# CLIII. *l*-MALIC DEHYDROGENASE AND CODEHYDROGENASE OF *BACTERIUM COLI*

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(*Received 19 June 1939*)

QUASTEL & WHETHAM [1924] stated that resting suspensions of *Bact. coli* will not reduce methylene blue in the presence of malate. However, den Dooren de Jong [1926] showed that a strain of *Bact. coli* can grow in an inorganic medium containing $NH_3$ with malic acid as the sole source of carbon. Booth & Green [1938] found a malic dehydrogenase in the cell-free juice obtained by crushing *Bact. coli* in the bacterial mill. The following paper establishes the existence of the enzyme in the organism, deals with its formation, extraction from the cell, properties, coenzyme requirements and with the identification of the oxidation product.

## *Methods*

The organism used was the stock strain of *Bact. coli* in use in this laboratory. Washed suspensions of the organism were prepared as described in previous papers. The enzyme activity was investigated first by the methylene blue technique. The following quantities were used in Thunberg tubes: 1 ml. phosphate buffer at *p*H 7·2; 0·2 ml. 0·5% methylene blue; 1 ml. *M*/10 *l*-malate (water in controls); 0·2 ml. 2*M* NaCN adjusted to *p*H 8; 1 ml. washed suspension of enzyme preparation and 0·5 ml. water, coenzyme solution or other addition as described below. The tubes were evacuated, equilibrated in a bath at 37°, the substrate tipped in from the hollow stopper and the time measured for the dye to be completely decolorized.

Where $O_2$ uptakes were studied, the experiments were carried out in Warburg manometers under conditions which are set out in detail later.

### *Demonstration of the enzyme in washed suspensions of* Bact. coli

This enzyme is difficult to demonstrate in the intact organism owing to its small amount or low activity. Its presence is best shown in the presence of

Table I. *Demonstration of malic dehydrogenase in* Bact. coli

| | Additions (ml.) to tubes | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| (*a*) *M*/25 buffer *p*H 7·2 | 1·0 | 1·0 | 1·0 | 1·0 | 1·0 | 1·0 | 1·0 | 1·0 |
| (*b*) 0·5% methylene blue | 0·2 | 0·2 | 0·2 | 0·2 | 0·2 | 0·2 | 0·2 | 0·2 |
| (*c*) *M*/10 Na malate | 1·0 | — | 1·0 | — | 1·0 | — | 1·0 | — |
| (*d*) 2*M* NaCN | 0·2 | 0·2 | 0·2 | 0·2 | — | — | — | — |
| (*e*) Cozymase 2 mg./ml. | 0·5 | 0·5 | — | — | 0·5 | 0·5 | — | — |
| (*f*) Water | — | 1·0 | 0·5 | 1·5 | 0·2 | 1·2 | 0·7 | 1·7 |
| (*g*) Bacterial suspension 9 mg. dry wt./ml. | 1·0 | 1·0 | 1·0 | 1·0 | 1·0 | 1·0 | 1·0 | 1·0 |
| Reduction time (min.) | 32 | ∞ | 54 | ∞ | 8 | 11 | 40 | 58 |

Additions (*a*)–(*d*) in quantities as in tube 1 remain the same in subsequent experiments.

NaCN and with the addition of coenzyme I (see later). The cyanide inhibits the high blank due to the large quantity of organism necessary and also acts by fixing the keto-group of the oxaloacetic acid formed [Green, 1936; Green & Williamson, 1937]. An experiment demonstrating the action of these additions is set out in Table I.

*Extraction of the enzyme.* For this purpose the wet-crushing mill of Booth & Green [1938] was used. The organisms obtained from the surface of broth-agar in 30 Roux bottles were washed off, centrifuged and washed twice, made up to 40 ml. (total dry weight of organism = about 3 g.) and circulated in the mill for 2½ hr. The crushed material was centrifuged for 30 min. at 3000 r.p.m. This gives an opaque juice *A* (25–30 mg./ml.) and a sediment *B*. *A* was further centrifuged for 20 min. at 11,000 r.p.m., giving a clear brown fluid $A_1$ (18–20 mg./ml.). $A_1$ was rendered water-clear by passage through a Seitz filter to give $A_2$ (3–4 mg./ml.).

