CCLVII. THE ROLE OF FUMARATE IN THE RESPIRATION OF *BACTERIUM COLI COMMUNE*

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SZENT-GYÖRGYI and his co-workers [1935; 1936] and Stare & Baumann [1936] have recently shown that certain simple organic molecules such as fumarate, oxaloacetate and pyruvate take part as catalysts in cellular respiration. The function of these catalysts in respiration can be conveniently formulated as transport of hydrogen and for our present purpose they will therefore be termed "hydrogen carriers".

An attempt is made in this paper to analyse the role of this type of hydrogen carriers in the respiration of *Bact. coli commune*. The experiments aimed at elucidating the following points:

1. Which substances act as hydrogen carriers in the cell?
2. The rate of hydrogen transport by a given carrier.
3. The nature of the chemical grouping donating hydrogen to the carrier.
4. The nature of the chemical grouping accepting hydrogen from the carrier.
5. The significance of a given carrier in cellular respiration.

These five points will be referred to later as the "programme" of this paper. Only in some special cases was it possible to find the answer to all five questions. In other cases the analysis had to remain incomplete owing to the experimental difficulties which arise from the complex and involved nature of the chemical organization of the cell.

§ I. THE PRINCIPLE OF THE METHOD

Neglecting the role of the enzymes concerned with the "activation of organic molecules" and the "activation of oxygen" we may describe the function of intermediary hydrogen carriers in respiration as follows:

(1) Substrate + carrier = reduced carrier + oxidized substrate.

(2) Reduced carrier + O_2 = carrier + H_2O.

"Carrier" and "reduced carrier" cancel out in the balance sheet of (1) and (2) and the net effect is the oxidation of the substrate:

(3) Substrate + O_2 = oxidized substrate + H_2O.

The function of carriers can best be studied when it is possible to separate reaction (1) from (2) and to investigate the partial steps singly. Such a separation can be realized, for experimental purposes, on the basis of the following considerations.

The partial reaction (2) can be eliminated, obviously, by working anaerobically. Withdrawal of oxygen, however, will also check reaction (1); for the physiological concentration of the carrier, like that of other catalysts, is low and the available quantity will be rapidly consumed. We add therefore carrier from outside to the system. If an excess of carrier is present, reaction (1) will take place independently of reaction (2), i.e. in the absence of O_2. By adding the carrier and working anaerobically we thus separate (1) from (2).

Since "oxidized substrate" in (1) is, in the majority of cases, carbon dioxide, the rate of (1) can be measured manometrically and this principle makes it therefore simple to test whether a given substance can act as carrier. We add the supposed carrier to the cells under anaerobic conditions and measure, suitably controlled, the carbon dioxide output caused by the substance. Additional proof, of course, may be obtained from the determination of the other reactants in (1), for instance of the reduced carrier, or of the "substrate".

A positive test, e.g. an increased carbon dioxide production, indicates that the substance can act as hydrogen acceptor but it does not yet suffice to establish its function as carrier. The identification of the carrier, i.e. point 1 of the programme, has to include three tests, namely the investigations of reaction (1) and of reaction (2) and the demonstration of the occurrence of the supposed carrier in the cells.

If the tests are carried out with quantitative methods we can calculate the absolute activity of the carrier from the data and answer point 2 of the programme. Of the two reactions (1) and (2) the slower limits the catalytic activity of the carrier. The absolute activity, however, is often of less interest than the activity of the carrier in relation to cellular respiration. If we want information about the possible function of a carrier in cellular respiration, i.e. point 5 of the programme, we have to compare the activity of the carrier with the total respiratory activity of the cell in the presence of molecular oxygen (reaction (3)). We combine, therefore, our first test with a parallel determination of the rate of the aerobic respiration. Since (3) has to be compared with (1) it is advisable to measure (3) and (1) in the same units, for instance in terms of carbon dioxide produced.

This procedure which is generally applicable to any carrier and to any cell provides data to settle points 1, 2 and 5 of the programme.

Point 3 can be investigated either by the identification of the products of oxidation, or by indirect methods explained in § III 14. Point 4 requires a study of the metabolic behaviour of the oxidized carrier in the cell. The most important point in this respect is the question whether the oxidized carrier reacts directly with "activated oxygen" or through the intermediation of other carriers.

§ II. General aspects of the experimental procedure

1. *Bacteria*

Several strains of *Bact. coli commune* were obtained from Prof. J. W. Edington and Dr J. MacA. Croll of the Department of Bacteriology of this University. The standard medium in which the bacteria were cultivated was a broth containing 1 % "lemco" meat extract, 1 % peptone (difco), 0·5 % yeast extract (difco) and 0·5 % NaCl. Aerobic cultures were grown in Roux culture flasks on broth containing 3 % agar. After 20 hr. incubation at 37° the culture was rinsed off with 0·9 % NaCl and washed at the centrifuge. "Anaerobic" cultures, which we grew in order to have an organism containing active hydrogenlyase, were cultivated in 1 l. boiling flasks, which were filled with medium up to the neck; 2 % glucose and 2 % sodium formate were added to the broth. With these two substrates a good crop of bacteria resulted since the medium remains fairly neutral during the incubation: the acid produced by glucose is neutralized by the bicarbonate formed from formate [Grey, 1924]. 1 l. broth yielded about 400 mg. dry bacteria in 20 hr.

