. $M/5$ phosphate buffer pH 7·2 (solution B). Both solutions contain an

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active lactic dehydrogenase. Usually the greater activity is found in solution B but the reverse has been observed in a few instances. Solutions A and B may be clarified extensively by prolonged centrifuging with little loss in activity. The enzyme is soluble but under certain conditions may be removed from solution by adsorption on impurities. The enzyme may be kept active for a long period in the form of a powder by drying *in vacuo* the centrifugates which are used to prepare solutions A and B respectively. In solution the enzyme retains the greater part of the original activity for 5–8 days when kept at 0°.

It is not necessary to use phosphate buffer exclusively. Bicarbonate, glycine and borate buffers are also satisfactory for most purposes.

Coenzyme. Baker's yeast is particularly rich in the coenzyme for the lactic dehydrogenase of animal tissues. The method of preparation follows closely that of Myrbäck [1933] for the preparation of cozymase. 9 kg. of yeast are crumbled into 6 litres of 2 % sulphuric acid maintained at 80°, and the mixture is vigorously stirred for 15 min. The yeast is filtered off on large Büchner funnels. 2 litres of saturated lead acetate (slight excess of reagent) are added to the filtrate and the precipitate filtered off and discarded. The filtrate is mixed with 700 ml. of saturated lead acetate, and 3·5 litres of hot saturated baryta are cautiously added to bring the *p*H to 9 (blue to thymolphthalein). The lead precipitate is filtered, washed thoroughly with water and decomposed in 3 litres of 0·6 *N* sulphuric acid. The lead sulphate is removed by filtration, and the filtrate is mixed with 300 ml. of a 25 % solution of phosphotungstic acid. Part of the precipitate is more or less colloidal and defies direct filtration. However, by impregnating the filter paper with a small amount of kieselguhr, all the precipitate is held back and the filtrate is perfectly clear. The filtration is extremely slow. The phosphotungstate precipitate is decomposed by suspending in 500 ml. of 10 % sulphuric acid and shaking the suspension vigorously with 2 volumes of a mixture of equal parts of amyl alcohol and ether. Usually an emulsion is formed which does not easily settle out. In that event, it must be centrifuged. The extraction is repeated with half the original volume of amyl alcohol and ether. The aqueous layer is filtered and the sulphuric acid removed with 1200 ml. of cold saturated baryta (final *p*H = 5). The deep yellow-brown colour observed in the previous stages is removed by the barium sulphate precipitate, and the filtrate now appears pale yellow. The volume is brought down by distillation *in vacuo* to 800 ml. and the slight precipitate filtered off. The *p*H is now adjusted to 7 with NaOH. This solution is stable for months when kept at 0°. It is advisable to use octyl alcohol as a preservative. 0·3 ml. or less of the coenzyme solution will activate 1 ml. of the lactic enzyme practically maximally.

The exact quantities used in different preparations are somewhat variable, and should be redetermined in each isolation experiment by small scale trials.

II. *The condition for linear oxidation.*

Suppose that given solutions of enzyme, coenzyme, lactate and methylene blue are mixed and the oxygen uptake measured. A rapid uptake is found to ensue for 5–10 min. and then the rate falls off sharply, so that at the end of 1 hour the final uptake is only slightly greater than at the end of the first few minutes. This effect can be explained in either of two ways: the enzyme is rapidly destroyed, or some product of the reaction inhibits the enzyme very strongly. It seemed more likely that the second alternative was the correct one and that the product of the oxidation of lactic acid, presumably pyruvic acid, was the agent responsible for the inhibition. Therefore if some means could be found of removing or fixing the pyruvic acid, the oxygen uptake should be linear for a

considerable period. Cyanide is known to combine with ketones and Fig. 1 shows that in presence of this reagent, the rate of oxidation does not fall off appreciably before the first half hour. The rate of oxidation is increased as the strength of the cyanide is increased until a maximum concentration is reached—beyond which the enzyme is inhibited. Apparently the equilibrium point of the reaction

$$\text{Pyruvic acid} + \text{HCN} \rightleftharpoons \text{pyruvic cyanohydrin}$$

is not entirely to the right of the equation, and it is only with fairly high concentrations of cyanide that most of the pyruvic acid is fixed.

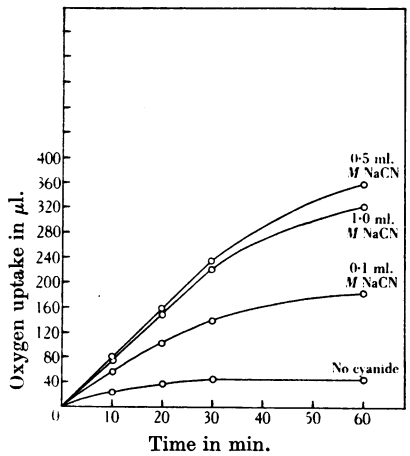

Fig. 1. Effect of cyanide on rate of oxidation of lactate. 1 ml. enzyme, 0·5 ml. coenzyme, 0·2 ml. 2*M* lactate and 0·1 ml. 0·5% methylene blue in each experimental manometer cup (water in the control cup). The lactate was placed in a dangling Keilin tube and was introduced after equilibration.

If the action of cyanide truly consists in combining with the pyruvic acid formed in the reaction, then the simultaneous addition of a large excess of pyruvate should abolish the cyanide effect whereas a small amount of added pyruvate should have little or no effect. Such is actually the case (Table I). It is

Table I. *Effect of pyruvate in presence and absence of cyanide.*

Each manometer cup contained 1 ml. enzyme, 0·2 ml. 2*M* lactate, 0·5 ml. coenzyme and 0·1 ml. 0·5% methylene blue.

	Oxygen uptake in μl.	
	10 min.	20 min.
Enzyme system + 0·5 ml. *M* NaCN	79	149
Enzyme system + NaCN + 0·5 ml. 0·05 *M* pyruvate	74	157
Enzyme system + NaCN + 0·5 ml. 0·5 *M* pyruvate	19	39
Enzyme system + 0·5 ml. 0·05 *M* pyruvate	6	9

interesting to note that 0·05 *M* pyruvate almost completely inhibits the oxidation of lactic acid in absence of fixing agent although this concentration of pyruvate in presence of cyanide is without effect.

Hydroxylamine and hydrazine can also act in the capacity of pyruvic fixatives providing they are used in non-toxic concentrations (Table II). Dimedon, ammonia and semicarbazide have no action.

The experiments with cyanide have an interesting physiological significance. If the oxidation of lactate is to proceed linearly in the animal cell, there must be

Table II. *Hydroxylamine and hydrazine as pyruvic fixatives.*

Control contains 1·5 ml. enzyme, 0·3 ml. coenzyme, 0·2 ml. 2*M* lactate and 0·1 ml. 0·5% methylene blue.

