THE UTILIZATION OF FUMARATE AND MALATE BY *ESCHERICHIA COLI* IN THE PRESENCE OF MOLECULAR HYDROGEN.

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(Two Text-figures.)

[Read 30th April, 1947.]

Introduction.

The reduction of fumarate by molecular hydrogen in the presence of washed suspensions of bacteria was first observed by Stephenson and Stickland (1931) using *Escherichia coli*. The phenomenon was studied by Krebs (1937) using a different strain of *E. coli*.

Fischer and Eysenbach (1937), using cell-free extracts of a strain of yeast, which did not appear to contain succinic dehydrogenase, showed that fumarate could be reduced enzymically with the hydrogen donated by certain reduced dyes of low E'_0 values. Later, Fischer, Roedig and Rauch (1939) obtained evidence that the enzyme responsible for this reaction, fumarate hydrogenase, was a flavoprotein.

Claren (1938), using washed suspensions of luminous bacteria, showed that fumarate was a hydrogen acceptor in the presence of molecular hydrogen, succinate being the product of the reaction. Under similar conditions, malate was also an acceptor. He observed that in these suspensions the Knallgas reaction did not occur in the absence of fumarate and concluded that the role of fumarate was catalytic. Succinate had the same effect as fumarate.

Hoberman and Rittenberg (1943) observed that hydrogenase of suspensions of *Proteus vulgaris*, as measured by the exchange reaction with heavy hydrogen, was inactivated by molecular oxygen and reactivated by incubation with fumarate, succinate and glucose.

The present study was undertaken to find out the relationship between hydrogenase and the enzyme systems activating fumarate and malate in washed suspensions of *E. coli*.

Methods.

The organisms were grown and suspensions prepared as described previously (Lascelles and Still, 1946*a*). Suspensions of about 5–7 mg./ml. dry weight were found to be most satisfactory.

In many instances experiments were carried out using suspensions treated with toluene as well as with normal suspensions. This treatment consisted in incubation of the cells with toluene for at least 2 hours in an atmosphere of hydrogen at 38°C. Similar preparations could be obtained by keeping the cells under hydrogen with toluene for 16 hours at room temperature, or, for a longer period, in the refrigerator. One effect of toluene was to minimize the reduction of fumarate by donators within the cells.

Some experiments were carried out with cell-free preparations of *E. coli*. These were prepared as previously described (Back, Lascelles and Still, 1946), using the technique of Kalnitsky, Utter and Werkman (1945).

Estimations of succinic acid were carried out by the method of Krebs (1937), after extraction of the acid from the reaction mixture with ether. Fumarate was estimated by the method of Krebs, Smyth and Evans (1940).

Otherwise, the techniques used were those described in another paper (Lascelles and Still, 1946*a*).

The Reduction of Fumarate.

Effect of Variation of H-ion Concentration.—The optimum pH using phosphate buffers was 6·0 for normal and toluene treated cells. Phthalate buffers were inhibitory, but this inhibition was not removed by addition of phosphate. Using cell-free extracts, the optimum pH was 6·8. The inhibitory action of phthalate was more marked in the instance of the extract. Acetate buffers of the same pH were not inhibitory.

The Course of the Reaction.—This varied with the cell preparation used. In the instance of the normal cells, the hydrogen uptake corresponded with about 60% of the theoretical uptake required for the complete reduction of the added fumarate to succinate. Analysis showed that approximately 90% of the fumarate disappearing was recoverable as succinate.

In the absence of KOH in the centre well of the manometer vessel, evolution of CO_2 was demonstrable. This evolution of CO_2 was most noticeable in an atmosphere of nitrogen. Krebs (1937) has studied this phenomenon in some detail.

