CCXCIV. THE MALIC DEHYDROGENASE OF ANIMAL TISSUES.

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THE following contribution is the fifth of a series devoted to the detailed analysis of individual dehydrogenase systems [cf. Ogston & Green, 1935, 1, 2; Green, 1936; Green & Brosteaux, 1936]. The immediate goal of these studies is a knowledge of the components of the various dehydrogenase systems, and an understanding of the mechanism by which these systems react with molecular oxygen. The ultimate goal is the reconstruction *in vitro* of series of parallel and coupled oxidations of the type living cells perform. The means by which the living cell integrates and co-ordinates the various oxidation systems has yet to be discovered.

All of the known dehydrogenases may be divided into three groups, viz. aerobic oxidases, cytochrome systems and coenzyme systems. Keilin & Hartree [1936] showed that the aerobic oxidases as a class react directly with molecular oxygen without requiring either a carrier or a coenzyme and produce H_2O_2 which can be utilized in coupled oxidations. The aerobic oxidases of animal tissues, e.g. uricase, amino-acid oxidase and xanthine oxidase are not generally distributed among the various organs and tissues but are restricted almost entirely to the liver and kidney. The general role of this class of oxidizing enzymes in cellular respiration must be therefore a minor one.

Ogston & Green [1935, 1, 2] and Green [1936] found that in animal tissues only the succinic and α-glycerophosphoric dehydrogenases depend upon cytochrome for their reaction with molecular oxygen. These two systems are found in all tissues in very considerable concentration. They do not require a coenzyme and do not react either with flavin or flavoprotein. The cytochrome catalysis involves the collaboration of two enzymes, the dehydrogenase and the indophenol oxidase. The former catalyses the reduction of cytochrome; the latter catalyses the oxidation of cytochrome by molecular oxygen. It is significant that the succinic and α-glycerophosphoric dehydrogenases as well as the indophenol oxidase are associated with particles which cannot be brought into solution.

The work of Warburg, Euler, Adler, Wagner-Jauregg and Green has made clear the characteristics of coenzyme dehydrogenase systems. This class of dehydrogenases is omnipresent in animal tissues, and there is good evidence that it accounts for the bulk of cellular respiration. Coenzyme dehydrogenase systems do not react directly with molecular oxygen. Between the substrate and oxygen, a coenzyme and a carrier intervene. The substrate in presence of the dehydrogenase reduces the coenzyme, reduced coenzyme reduces the carrier and reduced carrier then reacts with molecular oxygen. Flavin, flavoprotein or adrenaline can act *in vitro* as carriers in coenzyme systems, whereas cytochrome is completely inactive. Whether these carriers function physiologically has yet to be determined. There are only two coenzymes known to exist in animal tissues, viz. coenzyme I originally discovered by Harden & Young [1906] in yeast, and coenzyme II discovered by Warburg & Christian [1931] in horse blood corpuscles. They cannot replace one another in their respective catalytic capacities (with possibly one exception) despite their close similarity in chemical structure.

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The malic dehydrogenase offers a clear example of a coenzyme I system. Its properties are very similar to those of the lactic dehydrogenase—the prototype of the class. There is an extensive literature dealing with the malic dehydrogenase but little or no progress has been made in the way of isolating the enzyme and analysing the conditions and mechanism of the oxidation.

I. *Nomenclature.*

The great diversity of terminologies now in use among different investigators in the field of cellular respiration has rendered the literature almost unintelligible to the non-specialist. To avoid further confusion, the following list of synonymous terms is given—the preferred term being italicized.

Dehydrogenase: intermediary enzyme; colloidal carrier; oxidase; dehydrase; apodehydrase.

Flavoprotein: yellow enzyme; yellow pigment.

Coenzyme I: cozymase; coferment; fermentation coenzyme; codehydrase; lactic coenzyme; Harden & Euler coenzyme.

Coenzyme II: blood coenzyme; hexosemonophosphate coenzyme; coferment; codehydrase; Warburg coenzyme.

Coenzyme-dehydrogenase complex: holodehydrase; hydrogen transporting enzyme.

Carrier: this term refers to a substance which can be reversibly reduced and oxidised, regardless of chemical constitution or molecular dimensions. Thus cytochrome and flavoprotein as well as flavin and adrenaline are considered as carriers. The term should not be confused with colloidal carrier.

The practice of referring to the two oxidation coenzymes by numerals I and II was originally suggested by Euler & Adler. Its general acceptance is to be highly recommended.

II. *Preparation of the components.*

The malic dehydrogenase was prepared from the heart muscle of pig by the same method as was used by Green & Brosteaux [1936] for the preparation of the lactic enzyme. Since the concentration of malic enzyme in the heart muscle is extremely high, only two hearts need be used for the preparation of 300 ml. of very active enzyme solution (i.e. 100 ml. of solution A and 200 ml. of solution B). The Q_{O_2} of freshly prepared solution A or B varies from 300 to 800.

Coenzyme I was prepared from baker's yeast by the method of Green & Brosteaux [1936].

III. *The condition for linear oxidation.*

A mixture of dehydrogenase, coenzyme I, malate and methylene blue hardly takes up any oxygen. With addition of neutralized cyanide the oxygen uptake is rapid and linear for a considerable period, cf. Fig. 1. The maximum acceleration by cyanide is reached only at high concentrations. The control with cyanide and all components except malate is negative. There can be no doubt that the action of cyanide consists in reacting with the product of the reaction, presumably oxaloacetate—thereby preventing the complete inhibition of the enzyme by small concentrations of the reaction product. The fact that the cyanide effect is dependent upon the concentration indicates that the reaction of cyanide and oxaloacetate is not complete at low concentrations of the former.[1] The effect of cyanide on the malic system is exactly the same as that observed by Green & Brosteaux with the lactic system.

[1] Smythe [1932] found that cyanide catalyses the anaerobic decomposition and polymerization of methylglyoxal. The possibility therefore exists that the action of cyanide consists not in combining with oxaloacetic acid but in catalysing the conversion of oxaloacetic acid into other products.