	Oxygen uptake in μl.	
	10 min.	30 min.
Control	15	24
+0·5 ml. *M*/10 hydroxylamine sulphate	50	116
+0·5 ml. *M* hydroxylamine sulphate	6	21
+1 ml. *M*/20 hydrazine sulphate	32	77
+1 ml. *M*/2 hydrazine sulphate	0	0

some mechanism for the rapid removal of pyruvic acid, otherwise the oxidation would be prevented. Peters & Thompson [1934] have shown that pyruvic acid accumulates in avitaminous pigeon brain and this pyruvic acid largely disappears upon the addition of vitamin B_1. Peters and his co-workers have in their past work also established that the rate of oxidation of lactate in avitaminous brain is subnormal. Through the courtesy of Prof. R. A. Peters we were able to test the action of pure vitamin B_1 on the isolated lactic system. The findings were negative as far as showing that the vitamin either prevents the accumulation of pyruvic acid or protects the lactic enzyme against the poisoning action of pyruvic acid.

III. *The product of oxidation.*

2:4-Dinitrophenylhydrazine does not precipitate pyruvic acid in presence of excess cyanide. However, the hydroxylamine compound of pyruvic acid is much more easily decomposed. The isolation experiment was therefore carried out with hydroxylamine as the fixing agent. 200 ml. of enzyme, 30 ml. of coenzyme, 20 ml. of 2*M* lactate, 30 ml. of *M* hydroxylamine and 5 ml. of 0·5% methylene blue solution were mixed and aerated vigorously at 37° for 5 hours. After deproteinization with trichloroacetic acid and concentration *in vacuo* to 100 ml., 1 g. of 2:4-dinitrophenylhydrazine dissolved in 100 ml. of 2*N* HCl was added. The solution was allowed to stand at 0° for several hours before the crystalline precipitate was filtered off (yield 1·3 g.). After two recrystallizations from ethyl acetate the M.P. was found to be 215·5° which is identical with that of the hydrazone of pyruvic acid. The mixed M.P. was also 215·5°. The following are the analysis figures (Weiler). (C, 40·49; N, 21·07; H, 2·93%. $C_9H_8O_6N_4$ requires C, 40·28; N, 20·90; H, 3·01%.)

If pyruvate is the sole product of the oxidation of lactate, then for each mol. of lactate oxidized, 1/2 mol. of oxygen should be taken up. Fig. 2 shows the oxygen equivalence of lactate in presence of the enzyme system. Actually the limiting value of 1 atom of oxygen is not reached within 5 hours. The velocity of oxidation falls off very rapidly with decreasing concentration of substrate and in practice only as much as 70% of the theoretical uptake is obtained before complete destruction of the enzyme sets in. Since racemic lactate was used, the theoretical oxygen requirement was calculated on the basis that only half the lactate is oxidized.

IV. p*H*.

The lactic enzyme is unusually sensitive to the constituents of buffer mixtures, and since a variety of buffers is necessary to cover the *p*H range from 4 to 13, the *p*H curve accordingly appears discontinuous (Fig. 3). It must be abundantly clear that deductions from the shapes of *p*H curves as to the dissociation of enzyme substrate complexes are not always reliable.

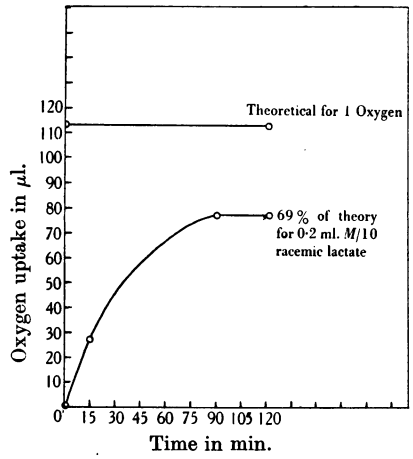

Fig. 2.

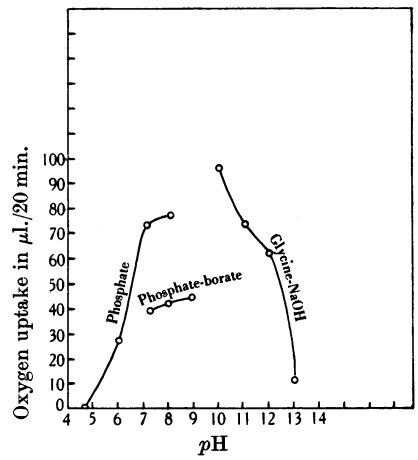

Fig. 3.

Fig. 2. The number of oxygen equivalents involved in the oxidation of lactate. The experimental manometer cup contained 1·5 ml. enzyme, 0·3 ml. coenzyme, 0·4 ml. *M* NaCN (neutral) and 0·1 ml. 1% pyocyanine hydrochloride. The control without lactate was completely negative. The lactate solution was prepared from pure lactic acid syrup, the composition of which was checked by titration.

Fig. 3. The effect of *p*H. After precipitation with acetate buffer, the enzyme was suspended in water and divided into 12 aliquot portions each of which was brought to a given *p*H. The final volumes after the adjustment of *p*H were the same in all cases. The manometer cups contained 0·5 ml. neutralized enzyme, 2 ml. *M*/5 buffer, 0·3 ml. coenzyme, 0·2 ml. lactate (in Keilin cups) and 0·1 ml. 0·5% methylene blue.

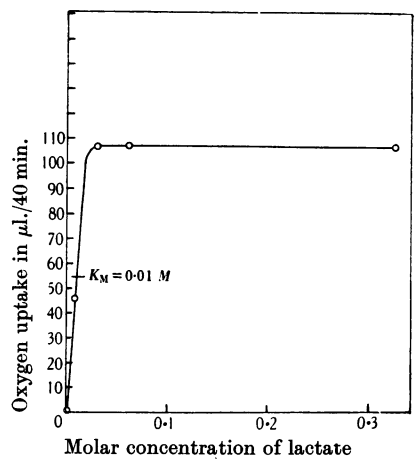

Fig. 4.

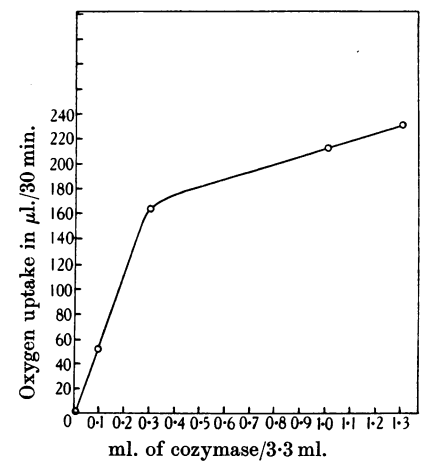

Fig. 5.

Fig. 4. Effect of the concentration of lactate on the rate of oxidation. 1·5 ml. enzyme, 0·3 ml. coenzyme, 0·1 ml. 0·5% methylene blue and 0·4 ml. *M* NaCN were placed in all the experimental cups.

Fig. 5. Effect of the concentration of coenzyme on the rate of oxidation. 1·5 ml. enzyme, 0·1 ml. 0·5% methylene blue, 0·4 ml. *M* NaCN and 0·2 ml. 2 *M* lactate were placed in all the experimental cups.

V. *Effect of concentration of lactate and coenzyme.*

Fig. 4 shows the dependence of the rate of oxidation on the concentration of lactate. Below $M/150$ the rate falls off rapidly, which means in effect that very small quantities of lactate are not oxidized with an appreciable velocity. The Michaelis constant is approximately $M/150$.

There is no critical concentration of coenzyme below which the rate of oxidation of lactate falls sharply (Fig. 5). The curve is linear at low concentrations of coenzyme and gradually approaches the value for the maximum rate of oxidation. It should be pointed out that an amount of coenzyme sufficient to saturate the enzyme in experiments with dilute methylene blue is insufficient when the methylene blue concentration is maximum for the activity of the enzyme.