After washing, stock suspensions of the bacteria in 0·9 % NaCl were prepared, and the concentration of the bacteria was determined by measuring

their dry weight. The stock suspensions were kept up to a week in the refrigerator, and during this period no decrease of their original metabolic activity was noted.

2. *Quantitative determination of succinic acid*

The method used is in principle that of Szent-Györgyi & Gözsy [1935]. Succinic acid is extracted from the solution with ether and determined by measuring the oxygen necessary for the oxidation of succinate to fumarate in the presence of succinic dehydrogenase.

(*a*) *Extraction.* Continuous extractors of the Kutscher-Steudel [1903] type with interchangeable glass joints were used. Ether was kept over sodium and freshly distilled immediately before the extraction. It is important that the ether should be free from peroxide, since peroxide in the extract reacts with the catalase in the succinic dehydrogenase preparation and thus interferes with the manometric determination. The solution from which succinic acid was to be extracted was acidified with 1/5 vol. of 50 % H_2SO_4 and 1/10 vol. 10 % sodium tungstate was added to prevent frothing during the extraction. The time necessary for complete extraction under our conditions was found to be about 30–60 min. It varies with the shape of the extractor, the depth of the aqueous layer, the rate of boiling and the temperature. The higher the temperature the less favourable are the conditions for extraction [Forbes & Coolidge, 1919]. In order to secure complete extraction we continued the process for 120 min. After extraction, 2 ml. of phosphate buffer (*M*/10, *p*H 7·4) were added to the extract and the ether was evaporated on the steam-bath, until the aqueous phase was reduced to about 1 ml. It is essential to remove the ether completely. The residual solution was quantitatively transferred to a graduated 5 or 10 ml. measuring cylinder. Small quantities of phosphate buffer (*p*H 7·4, *M*/10) were used for washings, so that the total volume did not exceed 2 or 3 ml. The extract should be neutral.

(*b*) *Succinic dehydrogenase.* Muscle tissue (pigeon's breast muscle, pig's heart) is finely minced in a Latapie mincer and thoroughly washed with distilled water which is filtered off through muslin. Usually 3 washings with 10 vol. of water are sufficient. The washed tissue, suspended in 5 vol. of phosphate buffer (*p*H 7·4, *M*/10) serves as the enzyme preparation. In order to secure a complete oxidation of succinate very active preparations must be used. The enzyme is inhibited by fumarate, and if the relative activity of the preparation is low the oxidation remains incomplete owing to the inhibitory effect of fumarate. The inhibitory effect appears to be less in a fresh preparation of the enzyme than in suspensions which have been kept for about a week. It is therefore advisable only to use fresh suspensions which have not been kept for more than 4 days.

(*c*) *Specificity.* So far, we know only four substances which are oxidized by this preparation, viz. succinate, methylsuccinate [Thunberg, 1933], α-glycerophosphate [Green, 1936, 1] and *d*(−)glutamic acid [Weil-Malherbe, 1937]. The rate of oxidation of α-glycerophosphate is very low, in pigeon muscle about 1/50 of that of succinate, in some other muscles even less. The oxidation of *d*(−)glutamic acid is also very slow. Since α-glycerophosphate and *d*(−)glutamic acid are not extracted with ether these substances do not interfere in our method. The presence in the washed muscle of further dehydrogenases is immaterial since they require coenzymes which are absent from the ethereal extract; the separation of succinate from the coenzymes is in fact the main object of the extraction.

That methylsuccinate is oxidized in the presence of succinic dehydrogenase was shown by Thunberg [1933] with the methylene blue technique. We find a measurable oxygen uptake under our experimental conditions if *dl*-methylsuccinate is added to the enzyme. The rate of oxygen uptake, however, is extremely slow, amounting to about 0·5–3 % of that of succinate oxidation. Moreover the affinity of the enzyme for methylsuccinate is very low. Increasing the concentration of the substrate from $M/500$ to $M/250$ doubles the rate of reaction, indicating that the enzyme is not saturated with substrate over this range of concentrations.

Since methylsuccinate has not been found in biological material and α-glycerophosphate and *d*(−)glutamate are separated from succinate by the ethereal extraction the actual specificity of the enzymic method is very high.

(*d*) *Manometric determination.* Conical cups provided with side-arms and a centre chamber are used. The side-arm should be large enough to hold 1–1·5 ml. liquid. Into the main part 4 ml. of the enzyme preparation are measured; an aliquot part of the extract (0·5–1·5 ml.) is placed in the side-arm and 0·2 ml. 2*N* NaOH in the centre chamber. A blank is necessary to eliminate the oxygen uptake of the enzyme. This correction is very small, about 1 or 2 μl. O_2 per ml. enzyme per hour.

(*e*) *Treatment of solution prior to extraction.* Szent-Györgyi & Gözsy [1935] recommend treatment with permanganate in order to remove substances which may be oxidized in the presence of washed muscle. We have, however, abandoned permanganate treatment since we find, as a rule, more succinate after permanganate treatment. Substances which we find to yield succinate on treatment with cold or hot permanganate in acid solution are glutamic acid, α-hydroxy- and α-keto-glutaric acids and it is probable that derivatives of these compounds such as glutathione react similarly. Since, on the other hand, no substance is yet known to react like succinate we discarded the permanganate treatment as it is unnecessary and introduces errors.