Other ketone reagents such as semicarbazide, hydrazine and hydroxylamine can be used instead of cyanide (cf. Table I).

Table I. *The effect of ketone fixatives.*

	μl. O_2	Time in min.
1. Enzyme system without fixative	25	60
+1·0 ml. *M*/2 semicarbazide (neutral)	214	60
+0·5 ml. *M*/2 semicarbazide	194	60
+0·2 ml. *M*/2 semicarbazide	147	60
2. Enzyme system without fixative	22	30
+1·0 ml. *M*/2 hydroxylamine (neutral)	35	30
+0·5 ml. *M*/2 hydroxylamine	52	30
+0·2 ml. *M*/2 hydroxylamine	57	30
3. Enzyme system without fixative	18	15
+1·0 ml. *M*/3 hydrazine (neutral)	141	15
+0·2 ml. *M*/3 hydrazine	94	15

The enzyme system comprised 0·5 ml. enzyme, 1 ml. coenzyme, 0·3 ml. 0·5% methylene blue and 0·2 ml. *M* malate. The total volume per manometer was 3·3 ml. The malate was tipped into the solution from a Keilin cup after equilibration of the manometer.

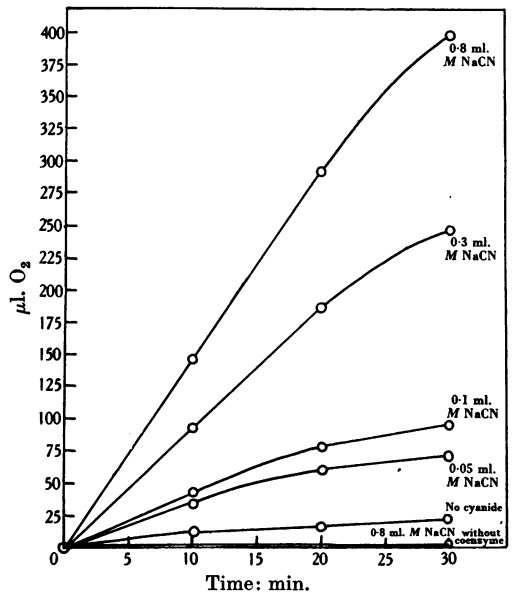

Fig. 1. The effect of cyanide. Each manometer cup contained 1·5 ml. enzyme, 0·5 ml. coenzyme, 0·2 ml. 0·5% methylene blue and 0·3 ml. *M l*(−)malate. The substrate was added from Keilin cups after equilibration.

The efficiency of a ketone-binding reagent in accelerating the oxidation depends upon three factors, viz. the toxicity to the enzyme, the speed of combination and the extent of dissociation of the addition compound. Cyanide is the most efficient reagent of those tested, with semicarbazide and hydrazine poor seconds.

IV. *The identity of the malic and fumaric dehydrogenase.*

Szent-Györgyi and co-workers [1935] have claimed that there exists in animal tissues a dehydrogenase specific for the oxidation of fumarate to oxaloacetate. The enzyme solution described in section II oxidizes both malate

and fumarate at approximately equal speeds. The question arose whether the same enzyme catalyses both oxidations. The following lines of evidence show conclusively that the so-called fumaric dehydrogenase is simply the malic dehydrogenase collaborating with fumarase—the enzyme which catalyses the conversion of fumaric into malic acid.

(*a*) If the fumaric and malic enzymes are identical, then the ratio

$$\frac{\text{rate of oxidation of fumarate}}{\text{rate of oxidation of malate}}$$

should be 1 or less but never appreciably more. The actual value of the ratio will obviously depend upon the concentration of fumarase. With a small amount of this enzyme present compared with the malic enzyme, the ratio should be very much less than 1. With excess of fumarase, the ratio should be approximately 1.

Table II is a summary of the ratios observed in a large number of preparations of different degrees of purity and from a variety of tissues and animals. The evidence is clear that the ratio never exceeds 1:3 and is usually much lower.

Table II. *The ratio* $\frac{\text{rate of oxidation of fumarate}}{\text{rate of oxidation of malate}}$ *in different preparations.*

Source of enzyme	Time of experiment in min.	Ratio
Pig heart, solution A	20	1·18
Pig heart, solution B	60	1·27
Pig heart, solution B (purified)	60	1·20
Pig heart, solution A (purified)	20	0·48
Pig heart, solution A (purified)	20	0·45
Rabbit skeletal muscle	30	0·77
Rabbit kidney	20	1·06
Rabbit liver	10	0·45
Rabbit heart	15	0·78
Rabbit brain	10	0·39
Rat kidney	20	1·20
Rat heart	20	1·11
Rat muscle	60	0·43
Rat liver	60	1·04
Pigeon breast muscle	60	0·73

Furthermore, on purification it is always the fumaric activity which is lost relatively to the malic and never the reverse. The presence of active fumarase in the stock enzyme solution from pig's heart was established by the polarimetric demonstration of large quantities of malic acid formed from added fumaric acid. 1 ml. of enzyme was able to convert 1 mg. of fumaric into malic in 1 min. In other words, the limiting factor in the oxidation of fumaric acid in unpurified preparations is not the conversion into malic but the oxidation of malic. On purification fumarase activity is relatively diminished, and the conversion of fumaric into malic becomes the limiting factor.

(*b*) If the malic and fumaric enzymes are identical, there should be no summation of the rates of oxidation in presence of malate and fumarate respectively. The following experiment shows that such is actually the case.

	μl. O_2	
	1st 10 min.	2nd 10 min.
Enzyme system + malate	344	210
Enzyme system + fumarate	119	181
Enzyme system + malate + fumarate	328	230

(*c*) The oxidation of fumarate, particularly with purified preparations, shows an initial lag period varying from a few minutes to as much as an hour (cf. Fig. 2).