Table II. *Malic dehydrogenase activity: washed suspension of* Bact. coli

| | Additions (ml.) to tubes | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 |
| (*a*) *M*/25 buffer *p*H 7·2 | 1·0 | 1·0 | 1·0 | 1·0 | 1·0 | 1·0 |
| (*b*) 0·5% methylene blue | 0·2 | 0·2 | 0·2 | 0·2 | 0·2 | 0·2 |
| (*c*) *M*/10 Na malate | 1·0 | 1·0 | — | 1·0 | 1·0 | — |
| (*d*) 2 *M* NaCN | 0·2 | 0·2 | 0·2 | 0·2 | 0·2 | 0·2 |
| (*e*) Cozymase 2 mg./ml. | — | — | — | 0·5 | 0·5 | 0·5 |
| (*f*) Water | 0·5 | 0·5 | 1·5 | — | — | 1·0 |
| (*g*) Bacterial suspension 7·2 mg./ml. | 1·0 | 1·0 | 1·0 | 1·0 | 1·0 | 1·0 |
| Reduction time (min.) | 68 | 59 | >180 | 24½ | 23½ | >180 |
| $Q_{MB}$ | 3·7 | 4·2 | <1 | 10·2 | 10·6 | <1 |

Table III. *Distribution of malic dehydrogenase in crushed material*

| Source of enzyme | Activity with cozymase added | Activity without cozymase |
|---|---|---|
| Washed suspension of organism | 100 | 27 |
| Crushed material | 122 | 2 |
| Washed sediment *B* | 65 | < 1 |
| Juice *A* | 185 | < 1 |
| Juice $A_1$ | 189 | < 1 |
| Juice $A_2$ | 190 | < 1 |

The sediment *B* was washed twice in distilled water on the centrifuge. The dry weight of all fractions was determined by drying to constant weight in a steam oven and the $Q_{MB}$ as malic dehydrogenase determined, where $Q_{MB}$ = $\mu$l. $O_2$ equivalent to the methylene blue reduced/hr./mg. dry weight of preparation. Table II shows the determination in full for the washed suspension of intact organism and Table III shows the activities of the various fractions expressed as % of that of the original washed suspension of organism (= 100) in the presence of cozymase and NaCN.

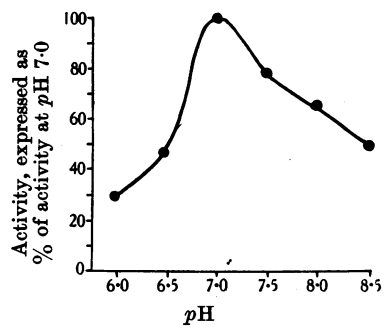


Fig. 1. Effect of *p*H on malic dehydrogenase activity (juice *A*).

The activity of the malic dehydrogenase is found to reside mainly in the juice, the sediment after washing being markedly less active than the original suspension while the final water-clear juice $A_2$ has an activity per unit dry weight of approximately twice that of the organism.

Fig. 1 shows the effect of *p*H on the activity of the malic dehydrogenase of juice *A* in the presence of cyanide and a constant excess of cozymase.

*Nature of the coenzyme.* The results in Table III show that a coenzyme is essential for the action of malic dehydrogenase with methylene blue. This coenzyme is present in the cozymase preparation from yeast [Green & Brosteaux, 1936] but this preparation contains considerable impurities. Accordingly the additions listed in Table IV were tested for their codehydrogenase activity in the place of the cozymase. Also the cozymase solution was boiled at *p*H 12 and then readjusted to *p*H 7 before testing, this treatment inactivating the coenzymes I and II [Green & Brosteaux, 1936]. From Table IV it appears that boiled

Table IV. *Nature of codehydrogenase*

Tubes were made up as usual with regard to additions (*a*)–(*d*). 1 ml. of juice $A_1$ was used as a source of malic dehydrogenase (*g*). 0·5 ml. of the following additions was used as source of codehydrogenase.

| Source of codehydrogenase | Reduction time |
|---|---|
| Water | > 4 hr. |
| Cozymase 2 mg./ml. | 12 min. |
| Cozymase previously boiled at *p*H 12 | > 4 hr. |
| Boiled organism 10 mg./ml. (Control on organism = >4 hr.) | 39 min. |
| Boiled organism 40 mg./ml. (Control on organism = >4 hr.) | 18 min. |
| *M*/600 nicotinamide | > 4 hr. |
| *M*/600 adenylic acid | > 4 hr. |
| *M*/600 ribose | > 4 hr. |
| *M*/600 (nicotinamide + ribose + adenylic acid) | > 4 hr. |

bacteria will act as a source of the codehydrogenase as also will the untreated cozymase solution, but not after treatment. The various decomposition products of coenzyme I are inactive so that the organism is unable to synthesize the coenzyme from these substances with sufficient rapidity to be active in these experiments.

In order to determine whether the active substance in boiled bacteria—which must presumably be the natural codehydrogenase—is identical with coenzyme I, the following experiments were carried out.