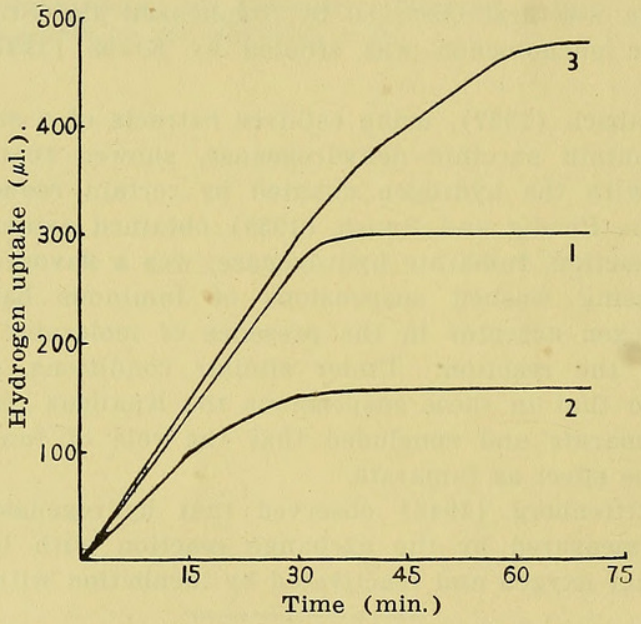

Fig. 1.—Course of hydrogen uptake in the presence of fumarate and malate.

Each manometer vessel contained: 1·0 ml. suspension dry wt. 7·4 mg./ml., 1·5 ml. M/5 phosphate buffer pH 6·0. 0·1 ml. M/5 fumarate and/or 0·1 ml. M/5 *dl*-malate was in the side arm. Distilled water was added to make a final volume of 3·0 ml. In the centre well was 0·2 ml. 20% KOH.

Curve 1.—Course of hydrogen uptake in the presence of M/150 fumarate.

Curve 2.—Course of hydrogen uptake in the presence of M/150 *dl*-malate.

Curve 3.—Course of hydrogen uptake in the presence of M/150 fumarate and M/150 *dl*-malate.

Using toluene-treated cells, the hydrogen uptake corresponded with 95–100% of the amount required for complete reduction of the fumarate to succinate (see Figure 1). Analysis showed that 95–100% of the fumarate disappearing was recoverable as succinate. Thus treatment with toluene eliminated most of the blank reduction of fumarate by hydrogen donators remaining in the washed cells. In addition, toluene treatment markedly accelerated the rate of hydrogen uptake in the presence of fumarate (see Table 1).

The toluene-treated cells did not show an evolution of CO_2, in the presence or absence of fumarate. The cell-free extracts behaved like the toluene-treated cells with respect to the correspondnce of hydrogen uptake to the amount of fumarate reduced to succinate; nor did these preparations show an evolution of CO_2.

It was shown that the reduction of methylene blue in the presence of succinate was greatly inhibited after the cells had been treated with toluene as described above.

TABLE 1.

The Effect of Treatment of Suspensions with Toluene.

Each manometer vessel contained 1·0 ml. suspension, 5·3 mg./ml. dry wt. 1·5 ml. M/5 phosphate pH 6·0; in the side arm was 0·1 ml. M/10 fumarate. Distilled water was added to make a final volume in each of 3·0 ml. In the centre well was 0·2 ml. 20% KOH.

Toluene Treatment.	Time of Incubation in Hydrogen.	Hydrogen Uptake (μl.). (Min.) 20	40	60	80	90	Theoretical Hydrogen Uptake.	Percentage Acceleration by Toluene (20′).
−	Nil	32	45	103	112	112	224	−
−	120 min.	36	76	106	112	112	,,	−
+	120 ,,	83	137	197	204	204	,,	130

Concentration of Fumarate.—The maximum rate of hydrogen uptake was obtained with M/300 fumarate, using normal cells, toluene-treated cells or the extract.

Artificial Carriers.—Unlike the reduction of nitrite and hydroxylamine by this strain of *E. coli* (Lascelles and Still, 1946*b*), reduction of fumarate by molecular hydrogen was independent of added carriers. The addition of cresyl blue, methylene blue or nile blue did not accelerate the hydrogen uptake. Benzyl viologen in concentration 0·002% did increase the rate of hydrogen uptake by about 50%. This was observed in the extracts also. As in the instance of nitrite and hydroxylamine reduction, this may be partly non-enzymic.

Dilution of the Suspensions.—The rate of hydrogen uptake in the presence of fumarate fell off sharply on diluting the suspensions. The activity of the diluted suspensions could be fully restored by the addition of boiled suspension. A preparation of diphosphopyridine nucleotide (DPN) obtained by the method of Williamson and Green (1940) was also active. Manganous ions in a concentration of M/300 restored some of the lost activity, but not to such a marked extent as the boiled suspensions or the DPN preparations. The addition of both DPN and manganous ions restored all the activity of the diluted suspensions.