This phenomenon can be explained satisfactorily only on the basis that the oxidation of malate does not proceed at an appreciable velocity until the malate reaches a certain minimum concentration, and that the lag period represents the time required for a weak fumarase to convert sufficient fumaric into malic acid. It is significant that after the lag period, the rate of oxidation of fumarate attains the value for the rate of oxidation of malate.

There are many other lines of evidence which could be adduced to prove the identity of the two dehydrogenases, such as similar behaviour towards inhibiting and accelerating agents, similar kinetics, etc. But they are not as conclusive as those mentioned in detail and will therefore be omitted from the argument in the interest of brevity.

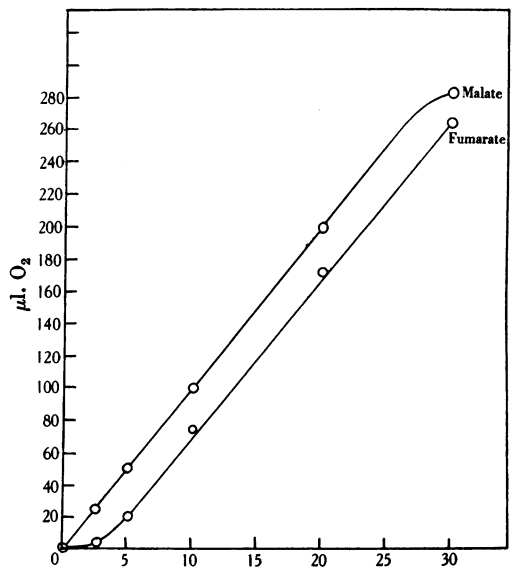

Fig. 2.

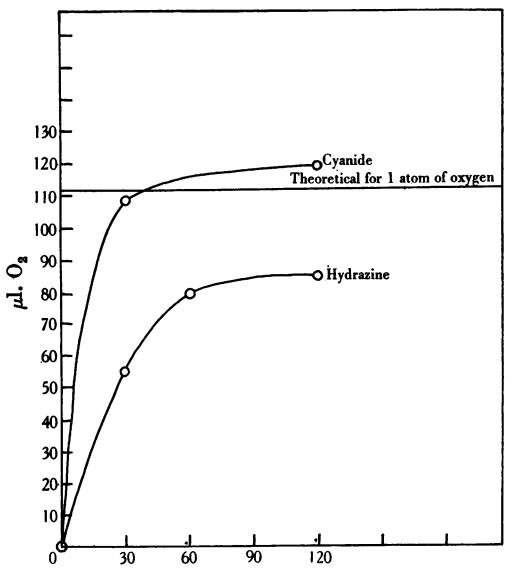

Fig. 3.

Fig. 2. The lag period in the oxidation of fumarate. Each manometer cup contained 1 ml. enzyme, 0·5 ml. coenzyme, 0·1 ml. 0·5% methylene blue and 0·5 ml. *M* NaCN (neutralized).

Fig. 3. The number of oxygen atoms. Each manometer cup contained 0·8 ml. enzyme, 0·8 ml. coenzyme, 0·2 ml. 0·5% methylene blue and 0·4 ml. *M*/40 *l*(−)malate. The concentrations of fixative were 0·5 ml. *M* NaCN and 1 ml. *M*/3 hydrazine respectively. The small control uptakes for the above mixtures without malate have been subtracted from the experimental uptakes.

V. *Product of oxidation.*

2:4-Dinitrophenylhydrazine does not react with oxaloacetic acid in presence of cyanide. The isolation was therefore carried out with semicarbazide as the fixing agent. 200 ml. of enzyme, 100 ml. of coenzyme, 20 ml. of *M* malate, 50 ml. of *M*/2 semicarbazide chloride (neutral) and 10 ml. of 0·5% methylene blue solution were mixed and aerated vigorously at 37° for 2 hours. After deproteinization with hydrochloric acid and filtration through kieselguhr-impregnated filter-paper to remove methylene blue, the solution was concentrated *in vacuo* to 100 ml. 1 g. of 2:4-dinitrophenylhydrazine dissolved in 100 ml. of 2*N* HCl was added. The solution was kept at room temperature for 24 hours before filtering off the crystalline precipitate (yield 0·5 g.). After two recrystallizations

from a mixture of ethyl acetate and ligroin by the method of Clift & Cook [1932], the M.P. was found to be 212° which is identical with that of the authentic 2:4-dinitrophenylhydrazone of oxaloacetic acid. The mixed M.P. was also 212°. The following are the analytical figures: (Weiler). (C, 38·78; N, 18·12; H, 2·39 %. $C_{10}H_8O_8N_4$ requires C, 38·34; N, 17·90; H, 2·58 %.)

The oxidation of malic to oxaloacetic acid requires 1 atom of oxygen. Fig. 3 shows that in presence of cyanide as the fixative, the theoretical uptake of oxygen for the oxidation of 0·1 ml. *M*/10 malate to oxaloacetate is reached within 30 min., whereas with hydrazine as the fixing agent, only 75 % of the theoretical is reached before the enzyme is completely inactivated.

If the sole chemical change is the oxidation of malate to oxaloacetate, then for each atom of oxygen absorbed, 1 molecule of oxaloacetic should be formed. Oxaloacetic acid can be estimated manometrically as CO_2 by the method of Ostern [1933] which involves the splitting of oxaloacetic acid by aniline in acid solution. Oxaloacetic acid after incubation with cyanide is not decomposed by aniline. However the semicarbazone is quite readily decomposed at 37° and the CO_2 liberation is complete in 3 hours. The experiments were carried out as follows. The complete enzyme system (enzyme, coenzyme, methylene blue and semicarbazide) was equilibrated in the manometer before the malate was introduced from a dangling Keilin tube. The oxygen uptake over a period of several hours was then measured. The manometer cup was then disengaged from the manometer and 0·3 ml. glacial acetic acid was added to deproteinize the enzyme solution. The aniline-citrate mixture (1 part of aniline to 3 parts of 50 % citric acid) was placed in a second Keilin tube and introduced after re-equilibration. The following table shows that for each atom of oxygen absorbed, approximately 1 molecule of CO_2 is liberated.