Table I. *Recovery of succinic acid*

Succinic acid added mg.	Observed O_2 uptake μl.	Calculated O_2 uptake μl.	% of theory
0·247	23·2	23·5	99
0·494	46·0	47·0	98
0·988	88·5	94·0	93
1·97	178·7	188·0	95
3·96	366·0	376·0	98
1·97	195·0	188·0	96
1·97	182·0	188·0	97
0·494	45·7	47·0	97
1·97	179·0	188·0	95

(*f*) *Recovery of succinate.* In Table I a few recovery experiments are recorded and it will be seen that the recovery of added succinate is satisfactory, the average being 96·5 %.

(*g*) *Malonate test.* Szent-Györgyi & Gözsy [1935] suggested that the specific inhibitory effect of malonate on succinic dehydrogenase might be used for the identification of succinate. This effect is indeed additional evidence establishing the identity of succinate. We have carried out the test in a great number of determinations, but as we find the inhibition always of the expected magnitude, there seems to be no other oxidizable substance but succinate present in our material. The test is therefore not necessary in routine determinations.

3. *Absorption of oxygen in anaerobic experiments*

In order to secure complete anaerobiosis a stick of yellow phosphorus was placed in the centre cup of the manometric flask.

If both carbon dioxide and oxygen were to be absorbed, alkaline hydrosulphite (1 g. $Na_2S_2O_4$ + 0·1 g. Na anthraquinone-β-sulphonate + 0·8 g. NaOH per 10 ml.) was used as absorbent.

4. *Calculations*

Deduction of blanks. Experiments on bacterial oxidations are often complicated by the fact that more than one oxidizable substrate is present in the cells. Apart from the substrate, added for experimental purposes, intracellular substrates may be present. The presence of such substrates is indicated by the respiration which occurs without the addition of a substrate. Even prolonged washing and aeration do not remove this "blank" respiration, and the question arises whether the blank respiration and the oxidation of added substances are independent of one another, and therefore additive, or whether they affect each other. If there is a competition between the two substrates, the added substrate will diminish or completely prevent the oxidation of the intracellular substrates so that the total oxygen consumption may be due to the oxidation of the added substrate. In a similar way it is necessary to consider how the oxidation of one added substrate is affected by the addition of a second substrate. We have tried to settle this problem experimentally by three methods. First we compared the effects on the oxygen consumption of the addition of two or more substrates. This method decides between additive and competitive oxidation of two added substrates but does not elucidate the behaviour of the blank respiration.

Experiments of this kind are given in Table II. Since *Bact. coli* oxidizes a great number of different substances there are very many experimental possibilities of combining various oxidizable substrates. We include in Table II only a few of those which we have carried out. It will be seen that in some cases the effects of adding several substrates are additive, in others they are competitive. The results may be summarized thus:

(1) No significant summation occurs if glucose or lactate is present together with acetate, glycerol or fumarate.

(2) Significant, but not complete, summation occurs in some experiments with lactate, succinate and formate, especially if two or all three of these substances are brought together.

(3) With most substrates the rate of oxidation increases with time, as observed previously by Cook & Haldane [1931]. With succinate, however, the rate is fairly constant, and with formate it falls off.

These facts will be further discussed in § VIII 1.

A second method for studying additive or competitive oxidations is the determination of the quotient (free CO_2 formed)/(O_2 used). With this method it can be decided, in some cases, whether the blank respiration competes with the oxidation of added substances. The ratio CO_2/O_2 of the blank respiration is usually about 0·9. If the added substrate is the salt of an organic acid, the ratio CO_2/O_2 differs often very considerably from that of the blank respiration. For instance, the carbon dioxide formed from acetate in the initial stages of the oxidation (see Table III) is not free but in the form of bicarbonate.

The ratio CO_2/O_2 can be determined manometrically according to the principles developed by Warburg & Negelein [1921]. Two suspensions, one in phosphate buffer and one in bicarbonate and CO_2 buffer of the same *p*H are used.

Table II. *Additive and competitive oxidations in* Bact. coli commune

40°. Phosphate buffer *M*/10 *p*H 6·5. Final concentration of the substrate 0·01 *M*. The substrate was added to the bacterial suspension at 0 min. (after equilibration). Each flask contained 1·5 mg. bacteria. The 40 min. figure equals therefore Q_{O_2}.

Substance added	μl. O_2 absorbed after 10 min.	20 min.	40 min.	60 min.
—	10	18	33·5	50
Glucose	39	88	200	328
Fumarate	17·5	46	106	162
Acetate	24·5	59	134	213
Glycerol	32	73	165	262
Succinate	49	103	207	302
Formate	31	59	99	126
Lactate	51·5	115	264	429
Lactate + glucose	61	136	301	466
Lactate + fumarate	50	129	268	424
Lactate + acetate	52	125	282	445
Lactate + glycerol	58	132	291	450
Lactate + succinate	77	170	362	538
Lactate + formate	67	146	306	472
Succinate + glucose	70	150	318	465
Succinate + fumarate	32	76	160	221
Succinate + acetate	51	105	207	282
Succinate + glycerol	76	165	343	495
Succinate + formate	69	138	252	342
Glucose + fumarate	44	104	228	354
Glucose + acetate	41	97	219	357
Glucose + glycerol	44	97	209	326
Glucose + formate	55	118	227	368
Formate + fumarate	47	101	180	241
Formate + acetate	45	92	175	254
Succinate + formate + lactate	86	186	372	—