VI. *Specificity of donator.*

The enzyme preparation in presence of the coenzyme oxidizes *l*(+)-lactate [180], β-hydroxypropionate [66], malate [200], fumarate [236], α- [24], β- [108] and γ- [6] hydroxybutyrate. The oxidations were tested for both anaerobically and aerobically with the same results. A comparison was made of the relative speeds of oxidation of these substrates in equimolar concentrations. The numbers in brackets refer to the oxygen uptake in μl./30 min. in presence of enzyme, coenzyme, methylene blue and cyanide. Fumarate, *l*(+)-lactate, malate and β-hydroxybutyrate are rapidly oxidized whereas α- and γ-hydroxybutyrates show only slight activity.

The following substances are not oxidized: *d*(−)-lactate, lactamide, malonate tartronate, glycerate, glycollate, mandellate, 3-phosphoglycerate, 2-phosphoglycerate, gluconate, α-glycerophosphate, acetate, alcohol, propionate, oxalate, acetaldehyde, pyruvate, citrate, serine, *iso*serine and glucose. It is interesting to note that lactamide ($CH_3.CH(OH).CONH_2$) and *iso*serine ($CH_2NH_2.CH(OH).COOH$) are not oxidized to the slightest extent although their chemical resemblance to lactic acid is very close.

The optical isomerides of lactic acid were prepared (*a*) by the morphine method and (*b*) by the resolution of the zinc ammonium salts. The *d*(−)-salt prepared by the two independent methods was inactive (Table III). We are grateful to the

Table III. *Oxidation of* d(−)- *and* l(+)-*lactate.*

Enzyme system: 1·5 ml. enzyme; 0·5 ml. coenzyme; 0·2 ml. 0·5% methylene blue and 0·4 ml. *M* NaCN (neutral).

	O_2 uptake in μl./30 min.	
	Morphine lactate	Zinc lactate
Enzyme system + 0·5 ml. *M*/5 *d*(−)-lactate	0	0
Enzyme system + 0·5 ml. *M*/5 *l*(+)-lactate	90	85

Distillers Co. Research Dept. for specimens of the pure *d*- and *l*-zinc ammonium salts.

The lack of chemical similarity between fumarate and lactate made it improbable that the same enzyme system was responsible for the oxidation of both substrates. To test that point, the following experiment was performed. The times for the reduction of methylene blue in presence of the original enzyme system and the various substrates were measured (Table IV). The enzyme was then purified by treating thrice with kaolin, and the times of reduction again measured. If the same enzyme were involved in all these oxidations, the ratio of

Table IV. *Specificity of the enzyme.*

Each Thunberg tube contained 1 ml. enzyme, 0·2 ml. *M* substrate, 1 ml. water and 1 ml. 20 mg./100 ml. methylene blue (in side-bulb).

	Reduction time of methylene blue		
	Original preparation	Treated 3 × with kaolin	Treated 3 × with kieselguhr
Lactate	3 min. 15 sec.	6 min. 30 sec.	5 min.
Malate	2 ,, 30 ,,	21 ,,	14 ,,
Fumarate	9 ,,	50 ,,	24 ,,
β-Hydroxypropionate	7 ,, 30 ,,	120 ,,	∞
β-Hydroxybutyrate	4 ,, 10 ,,	40 ,,	∞

the various rates should be the same regardless of the degree of purification of the enzyme preparation. The results accordingly indicate that the β-hydroxypropionate, fumarate, β-hydroxybutyrate and malate enzymes are not identical with the lactic enzyme. The same type of result is observed when the enzyme preparation is purified by treatment with kieselguhr. The extensive change after purification in the ratio of the rate of oxidation of lactate to the rate of oxidation of the other substrates is not a final proof of non-identity. The problem of the number of enzymes involved will be further considered in papers dealing with the fumarate and β-hydroxybutyrate enzymes.

A lactic enzyme can be easily prepared from an autolysed suspension of *Bact. coli* by the method of Stephenson [1928] and from baker's yeast by the method of Ogston & Green [1935]. A comparison was therefore made of the specificities of lactic enzyme preparations from different sources (Table V). The

Table V. *Specificity of the lactic preparations from heart,* Bact. coli *and yeast.*

	Heart	*Bact. coli*	Yeast
d(−)-Hydroxypropionate	0	+ + + +	+
l(+)-Lactate	+ + + +	+ +	+ + + +
β-Hydroxypropionate	+ + +	0	+ +
Malate	+ + + +	0	+ + +
α-Hydroxybutyrate	+	+ + + +	+ + + +
β-Hydroxybutyrate	+ + + +	0	0
γ-Hydroxybutyrate	+	+	0

0 indicates no oxidation. + indicates oxidation.

animal enzyme preparation is the only one to show complete optical specificity. *Bact. coli* enzyme preparation oxidizes *d*(−)-lactate more rapidly whereas the yeast preparation preferentially attacks *l*(+)-lactate. It is interesting to note that intact *Bact. coli* and yeast oxidize both isomerides at the same speed. That would suggest that there is an enzyme for each isomeride, and that in the method of preparation one is more easily destroyed than the other.

The facts that the *Bact. coli* enzyme preparation does not oxidize β-hydroxypropionate or malate and that the yeast preparation does not oxidize β-hydroxybutyrate agree with the above conclusion that these three substrates are not oxidized by the particular enzyme of heart which catalyses the oxidation of lactate. The possibility however still remains that the specificities of lactic enzymes from various sources are not all the same and that the inability of the *Bact. coli* lactic preparation to oxidize malate may constitute no proof that the heart lactic enzyme similarly lacks this property.

VII. *Respiratory carriers and the reaction with molecular oxygen.*

A solution containing the enzyme, coenzyme and lactate does not react with molecular oxygen. The addition of a trace of methylene blue, pyocyanine or any other suitable oxidation-reduction indicator immediately catalyses the reaction with oxygen. The lactic dehydrogenase must therefore be considered as anaerobic in the sense that the reaction with oxygen is not direct but requires the intermediation of some respiratory carrier.

Table VI. *Carriers for the aerobic oxidation of lactate.*

Each manometer contained 1·2 ml. enzyme, 0·3 ml. coenzyme and 0·2 ml. 2 *M* lactate.

	μl. O_2/30 min.	Fixing agent
Control + NaCN	0	NaCN
+ 0·1 ml. 0·5% methylene blue chloride	200	,,
+ 0·1 ml. 0·5% pyocyanine hydrochloride	310	,,
+ 0·1 ml. 0·5% flavin	152	,,
+ 1 ml. 10% yellow pigment	20	,,
+ 0·1 ml. 0·5% adrenaline	196	,,
Control + hydroxylamine	15	Hydroxylamine
+ 1 ml. 0·001 *M* cytochrome	15	,,
+ 0·5 ml. 0·5% glutathione (S–S)	16	,,
+ 0·5 ml. 0·5% ascorbic acid	25	,,
+ 0·5 ml. 0·5% ascorbic acid (without lactate)	8	,,

Table VI contains a list of substances which were tested as respiratory carriers. With the exception of methylene blue and pyocyanine, these substances are normal constituents of animal cells. Flavin and adrenaline show high catalytic activity; yellow pigment is only slightly active, whereas cytochrome *c*, glutathione and ascorbic acid are inactive. Hydroxylamine must be used as the fixing agent when cytochrome, glutathione and ascorbic acid are studied since cyanide interferes with their catalytic activity (i.e. the activity in other systems in which they are known to function).