	μl. O_2	μl. CO_2	Ratio $\frac{\mu l.\ CO_2}{\mu l.\ O_2}$
Control (no malate)	5	4	—
Determination 1	264	570	2·16
2	245	505	2·06
3	187	368	1·98

VI. p*H*.

Table III shows the dependence of the rate of oxidation upon the hydrogen ion concentration. The maximum rate is at *p*H 8. It is interesting that the oxidation is completely inhibited by a very slightly acid reaction (*p*H 6) whereas

Table III. *The effect of* p*H*.

Each manometer cup contained 0·5 ml. enzyme suspension, 3·3 ml. of *M*/2 buffer, 0·5 ml. *M* NaCN, 0·1 ml. *M* fumarate, 0·2 ml. 0·5 % methylene blue and 0·5 ml. coenzyme. The enzyme suspension was made with water rather than the usual phosphate.

*p*H ...	6·0	7·0	8·0	9·0	10·0	11·0	12·0	13·0
Buffer ...	Phosphate	Phosphate	Phosphate	Glycine	Glycine	Glycine	Glycine	Glycine
				μl. O_2				
15 min.	0	37·8	84·0	37·5	15·4	29·0	20·5	17·0
30 min.	6·0	87·5	196·0	97·0	34·0	54·0	77·5	37·2

a very alkaline reaction (*p*H 10–13) does not seriously interfere. The constituents of the buffer are probably as important as the *p*H in determining the speed of oxidation.

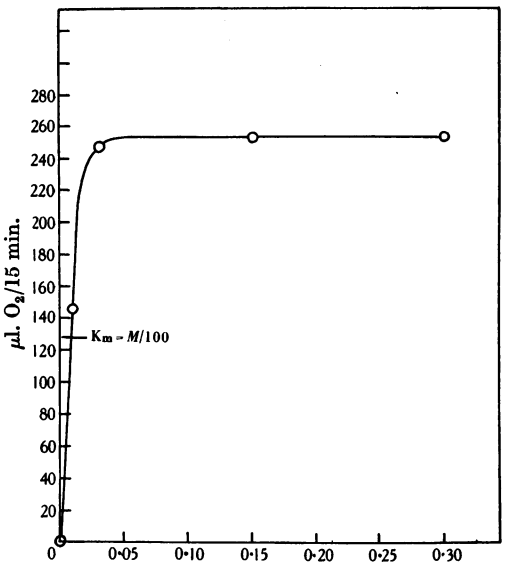

Fig. 4.

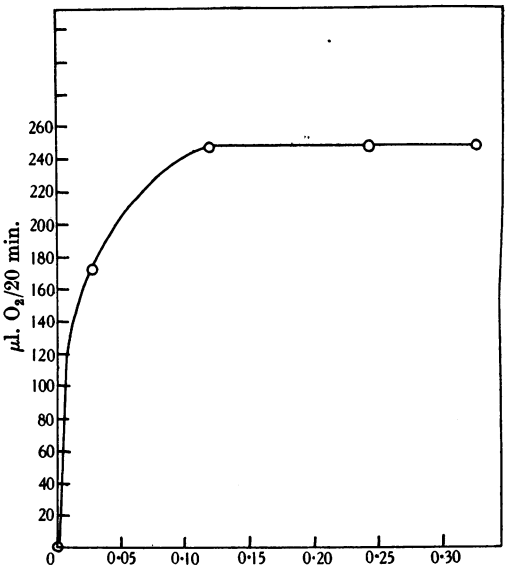

Fig. 5.

Fig. 4. The effect of fumarate concentration. Each manometer cup contained 1 ml. enzyme, 0·5 ml. coenzyme, 0·1 ml. 0·5% methylene blue and 0·5 ml. *M* NaCN.

Fig. 5. The effect of malate concentration. Each manometer cup contained 0·7 ml. enzyme, 0·5 ml. coenzyme, 0·7 ml. *M* NaCN and 0·1 ml. 0·5% methylene blue.

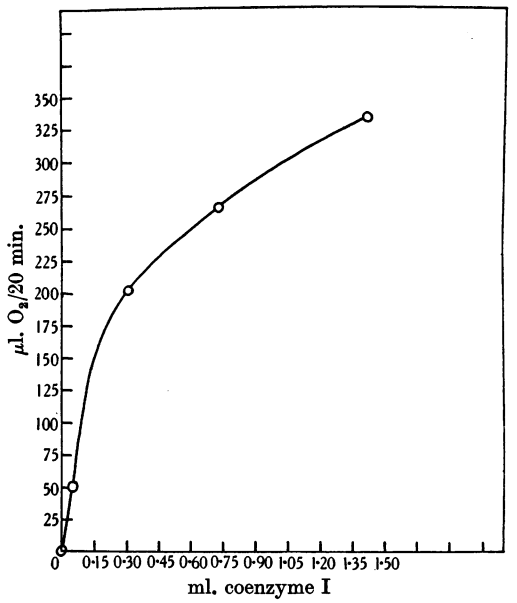

Fig. 6. The effect of coenzyme I concentration. Each manometer cup contained 1 ml. enzyme, 0·3 ml. *M* fumarate, 0·5 ml. *M* NaCN and 0·1 ml. 0·5% methylene blue.

VII. *Effect of concentration of substrate and coenzyme.*

The relation between the speed of oxidation and the molarity of substrate is shown in Figs. 4 and 5. The two sets of experiments with fumarate and malate respectively were not performed with the same enzyme preparation. Hence the absolute velocities are not comparable.

The rate of oxidation is proportional to the concentration of coenzyme over a very wide range of concentrations (cf. Fig. 6). The maximum asymptotic velocity would be reached only in presence of an enormous excess of coenzyme.