In phosphate buffer, with caustic soda in the centre cup, the change of pressure is due solely to the absorption of oxygen, since the carbon dioxide produced is absorbed. In the bicarbonate buffer, the change of pressure is due to oxygen consumption and carbon dioxide production. If the metabolic processes are identical in phosphate and bicarbonate buffer (which is generally the case) we can calculate the free carbon dioxide produced in the following way:

Let h_I = pressure observed in phosphate buffer,

h_{II} = pressure observed in bicarbonate buffer (during the same period),

$K^I_{O_2}, K^I_{CO_2}, K^{II}_{O_2}, K^{II}_{CO_2}$ = the constants for the manometer in which h_I and h_{II} are measured;

then (4) $$X_{O_2} = h_I K^I_{O_2},$$

(5) $$X_{CO_2} = \left(h_{II} - h_I \frac{K^I_{O_2}}{K^{II}_{O_2}}\right) K^{II}_{CO_2}.$$

As it is desirable in many cases to know also the change in bicarbonate, we determined the bicarbonate by adding an excess of acid to the solution at the beginning and at the end of the experiment. Two flasks, one for the initial and one for the final bicarbonate, are necessary. The complete experimental arrangement will be seen from Table III.

The experiment shows that the ratio (free CO_2)/O_2 is about 0·8 in the blank. It remains the same throughout the experiment. With acetate, the ratio

(total CO_2)/O_2 calculated for the total tissue is (244 μl. free CO_2 + 218 μl. bicarbonate)/470 μl. O_2 = 0·98. Of special interest is the time curve of the formation of free CO_2: very little free CO_2 is formed in the initial stages of the oxidation, while in the later stages the ratio (free CO_2)/O_2 is nearly 1·0. This shows that the oxidation of acetate proceeds by stages. In the first stage bicarbonate is set free, in the second stage carbon dioxide.

The fact that there is no free carbon dioxide formed in the presence of acetate in the initial stage shows that acetate inhibits the blank respiration. It is therefore not correct to deduct the blank respiration for the calculation of the ratio O_2 used/acetate added [*v.* Cook & Stephenson, 1928]. An accurate method of calculating this ratio is not available. An approximation is obtained on the assumption that the total oxygen consumed after addition of acetate is used for the oxidation of acetate as long as the rate of oxidation is maintained above the level of the blank oxidation. On the basis of this assumption it would be expected that the rate would fall to that of the control after absorption of 448 μl. oxygen and production of 224 μl. free CO_2 and 244 μl. bicarbonate. The time curve shows that this is approximately the case. We come thus to the conclusion that the oxidation of acetate is complete; this result, however, should not be generalized—each substrate has to be examined individually.

Table III. *Oxidation of acetate by* Bact. coli

Solutions: (1) Bacterial suspension in *M*/10 phosphate buffer *p*H 6·5; 1·66 mg. dry bacteria per ml.
(2) Bacterial suspension in NaCl (0·9%)—$NaHCO_3$ solution.
(3) Sodium acetate 0·05 mol.

	(1)	(2)	(3)	(4)	(5)
Main compartment	3 ml. solution 1	3 ml. solution 1	3 ml. solution 2	3 ml. solution 2	3 ml. solution 2
Centre cup	0·2 ml. 2*N* NaOH	0·2 ml. 2*N* NaOH	—	—	—
Side-arm (1) (Mixed after equilibration)	—	0·2 ml. solution 3	—	0·2 ml. solution 3	—
Side-arm (2)	—	—	0·2 ml. 5% H_2SO_4 (mixed at 120 min.)	0·2 ml. 5% H_2SO_4 (mixed at 120 min.)	0·2 ml. 5% H_2SO_4 (mixed at 0 min.)
Gas	O_2	O_2	5% CO_2 in O_2	5% CO_2 in O_2	5% CO_2 in O_2

Calculated O_2 uptake and CO_2 formation:

Time min.	In the absence of acetate: μl. O_2	In the absence of acetate: μl. CO_2	In the presence of acetate: μl. O_2	In the presence of acetate: μl. CO_2
10	− 12·9	+10·2	− 28·8	+ 1·6
20	− 27·2	+21·4	− 78·8	+ 8·6
40	− 50·3	+39·7	−185	+ 37·5
60	− 71·2	+56·1	−286	+ 60
80	− 90	+70·9	−376	+161
100	−110	+86·6	−445	+221
120	−126	+99·2	−470	+244
	μl. initial bicarbonate (per cup)		358	358
	μl. final bicarbonate (per cup)		364	576
	μl. change in bicarbonate		+ 6	+218

Calculated for complete oxidation of the added acetate: −448 μl. O_2; +224 μl. CO_2; +224 μl. bicarbonate.

A third method of studying the mutual effects on respiration of two competing substrates consists in determining a specific product of the oxidation. Such specific substances are for instance ammonia if amino-acids are oxidized [*v.* Krebs, 1935, 1] or bicarbonate if the salts of organic acids are oxidized. If

fumarate and glucose are the substrates the amount of bicarbonate formed indicates, under suitable conditions, the amount of fumarate oxidized. This method supplements the first method, which shows whether or not there is competition between substrates, but does not decide the extent to which one substrate inhibits the oxidation of the other. Experiments of this type are reported in § III.