The variation of the rate of oxidation with change in the concentration of pyocyanine, methylene blue and flavin respectively is shown in Figs. 6, 7 and 8. Since the experiments with each carrier were done at different times and with enzyme preparations of different activities, the final limiting velocities are not identical in the three cases. It is interesting that in all cases at least half the maximum velocity is reached at a concentration of 0·25 mg. or less per 3·3 ml. Pyocyanine functions maximally in even lower concentration. With excess of pyocyanine inhibition of the enzyme sets in.

Wagner-Jauregg *et al.* [1934, 1, 2, 3; 1935] and Euler & Adler [1934; 1935] have proposed the theory that the yellow pigment (yellow enzyme) forms an essential component of many dehydrogenase systems. The experimental basis rests entirely on the following observations with methylene blue tubes. The time of decoloration of methylene blue by various enzyme systems is considerably shortened by the addition of yellow pigment. The same holds true for the reduction of flavin. These facts they interpreted to mean that the reduction of both methylene blue and flavin proceeds through the yellow pigment as an intermediary. Our experiments with the lactic system show that this conclusion of Wagner-Jauregg and of Euler and Adler is definitely incorrect. From Figs. 7 and 8 it is clear that, within limits, the higher the concentration of flavin or methylene blue, the more rapid is the rate of oxidation of lactate. When the concentrations become very small—of the order of magnitude of the concentrations used in methylene blue experiments ($< 50\gamma$)—then the catalytic activities of

both flavin and methylene blue become enormously reduced and approach zero. In effect that means that very small quantities of both methylene blue and flavin are reduced extremely slowly by the lactic dehydrogenase system. Suppose that a substance could be found with a very low Michaelis constant (i.e. it

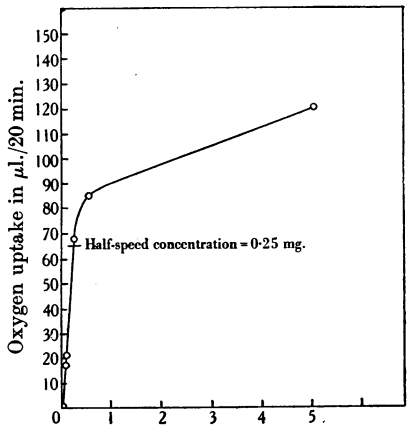

Fig. 6.

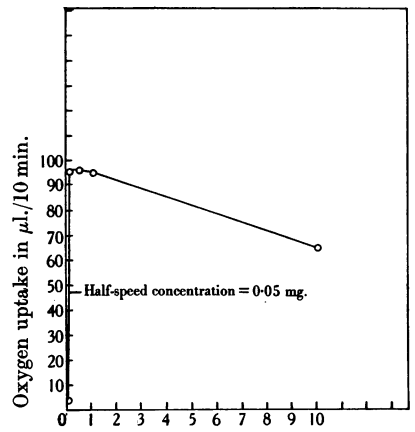

Fig. 7.

Fig. 6. Effect of concentration of methylene blue chloride on the rate of oxidation. 1·4 ml. enzyme, 0·3 ml. coenzyme, 0·4 ml. *M* NaCN and 0·2 ml. 2*M* lactate in all the experimental cups.

Fig. 7. Effect of concentration of pyocyanine hydrochloride on the rate of oxidation. 1·4 ml. enzyme, 0·3 ml. coenzyme, 0·2 ml. 2*M* lactate and 0·4 ml. *M* NaCN in all the experimental cups.

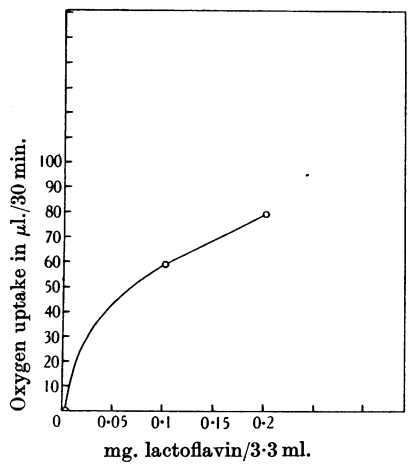

Fig. 8. Effect of concentration of lactoflavin on rate of oxidation. 1·5 ml. enzyme, 0·3 ml. coenzyme, 0·4 ml. *M* NaCN and 0·2 ml. 2*M* lactate in all the experimental cups.

was able to function maximally at high dilutions) which in the reduced form could reduce methylene blue or flavin. Then the addition of such a substance to the lactic dehydrogenase system or any of the other coenzyme systems would result in a more rapid reduction of flavin or methylene blue when they were present in small concentration ($<50\gamma$) but not when they were in a sufficiently

high concentration to allow the enzyme to work at maximum efficiency. Yellow pigment has the properties of this hypothetical substance. It can be reduced by the dehydrogenase system and in the reduced form can reduce in turn either flavin or methylene blue. Furthermore, the Michaelis constant is very low indeed, which means that the rate of reduction of yellow pigment is constant down to high dilution. The yellow pigment effect must therefore be interpreted in terms of affinity constants and offers no support of the theory that methylene blue or flavin is not reduced directly by coenzyme systems but only through the intermediation of the yellow pigment.

The curious fact is that even a large excess of yellow pigment shows a slight catalytic activity (Table VII) hardly comparable with the activity in presence of excess of either flavin or methylene blue. Furthermore, in aerobic experiments,

Table VII. *Effect of yellow pigment on enzyme purified with kaolin.*

1·5 ml. enzyme, 0·3 ml. coenzyme, 0·3 ml. *M* NaCN (neutral) and 0·2 ml. 2*M* lactate in each experiment.

	μl. O_2/30 min.
Control	0
+0·1 ml. 0·5% methylene blue	63
+0·5 ml. 5% yellow pigment	19
+yellow pigment+methylene blue	67
+0·5 ml. 0·5% lactoflavin	50
+lactoflavin+yellow pigment	48

the addition of yellow pigment does not increase the flavin effect over a wide range of flavin concentrations. This comparative inactivity of yellow pigment with the lactic system is rather unexpected and requires further study. The pigment was prepared from bottom yeast by the method of Warburg & Christian [1933]. We are indebted to Prof. O. Warburg for his generosity in placing at our disposal a sample of a relatively pure preparation. Both our preparation and the authentic gave the same results.

The analysis of the adrenaline effect will be dealt with in a separate communication by Green and Brosteaux. The essential facts may be summarized as follows. The lactic system reacts vigorously with oxygen in presence of adrenaline. If any of the components of the lactic system are omitted, e.g. the coenzyme or lactate, the adrenaline effect disappears. The results of many control experiments show conclusively that adrenaline is acting as a respiratory catalyst in the lactic system. The mechanism of the catalysis is very complex. Adrenaline is the precursor of the actual catalytic system. Upon addition of adrenaline to the complete lactic system, a red colour rapidly develops and persists throughout the reaction. This red substance can be reduced and oxidized reversibly.