VIII. *Specificity of donator.*

Only *l*(−)malate is oxidized by the dehydrogenase system as shown by the following experiment:

	μl. O_2/15 min.
Enzyme system + 0·5 ml. *M*/10 *l*(−) malate	180
Enzyme system + 0·5 ml. *M*/10 *d*(+) malate	0

The optical isomerides were prepared from inactive malic acid by the method of Dakin [1924] which depends on the different solubilities of the cinchonine salts. Inactive malic acid is oxidized to the extent of only 50 % as shown by the fact that it yields exactly half the theoretical uptake for complete oxidation. Fumaric acid is converted into *l*(−)malic acid—hence the oxidation to oxaloacetic acid is quantitative.

Green & Brosteaux [1936] considered the evidence for and against the identity of the lactic and malic dehydrogenases and concluded that the two enzymes were probably not identical. The correctness of this conclusion has been confirmed by an extensive comparison of the activities of the two enzymes in different preparations from animal sources. In many cases, a preparation extremely rich in the malic enzyme showed little or no lactic activity. The following results are illustrative:

	μl. O_2	
	Exp. 1	Exp. 2
Enzyme system + lactate	39	20
Enzyme system + malate	282	392

It is noteworthy that whereas the method of preparation in section II invariably yields a highly active malic enzyme, success with the lactic enzyme is not as constant. The freshness of the tissue, the thoroughness of grinding and other factors in the preparation are more important for lactic activity than for malic activity. There can be little doubt therefore that the lactic and malic enzymes are distinct.

The dehydrogenase system, in presence of unpurified maleic acid (commercial preparations), reacts vigorously with oxygen. It can be demonstrated that such preparations are rich in fumaric acid and that when precautions are taken to remove all traces of fumaric acid, maleic acid is not oxidized. Freshly distilled maleic anhydride is practically inactive with the enzyme system. Since the conversion of maleic into fumaric acid occurs to some extent at 37° it is of course impossible to obtain a completely negative result. Dihydroxymaleic acid is also not oxidized.

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

A mixture of enzyme, coenzyme, malate and cyanide does not react with molecular oxygen. Some reversibly reducible and oxidizable substance is required to link the dehydrogenase system with molecular oxygen. The malic

system must therefore be considered as anaerobic in the sense that the reaction with oxygen is not direct.

Table IV contains a comparison of the efficiencies of the various carriers which were found to be active. It is noteworthy that the adrenaline effect has a

Table IV. *The activity of various carriers.*

Enzyme system was composed of 1 ml. enzyme, 1·4 ml. coenzyme, 0·4 ml. 2*M* NaCN and 0·2 ml. *M* malate.

	μl. O_2	
	1st 10 min.	2nd 10 min.
Enzyme system	3	3
+ *M*/1000 lactoflavin	123	119
+ *M*/1000 adrenaline	44	247
+ *M*/1000 methylene blue	348	226
+ *M*/1000 pyocyanine	299	178

lag period. The oxygen uptake in presence of adrenaline for the second 10 min. should be compared with the first 10 min. results for the other carriers. Adrenaline shows catalytic activity comparable with methylene blue and pyocyanine and about twice the activity of lactoflavin. The detailed analysis of the adrenaline effect will be considered in a separate communication.

Figs. 7, 8, 9 and 10 show how the speed of oxidation depends upon the concentration of the carrier. It is impossible to determine the limiting velocity for the catalysis by flavin owing to its relative insolubility. Lactoflavin was added in the form of a homogeneous aqueous suspension which went into solution only on dilution with the other components.

Flavoprotein shows but slight activity as a carrier:

	μl. O_2 in 10 min.	Manometer gas
Enzyme system	0	Air
Enzyme system	3	Oxygen
+1 ml. 10% flavoprotein	22	Air
+1 ml. 10% flavoprotein	35	Oxygen
+0·2 ml. 0·5% methylene blue	231	Air

Green & Brosteaux [1936] also observed comparatively little effect with flavoprotein. The fact that increase of the partial pressure of oxygen leads to a larger uptake in presence of flavoprotein suggests that the reoxidation of leuco-flavoprotein is slow under the conditions of the experiment. It is presumably the spontaneous oxidation of leuco-flavoprotein which is the limiting factor and not the enzymic reduction of the oxidized form.

The reduction of added heart cytochrome *c* by the malic enzyme system was tested for spectroscopically. There was not the slightest indication of the reduced bands of cytochrome when the oxidized form was added to the enzyme mixture under anaerobic conditions. As a control, succinate was added under the same conditions and the immediate reduction of cytochrome *c* could be seen even with the naked eye. This confirms the rule that coenzyme systems do not react directly with cytochrome.

Oxidized glutathione did not act as a carrier with the malic system. Chemical estimation showed that no reduction of GSSG to GSH took place.

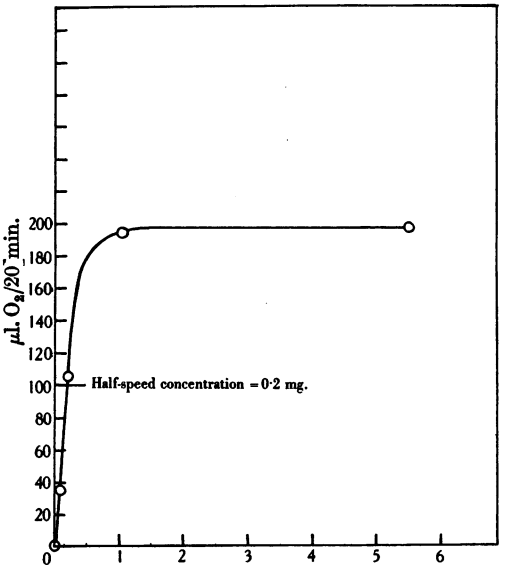

Fig. 7.