5. Method of measuring hydrogen transport by carriers

(*a*) *Preliminary method.* Before setting up a complete quantitative analysis of the carrier it is often desirable to test in a preliminary experiment whether or not a given substance can act as hydrogen carrier under given conditions. The anaerobic partial reaction (as (1)) substrate + carrier = reduced carrier + oxidized substrate is suitable for this preliminary test. In those cases in which "oxidized substrate" is CO_2, or an acid which liberates CO_2 from the bicarbonate of the medium, the rate of (1) can be measured manometrically, and a simple experiment, suitably controlled, therefore is often sufficient to decide the preliminary question (point 1 of the programme). The result may be verified by the determination of the reduced carrier, e.g. succinate in experiments with fumarate.

(*b*) *Complete method.* The data required for our purpose are the rates of the reactions (1), (2) and (3) and also the rates of other reactions in which the reactants of (1), (2) or (3) may take part. This is best made clear by an example such as the role of fumarate as hydrogen carrier in the oxidation of glucose. The reactions, the rates of which must be determined, are:

(1*a*) glucose + fumarate → succinate + CO_2,
(2*a*) succinate + O_2 → fumarate + H_2O,
(3*a*) glucose + O_2 → H_2O + CO_2,
(6) glucose → products of fermentation.

The reactions (1*a*) and (2*a*) concern points 1 and 2, whilst the other two reactions concern point 5 of the programme.

In arranging the experimental procedure of measuring the above rates of reaction we were guided by the following considerations:

(I) The rates of all reactions should be measured, for the purpose of the comparison, in commensurable units. Taking as example reaction (1*a*), i.e. the oxidation of glucose by fumarate and (3*a*), i.e. the oxidation of glucose by molecular oxygen, we may compare either the CO_2 formed in (1*a*) with the CO_2 formed in (3*a*), or the O_2 used in (3*a*) with the succinate formed in (1*a*). Experience shows that it is desirable to determine both sets of data.

(II) The determination of the carbon dioxide formed in reaction (1*a*) and (3*a*) is complicated by the facts that:

(*a*) Carbon dioxide may also arise from the bicarbonate of the medium if, for instance, glucose is split into fixed acids.

(*b*) Carbon dioxide may be bound if the substrate is a salt of an acid such as lactate, or acetate or a zwitterion such as alanine. In this case part of the respiratory CO_2 remains in the solution as bicarbonate. Because of these complications it is necessary to determine also the change in bicarbonate concentration in the medium.

(III) Controls have to be set up in order to see:

(*a*) Whether carbon dioxide is formed by other reactions in addition to reaction (1). In the case of reaction (1*a*) for instance, carbon dioxide may also be formed by fermentation of glucose.

(*b*) Whether carbon dioxide is the only gas formed.

The detailed experimental arrangement which furnishes the data necessary on the grounds of the preceding considerations is described in Table IV. The reaction between fumarate and glucose is taken as an instance.

Table IV. *Manometric arrangement for the simultaneous determination of reactions (1a), (2b), (3a) and (8)*

Reagents required:

1. Bacterial suspension in 0·9% NaCl, containing about 3–15 mg. dry bacteria per ml. The amount of bacteria used depends on the rate at which the substrate is metabolized.

2. "Phosphate saline", containing 100 ml. 0·9% NaCl, 4 ml. 1·15% KCl, 3 ml. 0·11 *M* $CaCl_2$, 1 ml. 3·84% $MgSO_4$ and 20 ml. 0·1 *M* phosphate buffer, prepared from Na_2HPO_4 and HCl; *p*H of this saline should be the same as that of the bicarbonate saline.

3. "Bicarbonate saline", containing 100 ml. 0·9% NaCl, 4 ml. 1·15% KCl, 3 ml. 0·11 *M* $CaCl_2$, 1 ml. 3·82% $MgSO_4$ and varying amounts of 1·3% $NaHCO_3$. The saline is saturated with a gas mixture containing 5% CO_2. The quantity of bicarbonate added is varied according to the *p*H desired; *p*H is calculated on the basis of $pK_1^{CO_2} = 6{\cdot}10$ and $\alpha_{CO_2}^{40°}$ (absorption coefficient) = 0·52. In order to facilitate the accurate determination of the change in bicarbonate it is advisable to work with low bicarbonate concentrations, viz. 0·0025–0·005 *M*; *p*H is thus 6·5–6·8.

4. 2*N* sodium hydroxide ("NaOH"); 0·2 ml. per flask.

5. 2*N* sulphuric acid ("H_2SO_4"); 0·2 ml. per flask.

6. Yellow phosphorus in sticks freshly fused.

7. 0·2 *M* sodium fumarate; 1 ml. per flask.

8. A neutral solution of the substrate, a suitable quantity dissolved in 0·2 ml. water ("substrate").

9. 0·2 *M* sodium succinate.