There is an additional factor concerned in the adrenaline effect with the lactic system. Its function is to catalyse the aerobic oxidation of adrenaline to the red substance. This oxidation depends upon the simultaneous oxidation of lactate to pyruvate. In absence of the specific factor, adrenaline has no action.

Any of the dehydrogenase systems which require the lactic coenzyme can utilize adrenaline as a respiration carrier. The adrenaline effect seems to be a general property of lactic coenzyme systems.

It is impossible from experiments *in vitro* to decide which of the natural carriers of animal source are involved in the oxidation of lactate within the living cell. There is presumably sufficient of flavin, adrenaline and yellow pigment in most cells to account for the observed rate of oxidation of lactate. But in the absence of experiments carried out on intact cells, no definite conclusions can be

drawn. The cyanide-insensitivity of the isolated lactate system in contrast to the cyanide-sensitivity of the lactate oxidation in tissue slices (Table VIII) may indicate that some carrier other than flavin, yellow pigment and adrenaline is

Table VIII. *Cyanide inhibition of lactate oxidation in tissue slices.**

Pigeon kidney	Q_{O_2}	Rabbit brain	Q_{O_2}
Control	−10·5	Control	− 5·9
+ *M*/500 cyanide	− 2·7	+ *M*/500 cyanide	− 1·3
+ *M*/125 lactate	−18·4	+ *M*/125 lactate	−10·9
+ lactate + cyanide	− 2·9	+ lactate + cyanide	− 1·7
		+ lactate + 0·5 mg. lactoflavin	−10·5
		+ lactate + lactoflavin + cyanide	− 1·0

* These experiments were kindly carried out for us by Dr L. F. Leloir.

involved physiologically. Another possibility remains that it is not the oxidation of lactate but that of pyruvate which is poisoned by dilute cyanide. The accumulation of pyruvate in presence of the reagent will thereby prevent the oxidation of lactate.

A comparison of the lactic dehydrogenase of animal cells with that of yeast reveals some very instructive differences. The former requires a coenzyme and reacts with flavin but not with cytochrome. The latter requires no coenzyme, and reacts with cytochrome but not with flavin [Ogston & Green, 1935]. It may be taken as a general rule that cytochrome is inactive in systems requiring a coenzyme. Conversely flavin is inactive in systems which do not require a coenzyme. There appears to be a fundamental distinction between the coenzyme-flavin systems and the cytochrome systems. Adrenaline shows the same behaviour as flavin in respect of its activity with the coenzyme and non-coenzyme systems. The three dehydrogenase systems which are known to react with molecular oxygen *via* cytochrome (yeast lactate, succinate and α-glycerophosphate of animal source) do not require a coenzyme. Conversely the dehydrogenase systems that react with flavin or yellow pigment (hexosemonophosphate, lactate, malate, glucose and alcohol) are incapable of reducing cytochrome with any appreciable velocity. The explanation of these curious facts will be dealt with in later sections.

VIII. *Reduced coenzyme and the mechanism of coenzyme action.*

The mechanism of the action of coenzymes in enzymic dehydrogenations—a long-standing biochemical puzzle—has become remarkably clear as the result of the recent brilliant study by Warburg *et al.* [1935]. Very convincing evidence has been adduced to show that the following series of events takes place in the reaction between hexosemonophosphate and oxygen:

(1) hexosemonophosphate + coenzyme $\xrightarrow{\text{enzyme}}$ phosphohexonate + reduced coenzyme,
(2) reduced coenzyme + yellow pigment → coenzyme + leuco-yellow pigment,
(3) leuco-yellow pigment + $\frac{1}{2}$ O_2 → yellow pigment.

The coenzyme is therefore to be considered as a highly specific carrier linking the dehydrogenase with the yellow pigment. The reversible reduction of the coenzyme can be effected not only enzymically but also with hydrosulphite. Reduced coenzyme is not autoxidizable. In the reconstructed system, the oxidation is brought about by yellow pigment.

The researches of Warburg *et al.* were confined to the coenzyme of red blood cells which is not identical with the coenzyme of lactic dehydrogenase (cf.

section X). It was therefore of interest to inquire how general is the behaviour of the Warburg coenzyme and to what extent the above scheme is applicable to the lactic enzyme. The results of the inquiry indicate that the mechanism of the action of the lactic enzyme closely parallels that of the Warburg coenzyme.

The lactic coenzyme prepared as in section I does not reduce methylene blue or flavin. However, by adding hydrosulphite to a neutral buffered solution of the coenzyme and by then oxidizing excess hydrosulphite in a stream of air, a solution of coenzyme is obtained which reduces methylene blue and flavin; e.g. 1 ml. reduced coenzyme reduced 0·2 ml. 0·02 % methylene blue in 10 min. and 0·1 ml. 0·05 % lactoflavin in 80 min. A trace of yellow pigment decreased the times of reduction of methylene blue and flavin to 1·5 and 10 min. respectively. Controls of buffer and of alkali-inactivated coenzyme treated in exactly the same way failed to show this reducing power. The presumption is therefore reasonable that hydrosulphite reduces the coenzyme and that reduced coenzyme is the agent responsible for the reduction of methylene blue. The catalytic effect of yellow pigment offers additional confirmation of this view.

The lactic coenzyme can also be reduced by the complete dehydrogenase system. If the coenzyme is incubated with a mixture of enzyme, lactate and cyanide for several minutes, and the mixture is then boiled and filtered through kieselguhr, a solution of reduced coenzyme is obtained which shows the same properties as that of reduced coenzyme prepared by the hydrosulphite method.

Assuming that Warburg's theory applies to the lactic coenzyme, it follows that any carrier which is active with the whole dehydrogenase system should be reduced by reduced coenzyme and conversely carriers which have no activity should not be reduced with any appreciable velocity. This prediction is in fact fulfilled. Reduced coenzyme can reduce methylene blye, pyocyanine, flavin, the red oxidation product of adrenaline and yellow pigment but has no action on cytochrome *c*. The evidence is therefore suggestive that in the reaction between lactate and oxygen, the coenzyme is reduced, and in turn reduces the carrier which finally reacts with molecular oxygen. The problem—which carriers of animal tissues are active with coenzyme dehydrogenase systems—simplifies to the problem—which carriers are reduced by the reduced coenzyme. It is predicable that all systems which require the lactic coenzyme will react with flavin, yellow pigment or adrenaline but not with cytochrome. The possibility of interaction is determined not by the enzyme but by the coenzyme which is the factor in common.

The suggestion has been made that the lactic dehydrogenase of yeast although independent of a soluble coenzyme is rigidly bound to its coenzyme and hence cannot be resolved into the two components by the ordinary methods. The suggestion is not tenable in view of the fact that the reduced coenzyme does not reduce cytochrome whereas the yeast enzyme reacts vigorously with molecular oxygen in presence of cytochrome. Similarly the reduced lactic coenzyme reduces flavin and yellow pigment whereas these two substances have no catalytic action on the yeast lactic system.

Several properties of reduced coenzyme are worth mentioning. Acid solutions rapidly destroy reduced coenzyme, although strong alkali at 100° has no effect. The reverse is true for the oxidized coenzyme. The reduced coenzyme shows a broad adsorption band at 330–340 mμ in the ultraviolet whereas the oxidized coenzyme shows only small absorption in this region. Dr E. van Heyningen kindly carried out the ultraviolet measurements. Lastly, reduced coenzyme is as active as oxidized coenzyme with the dehydrogenase system. This fact is consistent with the theory of the mechanism of coenzyme action.