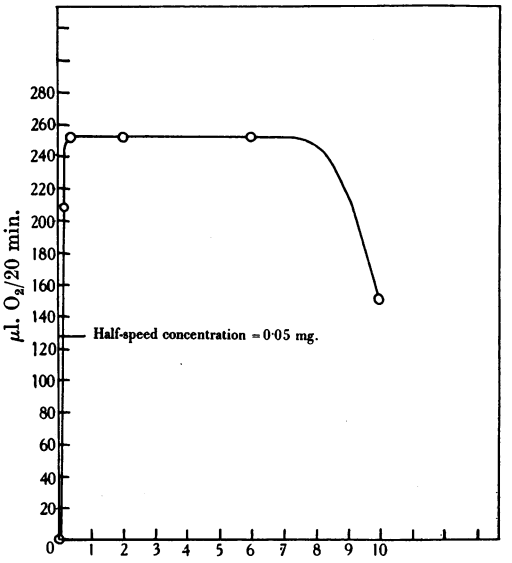

Fig. 8.

Fig. 7. The effect of methylene blue concentration. Each manometer cup contained 1 ml. enzyme, 0·5 ml. coenzyme, 0·5 ml. *M* NaCN and 0·3 ml. *M* fumarate.

Fig. 8. The effect of pyocyanine concentration. Details as for Fig. 7.

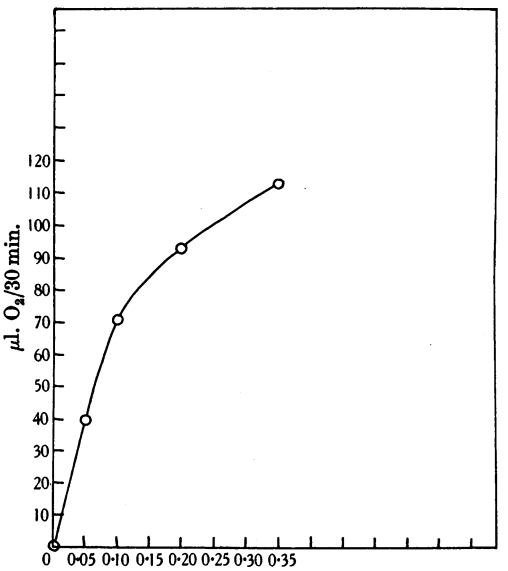

Fig. 9.

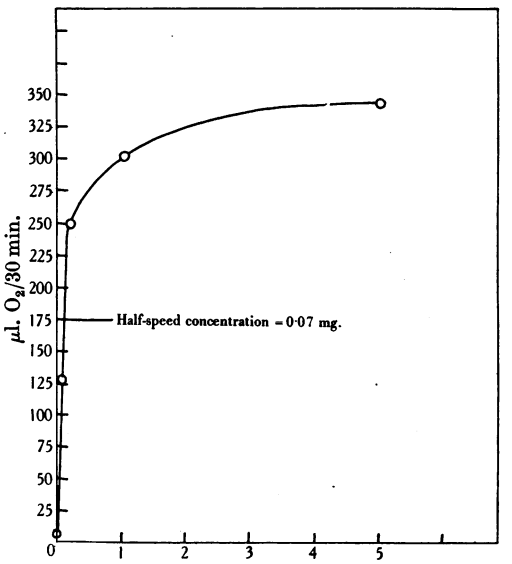

Fig. 10.

Fig. 9. The effect of lactoflavin concentration. Details as for Fig. 7.

Fig. 10. The effect of adrenaline concentration. Details as for Fig. 7.

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

Adler *et al.* [1936] and Green & Brosteaux [1936] independently demonstrated that coenzyme I, like coenzyme II, was reversibly reducible. Both groups of investigators agree on the main properties of reduced coenzyme such as insensitivity to strong alkali, sensitivity to acid, an absorption band in the ultraviolet with a peak at 340 mμ, rapid reaction with flavoprotein etc. The one important point of difference is the question whether the colourless reduced coenzyme can reduce methylene blue directly. Adler *et al.* claim that colourless reduced coenzyme can reduce methylene blue in acid but not in neutral solution. They describe the formation of a yellow substance by reduction of the coenzyme with hydrosulphite in alkaline solution. This substance, which is different from the colourless reduced coenzyme, can reduce methylene blue directly in alkaline solution. Green & Brosteaux reduced coenzyme I with hydrosulphite in neutral solution and showed with the vacuum tube technique that the solution of reduced coenzyme after removal of excess hydrosulphite by aeration could reduce methylene blue directly, albeit rather slowly. The addition of a small amount of flavoprotein increased the rate of reduction of methylene blue as much as thirtyfold. Hydrosulphite is oxidized to bisulphite which can reduce methylene blue. Controls however showed that the bisulphite present in solutions of reduced coenzyme could not account for the velocity with which methylene blue is reduced.

The controlled experiment was carried out as follows. 15 ml. of alkali-inactivated coenzyme and 15 ml. of untreated coenzyme were each mixed with 3 ml. *M*/5 phosphate buffer *p*H 7 and 1 ml. of a 0·5% hydrosulphite solution. After 10 min. incubation at 37°, both solutions were aerated vigorously for 20 min. The methylene blue reductions were carried out in Thunberg tubes, each tube containing 0·3 ml. 0·02% methylene blue, 1 ml. buffer *p*H 7 and 1 ml. of coenzyme solution:

	Time of reduction in min.
Alkali-inactivated coenzyme	>120
Alkali-inactivated coenzyme + 0·3 ml. 10% flavoprotein	>120
Coenzyme	30
Coenzyme + 0·3 ml. 10% flavoprotein	1

The rate of reduction of methylene blue by reduced coenzyme depends upon the concentration of methylene blue, e.g. 1 ml. of reduced coenzyme reduced completely 0·1 ml. of 0·05% methylene blue in 40 min. whereas it reduced ten times that quantity of methylene blue to the extent of 50% in the same time.

Oxidizing agents such as I_2 and H_2O_2 destroy the reduced coenzyme. No way has yet been found of oxidizing bisulphite to sulphate without destroying the reduced coenzyme at the same time.