Addition of M/3,000 manganous ions, M/300 magnesium ions, ferrous ions (as ferrous ammonium citrate) or of M/1,000 muscle adenylic acid did not result in any stimulation of the rate of hydrogen uptake by the diluted suspensions.

This problem will be studied at greater length using cell-free extracts of the bacteria.

Action of Inhibitors.—M/100 cyanide, M/50 hydroxylamine, M/50 hydrazine, and M/100 arsenite did not inhibit appreciably the hydrogen uptake in the presence of fumarate. In all cases the inhibitor was incubated with the cells or extract prior to addition of the fumarate.

Sodium malonate: M/30 malonate inhibited the system by about 20% using normal cells. The inhibition was much more marked after the cells had been treated with toluene and also with the cell-free extract. In these instances the inhibition was 50–70%. The degree of inhibition varied only slightly with variation in concentration of fumarate.

Sodium selenite: M/100 sodium selenite appeared to inhibit the hydrogen-fumarate system, especially with toluene-treated cells, but this compound was itself changed by the bacteria, as shown by the darkening in colour and the generation of a foul odour, suggesting selenium hydride.

Sodium fluoride: M/50 sodium fluoride was inhibitory to the extent of 40–50% using toluene-treated cells; the degree of inhibition was slightly less with normal cells and with the extracts. The effect was independent of the concentration of added magnesium ions.

Sodium pyrophosphate: M/50 pyrophosphate inhibited the toluene-treated cells by about 60%. The reaction in normal cells and in the extract was not inhibited appreciably. The inhibition was independent of the concentration of fumarate.

Carbon monoxide: This inhibitor was used in gas mixtures of hydrogen and carbon monoxide in the ratio $H_2/CO = 1:4$; this partial pressure of carbon monoxide

inhibited the uptake of hydrogen in the presence of fumarate by only 30%. The control experiments were carried out in atmospheres of hydrogen and nitrogen in the ratio of 1:4.

Sodium azide: M/100 sodium azide inhibited the hydrogen fumarate reaction in normal cells by about 30%; however, with the toluene-treated suspensions and in the extract, no inhibition was observed, even with M/30 azide.

Nitrophenols: The effect of certain nitrophenols was markedly changed by toluene treatment of the cells.

The toluene-treated cells and the cell-free extract were not inhibited by any of the four nitrophenols used. However, the rate of hydrogen uptake in the presence of fumarate with normal cells was almost completely inhibited by M/1,000 2:4-dinitrophenol, and partially inhibited by M/1,000 *m*-nitrophenol and *p*-nitrophenol, as shown in Table 2.

The action of *o*-nitrophenol was exceptional in that it brought about a marked increase in the rate of hydrogen uptake. In the absence of fumarate, these nitrophenols always caused a very small hydrogen uptake, not observed in their absence. This was not sufficient to account for the acceleration of hydrogen uptake by *o*-nitrophenol in the presence of fumarate.

TABLE 2.

Effect of Nitrophenols on Hydrogen Uptake in the Presence of Fumarate.

Contents of vessels as before. Suspension, dry wt. 6·3 mg./ml Nitrophenols were in contact with the cells during the equilibration period. 0·1 ml. M/4 fumarate was added from the side arm at zero. The toluene cells were stored with toluene for 60 hours at 5° C. in an atmosphere of hydrogen.

Toluene Treatment.	Concentration of Fumarate.	Nitrophenol.	Hydrogen Uptake (μl.). (Min.)			Percentage Change 40 Min.
			10	40	60	
–	M/120	Nil	38	207	343	—
–	,,	M/1000 DNP	0	3	9	−99
–	,,	M/1000 *m*–NP	37	179	283	−14
–	,,	M/1000 *o*–NP	92	334	459	+69
–	,,	M/1000 *p*–NP	8	104	163	−50
+	,,	Nil	117	376	504	–
+	,,	M/1000 DNP	113	380	515	0
+	,,	M/1000 *m*–NP	114	392	534	+4
+	,,	M/1000 *o*–NP	113	378	511	0
+	,,	M/1000 *p*–NP	124	411	548	+10

Ortho-phenanthroline: Gaffron (1945) has shown that *o*-phenanthroline inhibits the adaptation reaction and photoreduction in *Scenedesmus* by about 50%; this compound also stabilizes the adapted cells against reversion under the influence of strong light.