A thick washed suspension of the bacteria (20 mg./ml.) was boiled in distilled water for 5 min., cooled and the supernatant fluid spun off. The sediment of boiled cells was washed once and then resuspended in the original volume of water. The coenzyme activities of the sediment, supernatant and whole boiled suspension were compared with that of cozymase. From the results in Table V it is seen that the entire activity of the boiled suspension of bacteria resides in

Table V. *Distribution of codehydrogenase in boiled suspension of bacteria*

Tubes contained the standard quantities of additions (*a*)–(*d*). 1 ml. of juice $A_1$ was used as a source of malic dehydrogenase (*g*). 0·5 ml. of the following additions was used as source of coenzyme.

| Source of coenzyme | Reduction time |
|---|---|
| 1. Water control | > 4 hr. |
| 2. Cozymase 2 mg./ml. | 9½ min. |
| 3. Boiled organism 40 mg./ml. (Control on organism > 4 hr.) | 19 min. |
| 4. Supernatant fluid from (3) | 18 min. |
| 5. Sedimented cells from (3) | > 4 hr. |

the extracted supernatant fluid and this can therefore be used as a source of the codehydrogenase.

It has been shown that the malic dehydrogenase prepared from pig's heart-muscle requires coenzyme I specifically [Green, 1936]. We have shown that the supernatant fluid from boiled *Bact. coli* will also act as coenzyme for this system and the following data show that the ratio of codehydrogenase activity of coenzyme I/supernatant is constant for the pig's heart enzyme and the enzyme in the bacterial juice preparation (Fig. 2).

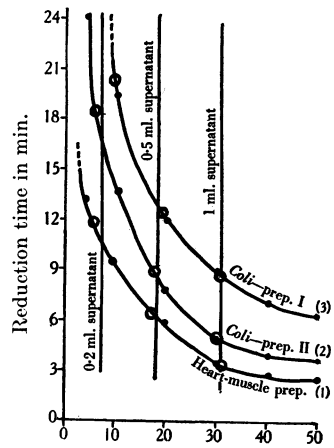


Fig. 2. (*a*) Effect of addition of cozymase on reduction time obtained with malic dehydrogenase preparations from *coli*-juice and heart muscle •—•. (*b*) Effect of addition of supernatant fluid from boiled organism on reduction time with the same preparations o—o. Demonstrates that active substance in the supernatant fluid is coenzyme I (see text).

Curve 1 shows the relation between the reduction time, under the standard conditions set out above, and quantities of coenzyme I added for the malic dehydrogenase preparation from pig's heart. Curves 2 and 3 show the same relation for two preparations of malic dehydrogenase from *coli*-juice (fraction *A*, Table III). In each case the reduction time was also determined in the absence of cozymase but with the addition of 1, 0·5 and 0·20 ml. of supernatant fluid obtained from a boiled suspension of bacteria (20 mg./ml.) by centrifuging off the whole cells and then passing the centrifugate through a Seitz filter. It is seen that not only does 1 ml. of supernatant correspond to the same amount of coenzyme I whether tested against the heart enzyme or the *coli* enzyme but also that this remains true on dilution. Further, halving the quantity of supernatant added is roughly equivalent to halving the quantity of coenzyme I. As coenzyme I is the only substance in the cozymase preparation which acts as co-dehydrogenase for the malic dehydrogenase preparation from heart [Green, 1936], it follows that the substance in the supernatant which is active as codehydrogenase for the malic dehydrogenase from *coli* must also be coenzyme I.

*Reduction of coenzyme I by the extracted enzyme.* The reduction of coenzyme I by the dehydrogenase extracted from the organism in juice *A* can be demonstrated as follows: 15 mg. cozymase preparation (15% coenzyme I) were dissolved in 2 ml. water and solid $Na_2HPO_4$ then added until $p$H = 8. Then 0·4 ml. *M* NaCN (neutralized to $p$H 8) and 1 ml. *M* Na malate were added. 2 ml. of juice *A* were then added and the whole incubated *in vacuo* at 37° for 30 min. At the end of that time, the reaction mixture was brought to the boil and the protein filtered off. The filtrate was diluted 1 in 5 and a sample examined in the Hilger Spekker spectrophotometer. The spectrum of reduced coenzyme I was obtained with a band at 345 m$\mu$, $\log I_0/I = 1{\cdot}0$. A similar experiment carried out with boiled enzyme gave $\log I_0/I = 0{\cdot}2$ at 345 m$\mu$.

*Coenzyme factor.* The juice *A* reduces methylene blue in the presence of *l*-malate and coenzyme I. In order to determine whether coenzyme factor [Dewan & Green, 1937; 1938] is a component of this system, the enzyme was purified in such a way that the factor would be destroyed. This was carried out as follows [Green & Dewan, 1938]: the juice was mixed at 0° with 3 vol. cold acetone. The precipitate was filtered off and washed with acetone and ether.

The dried powder was rubbed up with the original volume of water and the suspension dialysed overnight at 0°. The precipitate was centrifuged and discarded. The solution was then treated for 10 min. at 52°.

The $Q_{MB}$ of the preparation with the usual quantities of malate, coenzyme I and cyanide was 13. On the addition of 0·1 ml. coenzyme factor (for which we are indebted to Dr Straub of the Molteno Institute, Cambridge) the $Q_{MB}$ rose to 99. Coenzyme factor is thus an essential component of the system and is normally present in juice *A* unless special measures are taken to destroy it.