10. Gas mixture and gases: 5% CO_2 in O_2; 5% CO_2 in N_2; O_2.

No.	Main chamber	Centre chamber	Side-arm 1	Side-arm 2	Gas
1	3 ml. 2	NaOH	Bacteria	—	O_2
2	3 ml. 2; substrate	NaOH	,,	—	O_2
3	3 ml. 2; fumarate	NaOH	,,	—	O_2
4	3 ml. 2; fumarate; substrate	NaOH	,,	—	O_2
5	3 ml. 2	—	,,	H_2SO_4	5% CO_2 in O_2
6	3 ml. 3; substrate	—	,,	H_2SO_4	5% CO_2 in O_2
7	3 ml. 2; fumarate	—	,,	H_2SO_4	5% CO_2 in O_2
8	3 ml. 3; fumarate; substrate	—	,,	H_2SO_4	5% CO_2 in O_2
9	3 ml. 2	—	,,	H_2SO_4	5% CO_2 in O_2
10	3 ml. 2; fumarate	—	,,	H_2SO_4	5% CO_2 in O_2
11	3 ml. 2; fumarate	Phosphorus	,,	H_2SO_4	5% CO_2 in N_2
12	3 ml. 3; fumarate	,,	,,	H_2SO_4	5% CO_2 in N_2
13	3 ml. 2; fumarate; substrate	,,	,,	H_2SO_4	5% CO_2 in N_2
14	3 ml. 2	,,	,,	H_2SO_4	5% CO_2 in N_2
15	3 ml. 2	,,	,,	H_2SO_4	5% CO_2 in N_2
16	3 ml. 2; substrate	,,	,,	H_2SO_4	5% CO_2 in N_2
17	3 ml. 2; 0·3 sodium succinate	NaOH	,,	—	O_2

After thermal equilibration (t_0) side-arm 1 (bacteria) is mixed with the main chamber in all flasks; in flasks 9, 10, 11 and 14 side-arm 2 is emptied into the main chamber at the same time. After a period of 1–2 hr., during which the substrate has been metabolized, side-arm 2 is emptied in 5, 6, 7, 8, 12, 13, 15 and 16.

Seventeen manometers with conical flasks provided with a centre chamber and with one or two side-arms are required. The first 8 manometers are concerned with the measurement of reaction (3). The oxygen consumption is determined in cups 1–4 (blank, glucose, fumarate, glucose+fumarate). In 4–8 the corresponding carbon dioxide production is measured, according to the principles discussed in § II 4. These manometers also furnish the values of the final concentrations of bicarbonate. In 9 and 10 the initial bicarbonate is measured, with

and without fumarate. If the substrate is not exactly neutral a further manometer charged like 6 and acidified at the time t_0 is necessary in order to find the initial bicarbonate in the presence of substrate. This control is not required if glucose, or sodium acetate or other neutral substances serve as substrates.

The following flasks (11–16) are concerned with reaction (1*a*). An excess of fumarate is added to the bacterial suspension (1 ml. 0·2 *M*) in 11–13; 11 furnishes the blank carbon dioxide production and the final bicarbonate concentration; 12 gives the corresponding data in the presence of glucose and 13 the initial bicarbonate. 14–16 correspond to 11–13, but contain no fumarate. These 3 manometers are controls in which the carbon dioxide formation and the change in bicarbonate in the absence of fumarate are measured (reaction (6)).

In the last manometer, 17, the rate of reaction (2*a*) is determined.

No controls concerning the formation of hydrogen are included in this scheme since we deal in full with the question of hydrogen formation in § III 9. It may suffice to say at this point that *Bact. coli* does not produce hydrogen in the presence of fumarate and that carbon dioxide is the only gas formed if fumarate is present.

After the manometric experiment succinic acid formed is determined in the solution in 11, 12 and 13 and compared with the oxygen used in 3 and 4.

§ III. Fumarate as hydrogen carrier

1. *Preliminary experiments*

Fumarate is the most important hydrogen carrier in *Bact. coli* from the quantitative point of view. Table V shows an experiment which was carried out by the "preliminary method". The anaerobic CO_2 production taking place after addition of fumarate and another suitable substrate was followed for 1 hr.

Table V. *Oxidation of various substances by* Bact. coli *in the presence of fumarate*

Bicarbonate saline. *M*/80 bicarbonate; 5% CO_2 in N_2. Each flask contained 3 ml. bacterial suspension (5·5 mg. bacteria). Fumarate concentration *M*/20. Substrate concentration *M*/100. Fumarate and substrate were added to the bacterial suspension after the equilibration period.

No.	Substrates added	μl. CO_2 evolved after 10 min.	20 min.	40 min.	60 min.	μl. succinic acid formed during 60 min.
1	—	1	2·5	5	8	~0
2	Fumarate	7	13·5	26	35·5	130
3	Fumarate; *dl*-lactate	49	99	207	308	968
4	Fumarate; acetate	26	48	96	144	502
5	Fumarate; glycerol	37	98	248	427	1204
6	Fumarate; *dl*-glyceraldehyde	33·5	84	228	405	742
7	Fumarate; *l*(+)glutamate	33	59	121	183	760*
8	Fumarate; pyruvate	135	364	672	728	920
9	Fumarate; glucose	278	614	1272	1695	980
10	Fumarate; butyrate	22	28	40	52	316
11	Fumarate; acetoacetate	21	36	65	93	342
12	Fumarate; H_2	57†	140†	274†	411†	854
13	Glucose	221	488	955	1096	132
14	Glycerol	4	9	14	19·5	—
15	Glyceraldehyde	10·8	22	43	61	—
16	Acetoacetate	5	9	12	15	0
17	Pyruvate	49	146	358	516	164

* Part of the succinic acid formed may have been derived from glutamate.
† H_2 absorbed.

and succinate was then determined in the solution. Controls were set up with each substrate to see if CO_2 is produced in the absence of fumarate but only those controls have been included in Table V which gave increased CO_2 formation (lines 13–17).