The uptake of molecular hydrogen in the presence of fumarate was inhibited from 50–70% by M/500 *o*-phenanthroline. The same results were obtained with normal cells, toluene-treated cells, and with the extract. Shaking the cells or extract in oxygen in the presence of this inhibitor prior to determination of the hydrogen-fumarate activity (by replacement of the oxygen with hydrogen, and addition of fumarate to the reaction mixture) did not increase the inhibition.

Addition of M/300 ferrous ammonium citrate, or M/300 manganese sulphate or M/300 zinc sulphate to the cells or to the extract, after they had been shaken in hydrogen in the presence of M/500 *o*-phenanthroline, resulted in a partial reversal of the inhibition (see Table 3).

M/300 magnesium chloride and M/300 calcium chloride were ineffective.

Ferrous ions and zinc ions are known to form complexes with *o*-phenanthroline (Sandell, 1944).

A study was made of this inhibitor on other enzymic reactions involving molecular hydrogen, using the same strain of *E. coli*.

With methylene blue as acceptor, M/500 *o*-phenanthroline caused a slight acceleration in the rate of hydrogen uptake, when the methylene blue was added to the cells after

TABLE 3.

The Reversal of Inhibition by o-Phenanthroline.

Each vessel filled as before. Cells had been treated with toluene; dry wt. was 5·0 mg./ml. *o*-Phenanthroline (M/500) was in contact with the cells during the equilibration period of 10 minutes. In the side arms were 0·1 ml. M/4 fumarate and 0·1 ml. of M/10 metallic salt. The contents of the side arms were tipped into the main compartment at zero time.

Metallic Salt.	Concentration of *o*-Phenanthroline.	Hydrogen Uptake (μl.). 60 Min.	Percentage Inhibition by *o*-Phenanthroline.
Nil	0	223	—
Nil	M/500	47	79
M/300 ferrous ammonium citrate	0	217	—
Do.	M/500	151	32
M/300 $MnSO_4$	0	214	—
Do.	M/500	168	25
M/300 $ZnSO_4$..	0	216	—
Do.	M/500	160	28

they had been shaking in hydrogen in the presence of *o*-phenanthroline. But if the cells had been shaken in oxygen in the presence of *o*-phenanthroline subsequent determination of the rate of hydrogen uptake in the presence of methylene blue showed that *o*-phenanthroline now inhibited by about 80%. It was shown that the cells did not form an inhibitory compound while shaking with *o*-phenanthroline in an atmosphere of oxygen. Thus a suspension of *E. coli* was shaken with phosphate buffer and M/200 *o*-phenanthroline in an atmosphere of oxygen at 38°C. for 30 minutes; the cells were then removed from the reaction mixture by centrifugation, and aliquots of the supernatant were tested for their action on the hydrogen-methylene blue system with fresh cells. No inhibition was observed. But if the hydrogen-methylene blue reaction were carried out on the cells which had been shaken in oxygen with *o*-phenanthroline, strong inhibition was obtained. Control experiments were performed on suspensions which had been shaken in oxygen in the absence of *o*-phenanthroline.

The Knallgas reaction was inhibited by about 90% by M/500 *o*-phenanthroline. Gas mixtures containing H_2/O_2 in the ratio of 90/10 were used. Controls containing nitrogen instead of oxygen showed negligible uptakes of gas. This inhibition of the Knallgas reaction was partially reversed by M/300 zinc sulphate; this was added to the cells after they had been shaking with the gas mixture for 30 minutes in the presence of *o*-phenanthroline. Manganous ions and ferrous ions were not used, due to the blank O_2 uptake in the presence of these ions.

The rate of hydrogen uptake in the presence of potassium nitrate was inhibited from 50–60% by M/500 *o*-phenanthroline. This inhibition was partially reversed by M/300 ferrous ammonium citrate, $MnSO_4$ and $ZnSO_4$ under the same conditions already described for the reduction of fumarate.