*Coenzyme I in the bacterial cell.* Since coenzyme I is required for the functioning of the malic dehydrogenase of the organism, it is obvious from Table II that the cells as washed off agar contain some, but not an amount optimum for the activity of the malic dehydrogenase present. (Reduction time with cozymase = 23 min.; without cozymase = 68 min.) Lwoff & Lwoff [1937] showed that the *V* factor for *H. parainfluenzae* can be replaced by cozymase but that the organism grown in the presence of suboptimum amounts of coenzyme I dehydrogenates malate at a rate which can be increased if cozymase is added to the system. They studied the effect on the "index of codehydrogenase activity" of adding cozymase and its breakdown products to the growth medium and found that the organism is unable to synthesize coenzyme I from its various parts but requires the whole molecule supplied as such. To study whether a similar effect is obtained with *Bact. coli*, we adopted the "index of coenzyme saturation" ("i.c.s.") defined

$$\frac{\text{Activity without coenzyme I added}}{\text{Activity in presence of excess coenzyme I}} \times 100.$$

This can be regarded as showing the degree to which the organism is saturated with coenzyme in respect of the malic dehydrogenase.

In order to saturate the system in the case of malic dehydrogenase of *Bact. coli* grown on agar, it was necessary to add cozymase preparation equivalent to about 6 $\mu$g. coenzyme I per mg. dry weight bacteria to the washed suspension.

Before investigating the effect of growth conditions on the "i.c.s." it was necessary to determine whether this value varies in the washed suspension on standing and also whether it can be altered by incubating the washed suspension in the presence of cozymase.

Table VI. *Variation of "i.c.s." on* (a) *incubation alone, and* (b) *incubation with cozymase*

Bacterial suspension 7·2 mg./ml. Tubes prepared as before, Table II.

Reduction time in presence of excess cozymase = 24 min. 52 sec.
Reduction time in absence of cozymase = 63 min. 50 sec.
Index of codehydrogenase saturation = 27

| Treatment | "i.c.s." |
|---|---|
| Original washed suspension | 27 |
| Incubated alone 24 hr. at 37° | 27 |
| Incubated 3 hr. at 37° with cozymase, see text | 34 |
| Stood 3 hr. at 0° with cozymase | 29 |

Table VI shows that incubation of a washed suspension of the organism for 24 hr. produces no change in the value of the "i.c.s." At the same time 6 ml. of the bacterial suspension were incubated with 8 mg. cozymase preparation for 3 hr. It was then centrifuged, the cells washed once and the "i.c.s." determined. A small rise—27 to 34—was obtained but since cell division probably commenced during that time and, as will be shown later, growth in the presence of cozymase

results in an increase of "i.c.s.", this rise is probably not significant. Thus the "i.c.s." of the washed non-dividing suspension is constant and is not altered by incubation alone or in the presence of cozymase.

*Action of* Bact. coli *on coenzyme I.* Euler & Gunther [1936], Euler & Heiwinkel [1937] and Euler *et al.* [1937] have shown that cozymase is inactivated by certain animal and vegetable tissues. That this is not the case with *Bact. coli* was shown as follows: 5 test tubes were set up as follows:

| Tube | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| Cozymase 2 mg./ml. | 1 ml. | 1 ml. | 1 ml. | — | — |
| Bacterial suspension 5 mg./ml. | 1 ml. | — | — | 1 ml. | — |
| Boiled bacterial suspension 5 mg./ml. | — | 1 ml. | — | — | 1 ml. |
| Water | — | — | 1 ml. | 1 ml. | 1 ml. |

The tubes were incubated aerobically for 4 hr. at 37°, then boiled for 10 min. and 1 ml. from each tested for coenzyme activity, using the malic dehydrogenase preparation from *Bact. coli*. The results in Table VII show that the cozymase in tube 1, incubated in the presence of the organism, has suffered no appreciable inactivation in 4 hr. as the amount of coenzyme used is insufficient to saturate the enzyme even before treatment.

Table VII. *Action of* Bact. coli *on cozymase*

Tubes prepared as before with regard to additions (*a*)–(*d*). 1 ml. of juice *A* used as a source of malic dehydrogenase. 1 ml. from each of tubes 1–5 (see text) used in turn as source of coenzyme.

| Coenzyme addition from tube no. | Reduction time |
|---|---|
| None | >3 hr. |
| 1 | 8 min. |
| 2 | 8 min. |
| 3 | 8½ min. |
| 4 | >3 hr. |
| 5 | >3 hr. |

*Variation of "i.c.s." with age of culture.* Cultures were grown in caseinogen tryptic broth in Roux bottles and prepared in washed suspension at various periods during their growth. In each case the activity of the malic dehydrogenase in the absence of added coenzyme (curve 1), the activity in the presence