It will be seen that a variety of different substances are oxidized by fumarate in *Bact. coli*. The 10 substances recorded include glucose and various derivatives, lactate, glycerol and glyceraldehyde, an amino-acid (glutamate), fatty acids (butyrate and acetate) and ketonic acids (pyruvate, acetoacetate). All these substances reduce fumarate and liberate carbon dioxide. The formation of succinate is the more accurate measure of the rate of the dismutation since carbon dioxide may be evolved either by oxidation of the substrate or by decomposition of bicarbonate by acids formed. In those cases in which the substrates evolve CO_2 in the absence of fumarate there is an increase in CO_2 when fumarate is present.

Having thus established the fact that fumarate oxidizes anaerobically various substrates in *Bact. coli* we shall now apply the "complete method" for the quantitative investigation.

2. *Fumarate and glucose*

As the first example the role of fumarate in the oxidation of glucose will be examined. The experiment was arranged according to Table IV. The substrate solution contained 0·5 mg. glucose per flask. The organism was grown aerobically and 2·37 mg. bacteria were present in each flask. For the sake of brevity we omit the manometric readings and present the calculated results only (Table VI). The quantities of gas are expressed in μl. The figures given for carbon dioxide represent the total quantity (respiratory carbon dioxide + carbon dioxide liberated by acid from bicarbonate). The bicarbonate data at the foot of the table indicate the source of the carbon dioxide. The flask number given with the bicarbonate figure denotes the manometer in Table IV from which the figure was obtained. Succinic acid is also expressed in μl. (1 mM. = 22400 μl.).

The data of the aerobic part of the experiment are recorded in columns 1–4; they were calculated according to the formulae (4) and (5). The results of the anaerobic experiment are given in the columns 5–8; they were calculated in the usual way by multiplication of the pressure change with the constants for carbon dioxide. There are various difficulties in interpreting the blank figures since the methods discussed in § II 4 are not applicable to all cases in which a competition between metabolic processes may occur. Because of these uncertainties we present the experimental figures without corrections and avoid calculations as far as possible.

The experiment shows the following:

(1) If 0·5 mg. glucose is added to *Bact. coli* anaerobically, 152 μl. CO_2 are liberated. The amount of bicarbonate in the solution decreases by practically the same quantity (148 μl.). There is thus no formation of CO_2 from glucose (column 8).

(2) If 0·5 mg. glucose and an excess of fumarate are added to the suspension anaerobically, still more CO_2 (271 μl.) is liberated during the same time, but in this case there is only a slight (9 μl.) decrease in bicarbonate which is almost within the limits of error. The evolution of CO_2 therefore is due in this case to oxidation of the organic substances present, namely glucose or fumarate (column 6).

(3) Concurrent with this oxidation there is a reduction of fumarate to succinate. A comparatively slow reduction of fumarate occurs in the absence of

Table VI. *Fumarate and glucose in* Bact. coli commune

Column ...	1		2		3		4		5	6	7	8	9
	Oxidations by molecular oxygen								Anaerobic oxidation by fumarate		Anaerobic fermentation		Oxidation of succinate by molecular oxygen
Substrates added ...	—		Glucose		Fumarate		Glucose + fumarate		Fumarate	Glucose + fumarate	Blank	Glucose	
Calc. from manometer nos. ...	1 and 5		2 and 6		3 and 7		4 and 8						
Time min.	μl. O_2 used	μl. CO_2 formed	μl. O_2 used	μl. CO_2 formed	μl. O_2 used	μl. CO_2 formed	μl. O_2 used	μl. CO_2 formed	12 μl. CO_2 formed	13 μl. CO_2 formed	15 μl. CO_2 formed	16 μl. CO_2 formed	17 O_2 used
10	1·8	2·4	41·7	44·3	17·5	9·8	43·2	33·3	5·8	55	0·8	53·5	62
20	8·8	8·6	104	107	56·4	29·2	119	95	10·7	132	0·8	120	140
40	15·9	14·7	222	229	153	95	281	234	16·6	191	0·8	150	275
60	22·7	21·2	253	256	252	167	441	372	21·4	214	1·7	150	470
80	27·3	26·6	267	272	340	249	582	485	26·1	232	1·7	150	—
100	31·7	39·9	277	284	420	290	684	553	31·8	251	2·5	150	—
120	37	34·8	285	291	495	345	781	620	38·5	271	2·5	152	—
μl. initial bicarbonate		335 (flask 9)		335 (flask 9)		348 (flask 10)		348 (flask 10)	348 (flask 11)	348 (flask 11)	329 (flask 14)	329 (flask 14)	
μl. final bicarbonate		332 (flask 5)		335 (flask 6)		782 (flask 7)		890 (flask 8)	378 (flask 12)	339 (flask 13)	319 (flask 15)	181 (flask 16)	
Δ bicarbonate		−3		0		+434		+542	+ 30	− 9	− 10	−148	
Δ succinate		—		—		—		—	+165	+568	—	—	—

glucose (column 5) but glucose increases the rate of reduction considerably (column 6). This increase amounts to $568-165=403$ μl. We compare this figure with the reduction of molecular oxygen under analogous conditions (columns 3 and 4) and find that glucose causes an additional disappearance $781-495=286$ μl. Since the reduction of 2 mol. of fumaric acid is equivalent to reduction of 1 mol. of O_2 the rate of reduction of fumarate is $\frac{403}{2\times 286}$ or 71 % of the rate of reduction of molecular oxygen.