Green & Brosteaux pointed out that the rate of reduction of methylene blue by reduced coenzyme even in presence of flavoprotein was very much slower than the reduction of methylene blue by the enzyme-coenzyme-lactate system. This discrepancy is difficult to explain on the basis of the hypothesis that the reaction between the coenzyme and the carrier does not involve the enzyme. In the present work other difficulties have been found in the way of applying the new conception of the mechanism of coenzyme action to the case of coenzyme I.

One would expect that the properties of reduced coenzyme would be the same regardless of the mode of reduction. But in fact reduced coenzyme prepared by incubation with the enzyme and the substrate under anaerobic conditions shows only in a qualitative way the properties of reduced coenzyme

prepared by the hydrosulphite method. Whereas 1 ml. of the former can reduce 0·3 ml. of 0·02 % methylene blue in presence of flavoprotein within a minute, 1 ml. of the latter requires 30 min. or more. It is conceivable, as Euler and his group believe, that there is an equilibrium between reduced and oxidized coenzyme on the one hand and the reductant and oxidant of the enzyme system on the other—the equilibrium being in favour of the oxidized coenzyme. But this explanation cannot hold when a fixative like cyanide or semicarbazide is used to remove the oxidant. No increase in reducing power is obtained when the coenzyme is incubated with the enzyme, malate and fixing agent and thereby reduced to completion.

It remains for future research to determine whether the enzyme is involved in the reaction between the coenzyme and the carrier and whether coenzyme systems are carrier-specific. There can be no disputing the fact that the coenzyme is reversibly reduced and oxidized. But our knowledge of the mechanism of the interaction of reduced coenzyme with the other components in the catalytic system is still obscure.

XI. *The dismutation of fumarate.*

In the course of an attempt to study the reversibility of the oxidation of malic to oxaloacetic acid, it was observed that indicators as far apart in potential as methylene blue and flavin were not reduced to completion. The phenomenon therefore could not be attributed to an oxidation-reduction equilibrium. Analysis showed that the following series of events took place:

$$\text{(1) Malate} \underset{}{\overset{\text{fumarase}}{\rightleftharpoons}} \text{fumarate.}$$

$$\text{(2) Malate} + \text{methylene blue} \xrightarrow[\text{enzyme}]{\text{malic}} \text{oxaloacetate} + \text{leuco-methylene blue.}$$

$$\text{(3) Fumarate} + \text{leuco-methylene blue} \xrightarrow[\text{enzyme}]{\text{succinic}} \text{succinate} + \text{methylene blue.}$$

The indicator, methylene blue in this case, is linking two dehydrogenases, the succinic and the malic; and is being oxidized by the former and reduced by the latter. The phenomenon of partial reduction therefore offers an interesting example of linked carrier reactions between dehydrogenase systems [Green *et al.*, 1934]. Reduction is never complete since fumarate is oxidizing leuco-methylene blue at a rate comparable with the reduction of methylene blue by malate.

The proof of this interpretation lies in the demonstration of the anaerobic formation of oxaloacetic acid from fumaric acid which had been incubated with the enzyme, coenzyme, methylene blue and semicarbazide. Controls lacking fumaric acid and methylene blue were negative. There was a small formation of oxaloacetate in presence of fumarate and with no coenzyme. The oxaloacetate was determined manometrically by the method of Ostern [1933]. Table V shows the results of an experiment.

Table V. *The anaerobic formation of oxaloacetate from fumarate.*

The experiments were carried out anaerobically in Thunberg tubes. Each tube contained 1 ml. of *M*/2 semicarbazide in addition to the solutions stated. The total volume in all cases was 7·7 ml.

Enzyme ml.	Coenzyme ml.	Methylene blue (0·5 %) ml.	Fumarate *M* ml.	Oxaloacetate mg.
3	3	—	0·5	0
3	3	0·2	—	0
3	3	0·2	0·5	4·0

The succinic acid formed was demonstrated in the following way. After incubation, the enzyme mixture was deproteinized with 0·2 ml. glacial acetic acid and filtered. The filtrate was kept in a boiling water-bath for 5 min. It was then made alkaline to thymolphthalein with 6 *N* NaOH and again placed in a boiling water-bath for 5 min. This treatment in acid and alkali destroys all traces of the coenzyme whether it be in the reduced or oxidized form. The solution was carefully neutralized and 4·0 ml. were tested for succinic acid with 0·5 ml. enzyme preparation and 0·2 ml. 0·5% methylene blue. The pig's heart preparation is very rich in the succinic enzyme and is quite suitable as the source of the enzyme. Fumaric and malic acids are not oxidized under these conditions since no coenzyme is provided. Table VI contains the protocol of an experiment.

Table VI. *The anaerobic formation of succinic acid.*

1·18 mg. succinic acid are equivalent to 112 μl. O_2 since the oxidation of succinate to fumarate only involves 1 atom of oxygen.

Enzyme ml.	*M*/3 semi-carbazide ml.	Coenzyme ml.	*M* fumarate ml.	0·5% methylene blue ml.	Succinic acid formed mg.
3	1	2	0·2	0·1	3·2
3	1	2	0·2	0	0
3	1	2	0	0·1	0

The observation of Moyle [1924] that in muscle succinic acid accumulates under anaerobic conditions indicates that this anaerobic dismutation of fumaric acid is a physiological process and that some cellular carrier takes the place of methylene blue in the model system.

XII. *Specificity of coenzyme.*

Needham & Green in unpublished experiments quoted by Green & Brosteaux [1936] studied the question of the identity of the lactic coenzyme and cozymase. They concluded that although the parallelism of the occurrence and activity of the two coenzymes pointed to their identity the mechanism of the action of the coenzyme was not identical in both fermentation and dehydrogenation. The recent note of Meyerhof & Kiessling [1936] describing the isolation of cozymase-pyrophosphate from yeast maceration juice indicates that cozymase is concerned with phosphorylation as well as dehydrogenation. Such being the case, to call the lactic coenzyme, cozymase or *vice versa* is illogical. The suggestion by Euler & Adler of calling cozymase, coenzyme I overcomes this difficulty. Coenzyme I is to be understood as the coenzyme which functions either in alcoholic fermentation or in certain dehydrogenations.