Given an excess of enzyme, the limiting factor in the oxidation of lactate should be the rate of reaction between reduced coenzyme and the carrier. Since the enzyme is not involved in this process, it follows that reduced coenzyme should react with flavin or methylene blue at the same speed as in presence of the enzyme. Actually such is not the case. The rate of reduction of methylene blue by reduced coenzyme is much slower in absence than in presence of the enzyme. The ratio of activities is 1 : 10 in presence of yellow pigment and 1 : 90 in absence of yellow pigment. Furthermore, yellow pigment enormously accelerates the rate of reaction between reduced coenzyme and methylene blue or flavin but not so in the presence of the enzyme. These two discrepancies may mean that the carrier theory of coenzyme action requires modification in part.

IX. *Reversibility.*

The enzymic oxidation of hypoxanthine to uric acid, of hydrogen gas to hydrogen ion or of succinate to fumarate is reversible under suitable conditions [Green, 1934; Green & Stickland, 1934; Filitti, 1934; Quastel & Whetham, 1924; Borsook & Schott, 1931]. The equilibrium potential of the system hypoxanthine-xanthine oxidase-uric acid (to take one example) can be measured by determining to what extent this system reacts with benzylviologen. The degree of reduction of the indicator is a direct measure of the potential corresponding to the equilibrium point.

The question of the reversibility of a dehydrogenase system involving a coenzyme raises certain theoretical considerations. In the case of the xanthine oxidase, there is a direct interaction between hypoxanthine and benzylviologen, and between reduced benzylviologen and uric acid. This simple condition does not apply to the lactic dehydrogenase system of animal tissues. The reaction between lactate and the indicator is not direct but proceeds through the intermediation of the coenzyme. For a reversible state to be established, the following steps must be reversible:

(1) lactate + coenzyme $\rightleftharpoons$ pyruvate + reduced coenzyme,
(2) reduced coenzyme + indicator $\rightleftharpoons$ coenzyme + reduced indicator.

Clearly the reversibility of the oxidation of lactate to pyruvate will depend on whether the coenzyme system can come into equilibrium (*a*) with the enzyme system and (*b*) with the indicator system.

The potential of a mixture of lactate, pyruvate, coenzyme and indicator will be determined by the system in highest concentration. In practice the concentrations of lactate and pyruvate are arranged to be in great excess over those of coenzyme or indicator. Hence if an equilibrium potential can be measured, it will refer to the potential of the system lactate-pyruvate and will not be governed by either the coenzyme or indicator system.

Banga *et al.* [1933] claimed to have demonstrated the thermodynamic reversibility of the lactate system of pig's heart muscle. Using Janus green as the indicator of the equilibrium, they found E_0' at *p*H 7·0 to be $-0{\cdot}181$ v. We have attempted to reoxidize leuco-Janus green with pyruvate in presence of the enzyme system but the results thus far have been negative. Janus green is reduced with extreme slowness, and is therefore of questionable value for demonstrating reversibility. Lactoflavin however is reduced quite rapidly and appears to form an equilibrium with the lactate system. In presence of excess lactate, lactoflavin is reduced practically to completion whilst the leuco-form can be reoxidized by excess pyruvate.

Table IX. *Reduction of oxidation-reduction indicators by lactic system and reduced coenzyme at* p*H 7·0.*

Experiments were carried out anaerobically in Thunberg tubes.

	E_0' at *p*H 7·0 in v.	Lactic system	Reduced coenzyme
Methylene blue	+0·011	Reduced completely	Reduced completely
Ethyl capri blue	−0·060	,,	,,
Nile blue	−0·142	,,	,,
Lactoflavin	−0·210	,,	,,
Yellow pigment	?	,,	,,
Janus green (red to colourless)	−0·258	,,	,,
Benzylviologen	−0·359	No reduction	No reduction

Table IX compares the indicators reduced by the enzyme system plus coenzyme and by the reduced coenzyme alone. The parallelism is very striking. The possibility presents itself that the equilibrium observed between the enzyme system and lactoflavin may concern the coenzyme system primarily and may not be a measure of the potential of the lactate-pyruvate system.

The usual method of fixing the ratio of lactate to pyruvate and then determining the potential either colorimetrically or electrometrically is not applicable to the lactate system of heart. Since traces of pyruvate inhibit almost completely oxidation of lactate, the measurements with mixtures are concerned with kinetics and not with thermodynamic equilibria. The question of the reversibility of the lactate system of heart must be reconsidered in this new light. The testing of the reversibility of the lactic systems of bacteria apparently does not involve these difficulties [cf. Barron & Hastings, 1934; Wurmser & Mayer-Reich, 1933].

Reduced coenzyme can reduce flavin to completion. The addition of oxidized coenzyme does not result in reoxidation of leuco-flavin. If the lactate-pyruvate system were reversible, we should expect that the ratio of reduced coenzyme to oxidized coenzyme would determine the ratio of reduced to oxidized flavin.

X. *Specificity of coenzyme.*

Identity of heart and yeast lactic coenzymes. A lactic coenzyme can be prepared from heart muscle by the method of Banga *et al.* [1933]. The fact that the coenzymes from heart and yeast can replace one another in the lactic system of animal tissues constitutes little or no proof of their possible identity. The sole alternative to isolating the respective coenzymes and establishing their identity by direct chemical means is an extensive comparison of the chemical and physical properties of the two coenzymes.

Given the respective coenzyme solutions in known catalytic strengths, the original ratio of activities should persist through a variety of parallel treatments if the coenzymes are identical. The constancy of the ratio is the measure of identity. Table X contains a summary of the comparison. The times in minutes refer to the reduction of methylene blue unless otherwise stated. The original ratio of 0·8 varies between the limits of 0·5 and 1·0. Bearing in mind that the impurities in the two preparations are different and that they may influence to some extent the behaviour of the coenzyme, the small variations in the ratio cannot be considered significant. There is little doubt that the chemical and physical properties of the two coenzymes are very similar, and we may reasonably assume that they are identical.

Table X. *Comparison of heart and yeast lactic coenzyme.*

The quantity of 0·02% methylene blue used with the enzyme system was 1 ml. and with the reduced coenzyme 0·2 ml.

	Heart min.	Yeast min.	Amount of coenzyme in ml.	Ratio
(1) With enzyme system.				
Original coenzyme solutions	4	5	0·2	0·8
Treated in cold with alkali	5·5	5·5	0·5	1·0
Treated with boiling alkali	35	60	0·5	0·6
(2) Without enzyme system.				
Original reduced coenzyme	18	37	0·8	0·5
+yellow pigment	2	4·5	0·8	0·6
Treated with acid in cold (tested with yellow pigment)	50	100	2·0	0·5
Reduction of pyocyanine by original reduced coenzyme	6	10	0·8	0·6

Identity with the hexosemonophosphate coenzyme. Through the courtesy of Prof. Keilin and Prof. Warburg we were able to test the activity of a highly purified preparation of the hexosemonophosphate coenzyme in the lactic dehydrogenase system, cf. Table XI. The result was completely negative. It is

Table XI. *The effect of Warburg coenzyme.*

Control lactic system: 1·5 ml. enzyme, 0·2 ml. 0·5% methylene blue, 0·4 ml. *M* NaCN and 0·2 ml. 2*M* lactate. Control hexosemonophosphate system; 1 ml. dialysed yeast juice, 0·2 ml. 0·5% methylene blue, 0·2 ml. 0·33*M* hexosemonophosphate.