It is not possible yet to decide whether inhibition by *o*-phenanthroline denotes the participation of a metal in the catalytic systems.

Sodium iodoacetate: M/1,000 iodoacetate inhibited hydrogen uptake with fumarate by 98% and M/5,000 by 80%.

The Effect of Molecular Oxygen.—In previous studies of systems involving hydrogenase, it has been found that shaking of the cells with oxygen inactivates some part of the system (Lascelles and Still, 1946*a*). The reduction of fumarate by molecular hydrogen was also inhibited by previous incubation in oxygen.

The inhibitory effect obtained by preliminary incubation in oxygen was much more marked in the cell-free extract.

The Effect of Succinate and Maleate.—The effect of addition of these C_4-dicarboxylic acids on the hydrogen-fumarate reaction is summarized in Table 4.

Thus, under the conditions of these experiments, succinate, the product of the reduction, did not inhibit the forward reaction when added initially. The system does not reduce the stereo-isomer, maleic acid, under the conditions suitable for the reduction of fumarate. Nor is maleic acid an inhibitor of the reduction of fumarate.

TABLE 4.

Effect of Addition of Succinate and Maleate.

Contents of vessels as before. Suspension, dry wt. 7·4 mg./ml. Fumarate and other additions made from the side arm at zero time.

Concentration of Fumarate.	Additions.	Hydrogen Uptake (μl.). Min.		
		15	40	70
M/150	Nil	135	299	299
0	M/30 maleate	0	+2	+8
M/150	,,	124	316	318
0	M/30 succinate	+2	+4	+6
M/150	,,	135	265	300

THE REDUCTION OF MALATE.

Quastel and Whetham (1924) showed that washed suspensions of *E. coli* contained the enzyme, fumarase. It was considered likely that, in the present investigation, addition of malate to the washed suspensions would result in uptake of hydrogen. This was shown to occur under much the same conditions as held for the reduction of fumarate.

H-ion Concentration.—The optimum pH for the hydrogen-malate reaction was 6·0 with normal cells, and with the extract was 6·8.

The Course of the Reaction.—In normal cells, the hydrogen uptake per molecule of *dl*-malate added was about 30% of the theoretical amount required for the reduction of the *dl*-malate *via* fumarate to succinate.

In the extract, the uptake corresponded with 50% of that required for complete reduction of the *dl*-malate to succinate. These findings suggested that only one of the isomers in the *dl*-malate had been activated by the bacteria. Other workers have shown that fumarase from animal and bacterial sources is specific for *l*-malic acid and fumaric acid only.

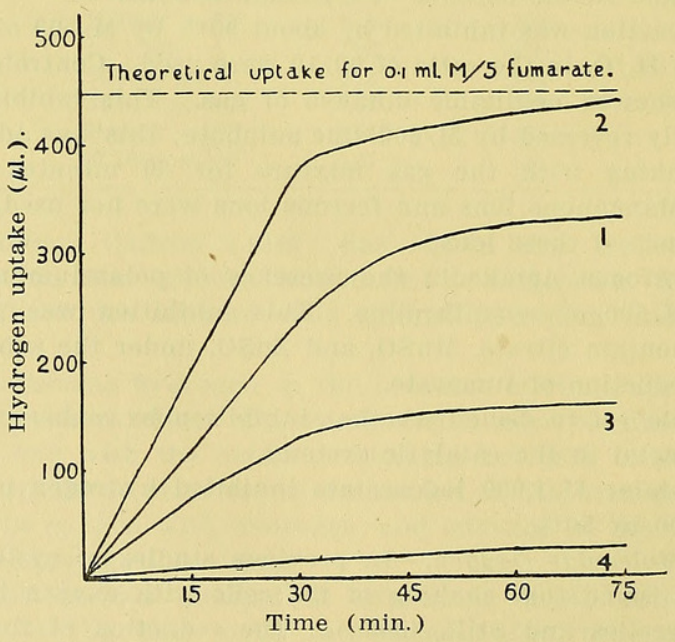

Fig. 2.—Course of hydrogen uptake in the presence of fumarate and malate with untreated and toluene treated cells.