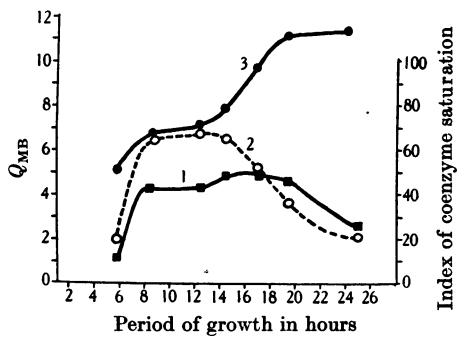


Fig. 3. Variation with age of culture of (1) enzyme activity ($Q_{MB}$) without added cozymase; (2) index of coenzyme saturation; (3) enzyme activity in presence of excess coenzyme I.

of excess coenzyme (curve 3) and the "i.c.s." calculated from these results (curve 2) were determined and the results are shown in Fig. 3. Curve 3 is a measure of the amount of enzyme formed, while curve 1 is the amount of that

enzyme activated by the coenzyme present in the organism. From this it appears that young cultures have relatively little coenzyme I and that the enzyme content increases until growth ceases, whilst the increase in coenzyme remains stationary after 8 hr. so that the "i.c.s." falls after the 14th hr. Wooldridge *et al.* [1936] and Wooldridge & Glass [1937] have shown that the activity of certain dehydrogenases varies during the growth period with *Bact. coli* and that, in general, this variation consists of an increasing activity during the early part of growth and a falling off in activity towards the end of active cell division. This is seen to be the case in curve 1 but, in this case, the apparent fall in activity is due to an insufficient amount of coenzyme synthesized by the organism.

Since there is an increase in "i.c.s." from the 6th to the 8th hr. of growth, one may deduce that the organism can synthesize coenzyme I during that period and the question arises whether the later fall in the "i.c.s." is due to a loss of this power or to the exhaustion of some essential factor in the medium which the organism is unable to synthesize with sufficient rapidity.

*Variations in "i.c.s." by alterations in and additions to the growth medium.* In the following experiments the organisms were harvested at the end of 24 hr. when the "i.c.s." has normally fallen to a value of 20–30. The media used were as follows:

1. Stephenson's inorganic medium + 2 % lactate + 2 % aspartate.
2. Tryptic broth agar.
3. Tryptic broth (strictly anaerobic).
4. Broth in Roux bottles.
5. As (4) + *M*/3300 adenine.
6. ,, *M*/3300 adenosine.
7. ,, *M*/3000 ribose.
8. ,, *M*/3000 nicotinamide.
9. ,, *M*/3000 each of adenosine, ribose, nicotinamide.
10. ,, 0·0002 % coenzyme I.
11. ,, 0·0005 % coenzyme I.

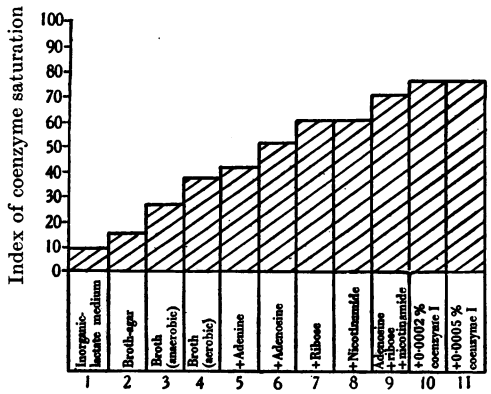


Fig. 4. Effect of additions to growth medium on the "i.c.s." of 24 hr. cultures.

In each case the "i.c.s." of the washed suspension was determined and the results are shown diagrammatically in Fig. 4. (1) shows that the power to synthesize coenzyme I from a simple medium is very restricted, about 4 times as much being synthesized in the caseinogen tryptic broth (4). The fall in "i.c.s."

shown in Fig. 3 is checked by the addition to the medium of coenzyme I (10) but the "i.c.s." never seems to reach 100, as increasing the quantity of coenzyme added to the medium (11) has no further effect. Addition of the various components of coenzyme I either singly or together does increase the value of the "i.c.s." to varying extents as shown in Fig. 4; ribose and nicotinamide being about equally effective while adenine has little effect.

These results suggest that the fall of "i.c.s." towards the end of growth in tryptic digest of casein is due to the exhaustion of some factor or factors used by the organism for the synthesis of coenzyme I which it is unable to synthesize with any rapidity for itself.

### *Oxidation of* l-*malate by* Bact. coli

A washed suspension of *Bact. coli* when shaken aerobically with *l*-malate in the presence of buffer, absorbs $O_2$. The $O_2$ uptake has been studied in Warburg manometers containing 1 ml. suspension of organism (dry wt. 1·5–2·0 mg./ml.), 1 ml. phosphate buffer *p*H 7·2 and 0·4 ml. *M*/60 Na malate in the main compartment and 0·2 ml. 10% NaOH in the centre cup. When corrected for the blank respiration of the organism, the malate is oxidized at a rate $Q_{O_2}=220$. Complete oxidation of malate would require 6 atoms of O per molecule. In the above experiment the total $O_2$ uptake amounted to 307 $\mu$l. which corresponds to an uptake of 4 atoms of O per molecule of malate; thus the oxidation is not carried to completion. With oxaloacetic acid as substrate, the organism carries out an oxidation corresponding to an uptake of 3 atoms O per molecule with a $Q_{O_2}=220$.