Coenzyme II was tested as an alternative coenzyme for the malic system. The following are the results.

	μl. O_2/40 min.
Enzyme system + 0·5 ml. coenzyme II (no malate)	5
Enzyme system + 0·5 ml. coenzyme II (malate added)	28
Enzyme system + 0·5 ml. coenzyme I (malate added)	258

The enzyme system contained 1 ml. enzyme, 0·2 ml. 0·5% methylene blue and 0·6 ml. *M* NaCN

The coenzyme II preparation used was about 30% pure and it is possible that traces of coenzyme I account for the slight effect on the malate oxidation. The point requires further study.

Trigonelline, nicotine, coramine, coramine methiodide and nicotine dimethiodide showed no activity as coenzyme. It seems highly probable that the nicotinamide molecule must be specifically linked with adenine, ribose and phosphoric acid in order to function as a coenzyme.

XIII. *Inhibitors.*

Table VII contains a summary of the effects of various reagents on the oxidation of malate. Oxaloacetic acid is the most powerful inhibitor found. Hence the indispensability of ketone-fixing reagents for studying the *in vitro* oxidation of malic acid. Pyruvic acid and acetoacetic acid are inhibitory but not

Table VII. *Inhibitors.*

Reagent	Concentration *M*	% inhibition	% acceleration
Oxaloacetic acid	0·18	100	—
	0·09	100	—
	0·018	96	—
Acetoacetic acid	0·3	39	—
	0·15	21	—
	0·03	0	—
Pyruvic acid	0·3	92	—
	0·06	76	—
	0·012	24	—
Muscle adenylic acid	0·0087	49	—
	0·0044	23	—
	0·00087	13	—
Yeast adenylic acid	0·0087	16	—
	0·0044	0	—
Adenylpyrophosphoric acid	0·0034	40	—
	0·0017	22	—
Iodoacetic acid	0·03	38	—
Arsenious acid	0·03	—	38
Pyrophosphoric acid	0·03	—	104
Urethane	0·017	10	—
Tartronic acid	0·06	24	—
Capryl alcohol	Saturated solution	18	—
Malonic acid	0·3	47	—
	0·03	11	—
Oxalic acid	0·075	30	—
Maleic acid	0·03	0	—

There is a considerable uptake by oxaloacetic acid solutions (non-enzymic) which must be allowed for in calculating the inhibition by oxaloacetic acid.

to as high a degree as oxaloacetic. Animal adenylic acid and adenylpyrophosphate inhibit presumably by competing with the coenzyme for the dehydrogenase. Yeast adenylic acid is much less inhibitory. Maleic acid contrary to the statements in the literature does not act as an inhibitor. The mechanism of the acceleration by arsenious acid and pyrophosphate is obscure.

All the inhibitor experiments were carried out with the enzyme system (enzyme, coenzyme, malate and methylene blue) in presence of cyanide as the fixative. Since oxaloacetic, acetoacetic and pyruvic acids combine with cyanide, the effective concentrations involved in the respective inhibitions must have been at least ten times as small as the added concentrations. Without cyanide, the oxygen uptake is not appreciable: hence inhibition experiments are not possible

without the use of fixing agents. It is possible, however, to calculate the actual inhibitory power of oxaloacetic acid from the limiting oxygen uptake of the enzyme system in absence of cyanide. Calculation shows that $M/1000$ oxaloacetic acid poisons the enzyme completely.

Table VIII. *The distribution of the malic dehydrogenase.*

Q_{O_2} malic dehydrogenase is the oxygen uptake in μl./hour/mg. dry weight of enzyme solution. The dry weight of the phosphate buffer is subtracted from the total dry weight.

	Q_{O_2} malic dehydrogenase		
	Rat	Rabbit	Pigeon
Brain	60·0	55·1	72·2
Kidney	10·8	23·0	124
Liver	40·6	29·4	60·5
Heart	218·0	56·7	189
Muscle	81·5	3·6	174

XIV. *Distribution of the enzyme.*

The method of estimation of the concentration of malic enzyme in various tissues is more or less that described by Green & Brosteaux [1936]. The tissue is minced finely with scissors and thoroughly washed with water. The last two washings are carried out with $M/50$ phosphate buffer of pH 7·2. The brei is then thoroughly ground with sand and $M/50$ phosphate buffer. After centrifuging the supernatant is tested directly for activity with 1 ml. coenzyme, 0·2 ml. 0·5 % methylene blue, 0·4 ml. $2M$ NaCN and 0·2 ml. M malate. The results are shown in Table VIII. The malic dehydrogenase is found in all tissues in extremely high concentration, particularly in heart. The chemical pathway leading from succinic to oxaloacetic acid must be of great importance in the intermediary metabolism of the various tissues.

SUMMARY.

The preparation of a highly active malic dehydrogenase from the heart muscle of the pig is described.

The product of oxidation, oxaloacetic acid, completely inhibits the oxidation even in extremely small concentration. The use of ketone fixatives such as cyanide, hydrazine, semicarbazide and hydroxylamine is essential in order to obtain a linear oxidation.

The catalytic system comprises the dehydrogenase, coenzyme I, carrier and malate. Methylene blue, pyocyanine, lactoflavin and adrenaline are the most active carriers, flavoprotein is only slightly active whereas cytochrome and glutathione are inactive.

The enzyme system specifically oxidizes $l(-)$malic acid to oxaloacetic acid—the latter being isolated as the 2:4-dinitrophenylhydrazone.

The so-called fumaric dehydrogenase is merely malic dehydrogenase collaborating with fumarase.

The malic enzyme is not identical with the lactic enzyme.

Fumaric acid has been shown to dismute anaerobically to form succinic and oxaloacetic acids. The dismutation depends upon the presence of the succinic and malic enzymes, coenzyme I and a suitable carrier.

The malic dehydrogenase is found in high concentration in the tissues of the rat, rabbit and pigeon.

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