Contents of vessels as in Figure 1; suspension, dry wt. 7·7 mg./ml.

Curve 1.—Course of hydrogen uptake in the presence of M/150 fumarate; cells kept in an atmosphere of hydrogen for sixteen hours at 21°C.

Curve 2.—Course of hydrogen uptake in the presence of M/150 fumarate; cells kept in an atmosphere of hydrogen with toluene for sixteen hours at 21°C.

Curve 3.—Course of hydrogen uptake in the presence of M/150 *dl*-malate; cells kept in an atmosphere of hydrogen for sixteen hours at 21°C.

Curve 4.—Course of hydrogen uptake in the presence of M/150 *dl*-malate; cells kept in an atmosphere of hydrogen with toluene for sixteen hours at 21°C.

Figure 1 shows the course of hydrogen uptake by normal cells in the presence of *dl*-malate and fumarate, separately and together.

Toluene treatment inhibited the hydrogen uptake in the presence of *dl*-malate almost completely; under the same conditions, the rate of hydrogen uptake in the presence of fumarate was markedly accelerated. Woolf (1929) showed that 2% cyclohexanol, after incubation with suspensions of *E. coli* for 15 hours, inhibited fumarase completely.

This action of toluene made it possible to examine the properties of the hydrogen-fumarate system uncomplicated by the fumarate-malate equilibrium.

Figure 2 compares the course of hydrogen uptake by toluene-treated cells and normal cells, in the presence of fumarate or *dl*-malate.

Concentration of dl-*Malate.*—The optimum concentration of *dl*-malate was found to be M/100 or greater.

Dilution of the Suspensions.—The hydrogen-malate system showed marked loss in activity when the suspensions were diluted. This system appeared to be more sensitive to dilution than the hydrogen-fumarate system. However, restoration of the lost activity was obtained under the same conditions as already described for the reduction of fumarate.

Inhibitors.—As shown in Table 5, the rate of uptake of hydrogen in the presence of malate was affected qualitatively by most of the inhibitors in the same manner as the reduction of fumarate.

TABLE 5.

Effect of Inhibitors on the Reduction of Malate.

Each manometer vessel contained 1·0 ml. suspension, about 6·5 mg./ml. dry wt., 1·5 ml. M/5 phosphate buffer pH 6·0. The inhibitor was incubated with the cells during the equilibration period. The side arm contained 0·1 ml. M/1 *dl*-malate. In the centre well was 0·2 ml. 20% KOH.

Inhibitor.	Concentration.	Percentage Inhibition.
Sodium cyanide	M/100	40
Do.	M/1000	0
Sodium azide	M/100	90
Do.	M/1000	23
Hydrazine	M/50	10
Hydroxylamine	M/50	10
Carbon monoxide	H_2: CO = 20 : 80	30
Sodium pyrophosphate	M/50	30
Sodium selenite	M/100	0
Sodium fluoride	M/50	100
Do.	M/100	100
Do.	M/300	88
Do.	M/600	0
Sodium malonate	M/10	35
2 : 4 DNP	M/1000	96
m–NP	M/1000	25
o–NP	M/1000	+37
p–NP	M/1000	91
o–Phenanthroline	M/500	100
Do.	M/3000	92
Sodium iodoacetate	M/1000	100
Do.	M/5000	75

Outstanding differences with respect to the action of inhibitors on the two systems were noted with sodium azide, sodium fluoride and *o*-phenanthroline. In these cases, the hydrogen uptake in the presence of malate was much more markedly inhibited than was the reaction with fumarate.

Attempts were made to reverse the inhibition by *o*-phenanthroline by addition of ferrous ions, manganous ions, zinc ions and magnesium ions. However, no diminution of the inhibition was obtained under those conditions which reversed to a marked degree, the inhibition by *o*-phenanthroline of the hydrogen-fumarate system.

Preliminary incubation with molecular oxygen inhibited the reduction of *dl*-malate by molecular hydrogen to the same degree as the hydrogen-fumarate system.

The uptake of hydrogen in the presence of *dl*-malate was more sensitive to M/30 maleate and to M/30 succinate than was the uptake in the presence of fumarate.

Discussion.