The addition of cozymase to the reaction mixture has no effect on either the $Q_{O_2}$ or the total $O_2$ consumption for the oxidation of malate. Since the addition of cozymase has a marked effect on the dehydrogenase activity of the organism but not on the $Q_{O_2}$ it follows that the rate of $O_2$ consumption is not limited by the velocity of reaction of the dehydrogenase: this is confirmed by the fact that oxaloacetic acid gives the same $Q_{O_2}$ as that for malate and it will be shown later that oxaloacetic acid is the product of the dehydrogenation of malic acid.

The oxidation of malate by the whole organism is completely inhibited by the presence of $10^{-4}$ *M* cyanide. Of other keto-fixatives tried, semicarbazide (*M*/100) is without effect; hydroxylamine (*M*/500) is toxic and hydrazine is itself partially oxidized.

### *Oxidation of* l-*malate by malic dehydrogenase extracted from* Bact. coli

The enzyme was extracted from the washed suspension of organism as described above and the source used throughout the following experiments was the juice *A* obtained by centrifuging the crushed material for 30 min. at 3000 r.p.m. To obtain oxidation of malate by the juice in air, the following components are necessary:

malate—enzyme—coenzyme I—coenzyme factor—cyanide—methylene blue—$O_2$.

It has been shown that the coenzyme factor is contained in the juice *A* with the dehydrogenase. Figs. 5–8 show the effect of varying the concentration of the various components on the rate of $O_2$ consumption, using 1 ml. of juice *A* as the source of enzyme in each case. The extracted enzyme has a low affinity for malate, the optimum concentration being *M*/14 (Fig. 5). The work previously described has shown that coenzyme I is required by the system and that juice *A* contains insufficient to give rise to appreciable activity. Fig. 6 shows that maximum activity is obtained with the enzyme preparation by the addition

of 150 $\mu$g. coenzyme I/ml. preparation. The system will not react with $O_2$ in the absence of a carrying system which can be supplied by a high concentration of

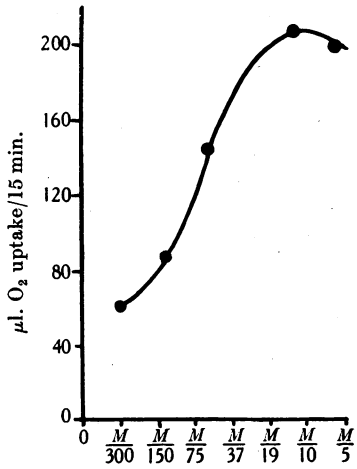


Fig. 5.

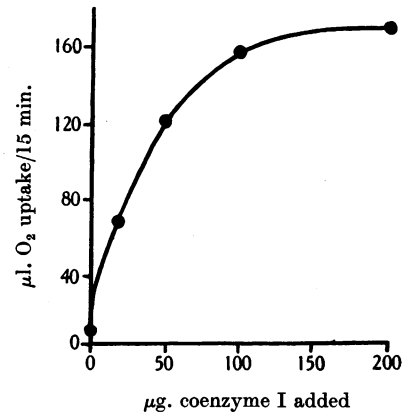


Fig. 6.

Fig. 5. Effect of substrate concentration. Manometers contain 1 ml. juice *A*; 0·5 ml. *M*/5 phosphate buffer; 0·5 ml. 0·5 % MB; 0·5 ml. cozymase; 0·1 ml. 2 *M* NaCN.

Fig. 6. Effect of coenzyme concentration. Manometers contain 1 ml. juice *A*; 0·5 ml. *M*/5 phosphate buffer; 0·3 ml. *M*/2 malate; 0·5 ml. 0·5 % MB; 0·1 ml. 2 *M* NaCN.

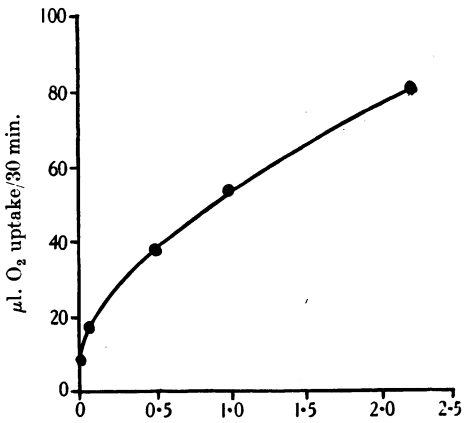


Fig. 7.