	μl. O_2/30 min.
Control lactic system without coenzyme	10
+0·5 ml. lactic coenzyme	220
+0·5 ml. Warburg coenzyme	10
Control hexosemonophosphate system without coenzyme	0
+0·5 ml. lactic coenzyme	0
+0·5 ml. Warburg coenzyme	171

interesting that the malate and β-hydroxybutyrate systems are also not activated by the hexosemonophosphate coenzyme (Table XII). Considering the similarity in the chemical constitution and in the mode of action of the lactic and hexosemonophosphate coenzymes, the observed specificity is most extraordinary.

Table XII. *Effect of Warburg coenzyme on oxidation of malate and β-hydroxybutyrate.*

1 ml. 0·02% methylene blue, 0·2 ml. 2*M* substrate, 1 ml. lactic enzyme preparation.

	Reduction time of methylene blue min.
Enzyme + malate	∞
Enzyme + malate + 0·5 ml. lactic coenzyme	2·5
Enzyme + malate + 0·5 ml. Warburg coenzyme	∞
Enzyme + malate + β-hydroxybutyrate	∞
Enzyme + malate + 0·5 ml. lactic coenzyme	2·7
Enzyme + malate + 0·5 ml. Warburg coenzyme	∞

Identity with cozymase. Needham & Green (unpublished experiments) have made a systematic comparison of the activities of various coenzyme preparations from yeast and heart with regard to (1) alcoholic fermentation and (2) the oxidation of lactate by the animal dehydrogenase system. In general the ratio of the activities in the two processes was constant for preparations derived from

various sources and subjected to various chemical and physical treatments. There was however one notable exception, viz. a coenzyme solution which was very active in catalysing fermentation but which apparently had little effect on the oxidation of lactate.

Both cozymase and the coenzyme of lactic oxidation are rapidly destroyed by alkali. On reduction with hydrosulphite, the lactic coenzyme becomes insensitive to alkaline treatment. Needham & Green tested reduced coenzyme boiled with alkali as the coenzyme of fermentation and found the activity unimpaired. This striking similarity in the chemical properties of lactic coenzyme and cozymase suggests the identity of the two coenzymes.

Needham & Green considered the relative magnitudes of the cozymase and lactic coenzyme catalyses. They found that with excess of enzyme in each case (and with the same amount of coenzyme) about 20 mols. of CO_2 are liberated in fermentation for 1 mol. of oxygen absorbed in lactate oxidation during the same period. This disparity in rates contrasts with the similarity in rates observed when a limited amount of coenzyme is used with excess of the various dehydrogenase systems which require in common the lactic coenzyme. The suggestion is therefore obvious that although the lactic coenzyme and cozymase are almost invariably concomitant and may be associated in the same molecule, yet the mechanisms of their respective actions are different. Possibly the one case where the activity of cozymase was not associated with significant lactic coenzyme activity may be explained on the basis that the original coenzyme molecule underwent some change whereby one function was impaired and not the other.

For the present in view of these results of Needham & Green, it is preferable not to designate the lactic coenzyme as cozymase. The final decision of nomenclature must be deferred until the question of identity is settled conclusively.

Synthetic coenzymes. Warburg *et al.* [1935] demonstrated that the hexose-monophosphate coenzyme contains 2 mols. of ribose, 3 of phosphoric acid and 1 of adenine and nicotinamide. In the reversible oxidation and reduction of the coenzyme, it is the nicotinamide moiety which is directly involved. Warburg *et al.* showed that trigonelline (methylbetaine of nicotinic acid) behaves like the coenzyme in many respects, e.g. it can be reversibly reduced with hydrosulphite and in the reduced form is not autoxidizable. Furthermore, yellow pigment can oxidize the reduced form. More recently, Karrer & Warburg [1936] have prepared several nicotinic derivatives and have tested their ability to be reduced by hydrosulphite and to be oxidized by yellow pigment.

The question arose whether any simple nicotinic acid derivative could replace the coenzyme in the complete dehydrogenase system. The following substances were tested: nicotinic acid, nicotinamide, nicotine, diethyl ester of nicotinamide (coramine), trigonelline and the methiodides of the first four mentioned. There was no indication of any activity except for a very slight acceleration by trigonelline of the reduction of methylene blue by the lactic enzyme system. The effect however was far too small to merit serious consideration. It appears therefore that the lactic coenzyme is extremely specific and is not replaceable by substances of similar chemical behaviour and constitution.

XI. *Purification.*

Solutions A and B of lactic enzyme prepared according to section I may be extensively purified by the following treatments with either kaolin or kieselguhr. 20 ml. of enzyme solution are shaken vigorously with 1 g. of the adsorbent and then centrifuged. After two or three such treatments, the enzyme solution

Table XIII. *Purification of lactic enzyme.*

Tests were made with 1 ml. enzyme, 0·5 ml. coenzyme, 0·4 ml. *M* NaCN (neutral), 0·2 ml. 0·5% methylene blue and 0·2 ml. 2*M* lactate.

The preparations of solutions A and B are described in section I.

Solution A.	Q_{O_2}	Solution B.	Q_{O_2}
Original after centrifuging	167	Original after centrifuging	10
Treated twice with kieselguhr	457	Treated thrice with kaolin	18
		Treated thrice with kieselguhr	34

becomes almost water clear. Table XIII shows the extent of the purification as measured by the Q_{O_2}. The absolute value of the Q_{O_2} depends upon how fresh are the hearts used in the preparation of the enzyme and how quickly the experiment is carried out after the preparation is completed. The greatest fall in activity is in the first 24 hours. After that period the loss in activity is much more gradual. Often solution A is less active than B. The ratio of activities is variable and depends upon the particular heart.

The purified enzyme is no longer precipitated completely by acetic acid and furthermore undergoes rapid destruction in presence of acetic acid. No way has yet been found of obtaining the purified enzyme in the dry form.

When the Q_{O_2} determinations are made, the use of phosphate buffers must be avoided. The precipitates for solutions A and B respectively are suspended in water and the *p*H adjusted to 7·0 with dilute NaOH. This procedure eliminates the use of phosphate which introduces a large error in determining the dry weight of the enzyme preparation.

Table XIV. *Inhibitors of lactic enzyme.*

All the experiments were carried out with cyanide as the fixative except in the case of pyruvate.

	Final concentration *M*	% inhibition
Octyl alcohol	Saturated aqueous solution	0
Ethylurethane	0·16	33
Pyrophosphate	0·08	0
Cyanide	0·33	0
Arsenious acid	0·33	0
Iodoacetate	0·02	0
Pyruvate	0·04	100
Tartronate	0·03	60
d(−)-Lactate	0·03	0

XII. *Inhibitors.*

None of the usual respiration inhibitors affects the lactic enzyme appreciably (Table XIV). Urethane in high concentration produces only 30% inhibition. Pyruvate and tartronate inhibit by virtue of their ability to be adsorbed on the active enzyme surface, thus preferentially displacing lactate. It is interesting that *d*(−)-lactate does not show this phenomenon of competitive inhibition. That rather indicates that even the purely physical process of the adsorption of a substrate on the enzyme molecule may involve stereochemical specificity.