Studies with inhibitors would seem to suggest that the part of the system activating fumarate rather than that activating the hydrogen was the factor limiting the rate of hydrogen uptake. Thus cyanide, carbon monoxide and hydroxylamine inhibited strongly the reduction of methylene blue by molecular hydrogen but did not inhibit the hydrogen-fumarate or the hydrogen-malate systems to any marked extent. In previous studies on other reactions linked with hydrogen oxidation, similar observations have been made. The QH_2 values when methylene blue was the acceptor were much higher than those observed when either molecular oxygen, nitrate, fumarate or malate were used. It can be assumed that all these acceptors, except methylene blue, required the participation of other enzymes besides hydrogenase.

Hoberman and Rittenberg (1943) have put forward the view that hydrogenase is a ferrous porphyrin enzyme. Of the systems linked with hydrogenase, Granick and Gilder (1945) have produced evidence indicating that the nitratase of *Haemophilus influenzae* may well be an iron porphyrin enzyme. In general, one may say that there is not a great deal known about the enzymes and carriers of all these systems involving the oxidation of molecular hydrogen.

Doubts about permeability invariably introduce difficulties in assessing the value of studies on the enzymes of unicellular organisms. Lynen (1942) has drawn attention to the impermeability of yeast cells to malonate and succinate within the usual pH range. He overcame this difficulty by using a very acid pH. The marked accelerating effect of toluene treatment on the reduction of fumarate by molecular hydrogen in the presence of cells of *E. coli* in this investigation may be due partly to its action on the cell membrane. This is further suggested by the observations on the cell-free extract. The properties of the hydrogen-fumarate system resembled, in this instance, more closely the behaviour of the toluene-treated cells than the normal cells.

The marked difference with respect to the effect of toluene treatment between the hydrogen-fumarate and the hydrogen-malate systems may be due to sensitivity of fumarase to such treatment.

In view of the work of Clifton and Logan (1939), the effect of 2:4 dinitrophenol on the hydrogen-fumarate system was of interest. They found that the rates of oxidation of fumarate and succinate were inhibited markedly by DNP, whereas the rates of oxidation of other substrates tested were affected to a much smaller extent.

In the present investigation, the hydrogen-fumarate system in the extract and in the toluene-treated cells was unaffected by DNP; but, with normal cells, DNP was strongly inhibitory. Sodium azide inhibited the system in the normal cells but to a smaller extent. It exerted no inhibition with the extract or with toluene-treated cells.

Results obtained to date with *o*-phenanthroline do not give any clear indication of its mode of action. Its most pronounced action was on the hydrogen-malate system, which was completely inhibited by M/500 *o*-phenanthroline. This fact, together with the very marked dilution effect on the system, suggested the possibility of a metal component in the enzyme, fumarase. Laki and Laki (1941) have obtained a crystalline fumarase from beef heart, but do not mention the involvement of a metallic ion in this enzyme.

Summary.

The reduction of fumarate and *dl*-malate by molecular hydrogen in the presence of washed suspensions of *E. coli* has been studied.

In general, the properties of the two systems were very similar.

Treatment of the cells with toluene gave a material most suitable for the investigation of the reduction of fumarate. This treatment diminished reduction of fumarate by endogenous hydrogen donators and accelerated the rate of hydrogen uptake in the

presence of fumarate but inhibited strongly the reduction of *dl*-malate by molecular hydrogen.

The inhibitors studied included DNP and *o*-phenanthroline. M/1,000 DNP strongly inhibited the rate of hydrogen uptake in the presence of fumarate by washed cells. This inhibition was not observed with the toluene treated cells. M/500 *o*-phenanthroline inhibited the rate of hydrogen uptake in the presence of fumarate strongly, and completely inhibited the reaction with malate.

A few experiments were carried out with cell-free extracts of the bacteria. In general, the action of inhibitors in these instances closely resembled the behaviour of the toluene-treated cells.

ACKNOWLEDGEMENTS.

The authors wish to acknowledge the assistance of Mr. K. J. Back, who prepared the cell-free extracts of *E. coli* used in this investigation. They are grateful to Mr. G. K. Hughes, of the Department of Organic Chemistry, University of Sydney, for a gift of some *o*-phenanthroline.

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