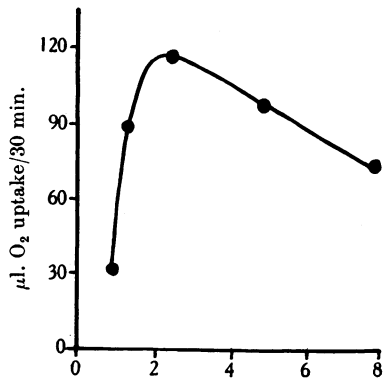


Fig. 8.

Fig. 7. Effect of methylene blue concentration. Manometers contain 1 ml. juice *A*; 0·5 ml. *M*/5 phosphate buffer; 0·5 ml. cozymase; 0·1 ml. 2 *M* NaCN; 0·3 ml. *M*/2 malate.

Fig. 8. Effect of cyanide concentration. Manometers contain 1 ml. juice *A*; 0·5 ml cozymase; 0·2 ml. 0·1 % MB; 0·3 ml. *M*/2 Na malate; 0·5 ml. *M*/5 phosphate buffer.

MB (Fig. 7). Further, as Green [1936] has shown, the malic dehydrogenase is inhibited by the oxaloacetic acid formed and an $O_2$ uptake cannot be obtained unless a keto-fixative is present. HCN is the most efficient fixative and has

an optimum concentration, under the experimental conditions, of $M/16$ (Fig. 8). Semicarbazide, hydroxylamine, hydrazine and bisulphite proved ineffective in this case, no significant $O_2$ consumption being obtained in their presence.

The malic dehydrogenase extracted from *Bact. coli* thus differs from that extracted from heart muscle by Green [1936] in its reactions with $O_2$, in being inhibited by high concentrations of cyanide (Fig. 8) and in being unable to react in the presence of semicarbazide, hydroxylamine or hydrazine.

Table VIII shows the $O_2$ uptake obtained with the complete system and that no reaction is obtained if any one of the components is missing. The $O_2$ uptake under optimum conditions is not linear, the $Q_{O_2}$ being halved in about 20 min. This effect cannot be altered by reducing the $O_2$ tension as has been shown by Gale [1939] for formic dehydrogenase. Owing to the low affinity of the extracted enzyme and the rapid deterioration of its activity, it is difficult to obtain the theoretical $O_2$ uptake. If the cyanide acts by fixing the oxaloacetic acid formed by the dehydrogenation, then an $O_2$ consumption should be obtained corresponding to an uptake of 1 atom $O_2$ per molecule malate. Fig. 9 gives an experiment in which this was realized.

Table VIII. *Oxidation system for extracted malic dehydrogenase*

| Manometer | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| | Additions in ml. | | | | |
| Juice *A* | 1 | 1 | 1 | 1 | 1 |
| $M/5$ phosphate *p*H 7·2 | 0·5 | 0·5 | 0·5 | 0·5 | 0·5 |
| $M/2$ Na malate | — | 0·3 | 0·3 | 0·3 | 0·3 |
| 0·5% methylene blue | 0·5 | — | 0·5 | 0·5 | 0·5 |
| 2 $M$ NaCN (neutral) | 0·1 | 0·1 | — | 0·1 | 0·1 |
| Cozymase 2 mg./ml. | 0·5 | 0·5 | 0·5 | — | 0·5 |
| Water | 0·3 | 0·5 | 0·1 | 0·5 | — |
| $\mu$l. $O_2$ uptake/15 min. | 0 | 14 | 0 | 0 | 196 |

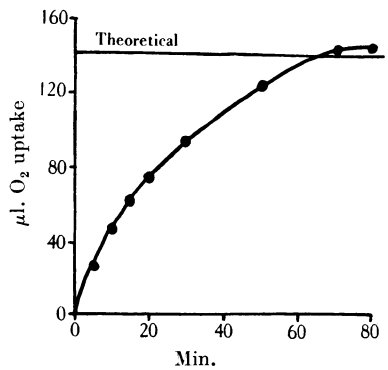


Fig. 9. Oxidation of *l*-malate by extracted enzyme system showing uptake of 1 atom of O/mol. malate oxidized. Manometer contains 1 ml. juice *A*; 0·5 ml. $M/5$ phosphate buffer; 0·5 ml. 0·5 % MB; 0·5 ml. cozymase; 0·1 ml. 2 $M$ NaCN and 0·5 ml. $M/40$ Na malate.

*Product of the oxidation.* Since cyanide is the only fixative which will allow of an appreciable rate of reaction, the keto-acid produced by the dehydrogenase has to be obtained first as the cyanohydrin. An experiment was set up as follows: 30 ml. of juice *A* were mixed with 20 ml. 0·5 % MB, 3 ml. 2 $M$ NaCN and 25 ml. $M/5$ phosphate buffer at *p*H 7·2 in which were dissolved 1·5 g. malic acid (neutralized) and 100 mg. cozymase preparation. The whole was divided into three

lots and incubated for 2 hr. at 37°, $O_2$ being bubbled throughout. At the end of that time the material was collected and cooled in ice. It was then deproteinized by treatment with colloidal $Fe(OH)_3$ and filtration through kieselguhr. Final traces of methylene blue were removed by shaking with a little kieselguhr followed by filtration.