XIII. *Distribution of the enzyme.*

The quantitative estimation of the amount of any particular enzyme present in a tissue involves difficulties which at the moment seem insuperable. Using the tissue slice technique, the difference between the Q_{O_2} in presence and in absence

of lactate provides the measure of the amount of enzyme present. The results however are very unsatisfactory for the following reasons: (1) the tissue may be saturated with lactate and hence the addition of more lactate produces no increase; (2) the missing component of the lactic system may not be the enzyme but the coenzyme. Hence with slices like those of muscle which allow the coenzyme to leak out into the surrounding medium, the fact that addition of lactate does not increase the Q_{O_2} has no bearing on the question whether there is any lactic enzyme present.

We have adopted the following method for estimating the amount of lactic enzyme present in various tissues. The organs of freshly killed animals only are used. After coarse mincing with scissors, the tissue is washed exhaustively with tap water to remove soluble components and then thoroughly ground with sand and a known volume of $M/100$ phosphate buffer pH 7. The success of the method depends entirely on the completeness of the grinding process. The sand and insoluble debris are filtered off through muslin; the filtrate is centrifuged and tested directly. The entire procedure is a matter of 10–15 min. The filtrate contains only the enzyme and must be supplemented with coenzyme, lactate, methylene blue and cyanide in order to reconstruct the whole system. A control with all the components except lactate must be carried out to correct for the blank of the enzyme.

The Q_{O_2} refers to the oxygen uptake in μl./hour of 1 mg. dry weight of the enzyme preparation. The phosphate of the buffer must be allowed for in determining the dry weight. The question arises whether the Q_{O_2} should be calculated on the basis of the original dry weight of tissue which corresponds to 1 mg. dry weight of enzyme. The Q_{O_2} values for tissues calculated on the basis of the original tissue dry weight are of course numerically different from the standard Q_{O_2} values. However, the relative activities of the enzyme in the various tissues are approximately the same regardless of the method of calculation, cf. Table XV.

Table XV.

	O_2 uptake in μl./hour/mg. dry wt. of tissue	O_2 uptake in μl./hour/mg. dry wt. of extract	Ratio
Muscle	1·1	6·7	6
Heart	1·3	7·4	6
Brain	2·0	12·2	6
Liver	0·2	1·0	5

Table XVI. *The distribution of the lactic enzyme in rat, rabbit and pigeon.*

$Q_{O_2\ lactate}$ = oxygen uptake in μl./hour/mg. dry weight of enzyme extract.

Enzyme extract tested with 0·5 ml. coenzyme, 0·3 ml. M NaCN (neutral), 0·2 ml. 0·5% methylene blue and 0·2 ml. 2 M lactate.

These values of the $Q_{O_2\ lactate}$ are the maxima found in repeat experiments.

	Rat	Rabbit	Pigeon
Kidney	79	9	3·1
Heart	111	15	4·5
Brain	73	32	0·4
Muscle	28	30	7·3
Lung	—	3·4	0·9
Liver	—	1·2	1·8

The distribution of the enzyme in various animals is shown in Table XVI. There can be little doubt that the lactic enzyme is an essential part of the

enzymic equipment of all animal cells. It is interesting that the highest concentrations of the enzyme were found in the tissues of the rat compared with those of rabbit and pigeon. This is to be expected on the basis of the Q_{O_2}/body weight relation.

The tissues of the rabbit fail to yield active extracts of the lactic enzyme when ground with sand and distilled water. Activity is only obtained when salts, preferably phosphates, are present in the surrounding fluid. This effect of phosphate is confined to the tissues of the rabbit. In the absence of phosphate no activity can be found either in the residue or in the extract. It appears that enzyme destruction occurs under these conditions.

XIV. *The classification of dehydrogenases.*

The experimental investigations of Warburg & Christian [1931; 1933], Keilin & Hartree [1936], Euler & Adler [1934; 1935], Wagner-Jauregg *et al.* [1934; 1935], Ogston & Green [1935] and Green [1936] have provided the groundwork for a systematic classification of dehydrogenase systems.

I. *Aerobic oxidases:*

Characteristics: react directly with molecular oxygen; produce H_2O_2; do not require coenzyme.

Examples: uricase, amino-acid oxidase, xanthine oxidase.

II. *Cytochrome dehydrogenase systems:*

Characteristics: do not react directly with molecular oxygen; can react through cytochrome but not flavin or yellow pigment; do not require coenzyme.

Examples: α-glycerophosphate, succinate, lactate (yeast).

III. *Coenzyme dehydrogenase systems:*

Characteristics: do not react directly with molecular oxygen; react with flavin or yellow pigment but not cytochrome; require a coenzyme.

A. Hexosemonophosphate coenzyme systems:

Examples: glucose (liver) and hexosemonophosphate of yeast or red blood cells.

B. Lactic coenzyme systems:

Examples: lactate (animal tissues), malate, fumarate, β-hydroxybutyrate, glucose (liver).

The glucose system of liver is the only one known to work with either of two coenzymes [Euler & Adler, 1935].

Summary.

The lactic dehydrogenase system of animal tissues comprises the following components: enzyme, coenzyme, lactate and carrier.

The product of oxidation—pyruvic acid—inhibits almost completely the oxidation of lactic acid. Ketone-fixing agents, such as cyanide and hydroxylamine, combine with pyruvic acid and allow the oxidation of lactic acid to proceed linearly for a considerable period.

The effects of *p*H and of concentration of coenzyme and substrate upon the rate of oxidation have been studied.

The lactic enzyme is found associated with the enzymes for the oxidation of malate, fumarate, β-hydroxypropionate and β-hydroxybutyrate. Methods of separating partially the lactic enzyme from the others are described.

The lactic enzyme catalyses the oxidation of *l*(+)-lactate to pyruvic acid which can be isolated as the 2:4-dinitrophenylhydrazine derivative. *d*(−)-Lactate, lactamide and *iso*serine are not oxidized.

Among the natural carriers, flavin, adrenaline and to a slight extent yellow pigment (flavoprotein or yellow enzyme) can function as carriers for the reaction with oxygen. Cytochrome, ascorbic acid and glutathione are inactive.

The lactic coenzyme can be reduced with hydrosulphite or by the enzyme system. Reduced coenzyme can reduce flavin, methylene blue, yellow pigment and the red substance formed from adrenaline. It does not however reduce cytochrome. Reduced coenzyme is unstable in acid solution but not in alkaline.

The Warburg scheme of coenzyme action is found to apply to the lactic system. The coenzyme functions as a highly specific carrier between lactic acid and the oxygen transporter.

The coenzyme of the hexosemonophosphate dehydrogenase cannot replace the lactic coenzyme in the lactic system.

The enzyme preparation may be purified extensively by treatment with kieselguhr and kaolin. Water-clear solutions can be prepared with Q_{O_2} values from 400 to 500.

The lactic enzyme is widely distributed in animal tissues. Its quantitative estimation has been carried out in the tissues of pigeon, rabbit and rat.

A new classification of dehydrogenase systems is presented based on recent research.

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