The cyanohydrin will not react with 2:4-dinitrophenylhydrazine and warming with acid or alkali results in its decomposition. The procedure finally adopted [cf. Green & Williamson, 1937] was to add 10% NaOH until the final concentration was $N/5$ NaOH, and after leaving in ice for 5 min. quickly pour into an acid solution of 2:4-dinitrophenylhydrazine (0·5%) containing sufficient HCl to make the final acidity $2N$. The mixture was then left to stand at room temperature for 24 hr. when the 2:4-dinitrophenylhydrazone slowly crystallized and was filtered off (crude yield 120 mg.). The substance was recrystallized [Clift & Cook, 1932] by dissolving the dry crystals in the minimum ethyl acetate, adding ligroin until turbid and leaving to crystallize. After two recrystallizations by this method, it was washed with ligroin and dried *in vacuo*: M.P. 212° decomp. Found (Weiler): C, 37·93%; H, 3·04%; N, 17·8%. Calc. for oxaloacetic 2:4-dinitrophenylhydrazone: C, 38·34%; H, 2·58%; N, 17·9%.

*Reversibility of the dehydrogenase.* It has been shown that the extracted enzyme reduces coenzyme I when incubated in the presence of malate. It remains to show the oxidation of reduced coenzyme I in the presence of the enzyme and oxaloacetic acid.

Reduced coenzyme I was prepared as follows: 30 mg. cozymase preparation (15% coenzyme I) were dissolved in 10 ml. $M/2$ $NaHCO_3$, 20 mg. $Na_2S_2O_4$ crystals added and the whole incubated anaerobically for 30 min. The solution was then aerated vigorously for 15 min. to oxidize the excess hydrosulphite. 1 ml., diluted 1/5, was taken for estimation by means of the Hilger Spekker spectrophotometer. Log $I_0/I = 1·0$ at 345 mμ.

4 ml. of the reduced coenzyme solution were then incubated with 2 ml. juice *A* and 2 ml. $M/40$ Na oxaloacetate at 37° *in vacuo* for 20 min. The reaction mixture was then brought to the boil, the protein filtered off, the filtrate diluted 2/5 and its spectrum examined. Log $I_0/I = 0·5$—the absorption in this case being due to a non-specific absorption in the reaction mixture. A control performed with boiled enzyme and reduced coenzyme gave a well defined absorption band at 345 mμ, log $I_0/I = 0·9$.

Thus incubation of reduced coenzyme with the enzyme in the presence of oxaloacetate results in oxidation of the reduced coenzyme.

## Summary

1. Washed suspensions of *Bact. coli* contain malic dehydrogenase.

2. When suspensions of *Bact. coli* are crushed in the Booth-Green mill, the malic dehydrogenase is extracted from the cell and resides mainly in the liquid fraction of the crushed material.

3. The malic dehydrogenase of the *coli*-juice requires the addition of coenzyme I and coenzyme factor (diaphorase) for its action with methylene blue.

4. When suspensions of *Bact. coli* are boiled, coenzyme I is liberated from the cell and passes into the supernatant fluid obtained by centrifuging.

5. *Bact. coli* does not inactivate cozymase at a significant rate when incubated with it.

6. Organisms grown on agar or in tryptic broth do not contain the optimum amount of coenzyme to saturate their malic dehydrogenase.

7. The "index of coenzyme saturation" varies with the age of the culture, young cultures having a low value (20 %) which rises by the 10th hr. of growth to 65 %, remains steady until the 14th hr. and then falls until growth ceases—having a value of 20 % by the 24th hr.

8. Addition of coenzyme I and, to a smaller extent, ribose and/or nicotinamide to the growth medium prevents this fall of "i.c.s." with age of culture.

9. *Bact. coli* oxidizes *l*-malate aerobically, taking up 4 atoms of O per molecule malate oxidized. The oxidation is inhibited by $10^{-4}$ *M* cyanide.

10. The malic dehydrogenase extracted from the cells will oxidize *l*-malate aerobically in the presence of the following system: malate—dehydrogenase—coenzyme I—coenzyme factor—cyanide—methylene blue—$O_2$. The effect of variation of concentration of these factors is studied.

11. Under such conditions 1 atom of O is taken up per molecule of malate oxidized and the product of the oxidation is oxaloacetic acid, which has been isolated as the 2:4-dinitrophenylhydrazone.

12. The dehydrogenase is reversible, reduced coenzyme I being oxidized by the enzyme in the presence of oxaloacetic acid.

The authors wish to express their thanks to Dr D. E. Green for his advice and to Prof. Sir F. G. Hopkins for his interest in this work.

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