(4) Comparing the oxidation of glucose by fumarate with that by molecular oxygen in terms of the carbon dioxide produced we obtain the following figures:

CO_2 produced anaerobically in the presence of fumarate: $271-38{\cdot}5=232{\cdot}5$ μl.
CO_2 produced aerobically: $620-345=275$ μl.

The rate of the anaerobic oxidation by fumarate is thus $\frac{232{\cdot}5}{275}$ or 85% of the rate of the aerobic oxidation.

(5) The course of the CO_2 production in the aerobic oxidation and that of the oxidation by fumarate do not run parallel in this example, since there is an initial excess of carbon dioxide in the anaerobic experiment. This excess is due to decomposition of bicarbonate by acids formed from glucose. The acids formed by fermentation disappear later under the influence of fumarate and in the balance sheet there is no production of fixed acid. It is of interest to note that fumarate unlike molecular oxygen does not suppress the "anaerobic" glycolysis.

(6) The oxidation of succinic acid by molecular oxygen proceeds faster than the oxidation of glucose under analogous conditions (column 9).

In summing up these results we may say that fumarate oxidizes glucose anaerobically to form carbon dioxide and that the rate of this oxidation is 71–85 % that of the oxidation of glucose by molecular oxygen under similar conditions.

This experiment is a typical example out of a series of 12 similar experiments. We varied the strain and the condition of the cultivation. The results were essentially the same in all experiments. The variations concerned the relative rates of the reactions (1*a*), (2*a*) and (3*a*). The rate of (1*a*) was 60–100 % of that of (3*a*) and the rate of (2*a*) varied between 60 and 130 % of that of (3*a*).

In view of these variations and also of the uncertainty in the calculations we do not wish to emphasize the exact numerical value of the calculated figures. These variations and uncertainties, however, cast no doubt on the order of magnitude of the ratio (oxidation of glucose by fumarate)/(oxidation of glucose by O_2) in *Bact. coli* and it will suffice for the further considerations to know the order of magnitude.

Before we turn to the study of the remaining points of the programme (§ III 11) a number of analogous reactions will be described.

3. *Fumarate and malate*[1]

(*a*) *Fermentation of fumarate.* If fumarate is added to *Bact. coli* anaerobically as already mentioned in the preceding section, carbon dioxide and succinic acid are formed, CO_2 arising by oxidation, succinic acid by reduction. Since the complete oxidation of 1 mol. of fumarate or malate is equivalent to the reduction

[1] Since fumarate and malate are interconvertible in *Bact. coli* it is not possible to decide which of the two substances reacts in a given case. It is probable from the work of Green [1936, 1, 2] that fumarate is not oxidized as such but only after hydration to malate, and that malate is not reduced as such, but only after dehydration to fumarate. We shall therefore speak of malate if oxidations are in question, and of fumarate if reductions are in question.

of 6 mol. of fumarate, the expected equation for the fermentation is as follows:

(7) 7 fumarate = 6 succinate + $2CO_2$ + 2 bicarbonate.

The expected yield of succinate, expressed in terms of fumarate fermented, is thus 85·6 %; the yield of both CO_2 and of bicarbonate is 28·6 %. We found the following figures in an experiment in which 19·5 mg. washed cells were incubated at 40° with 0·1 ml. 0·2 *M* fumarate in a manometric flask until the evolution of gas ceased (6 hr.):

Fumarate added and fermented	448 μl.
Succinate formed	406 μl. (90·8 %)
HCO_3^- formed	82 μl. (18·3 %)
CO_2 formed	170 μl. (38·0 %)

The figures show that slightly more fumarate is reduced and less is oxidized than would be expected according to scheme (7). The deviations from the theory are due to the fact that fumarate whilst reacting according to (7) may simultaneously oxidize such intracellular substrates as may be present, and succinate and free CO_2 are formed in these reactions. In order to reduce these side-reactions cells were used for the above experiment which had been aerated at room temperature for 24 hr. Since it is impossible to remove oxidizable substances from the cells completely, it is not surprising to see that equation (7) is only approximately verified.

The products found account completely for the fumarate added. Out of 448 μl. added, 406 μl. are recovered as succinate and $\frac{82}{2} = 41$ μl. as bicarbonate. There are no significant quantities of formate and acetate formed under our conditions by *Bact. coli* and the fermentation of fumarate in *Bact. coli* is in this respect unlike that in *Aerobacter aerogenes* [Barker, 1936].

We have not studied further details of the fermentation of fumarate under different conditions in *Bact. coli*, since it will suffice for the purpose of this paper to deal with two aspects of the problem, the first being the quantitative determination of the ratio

(8) $\dfrac{\text{Rate of anaerobic oxidation of malate}}{\text{Rate of aerobic oxidation of malate}}$,

and the second being the effect of certain other metabolites on the rate of the oxidation of fumarate or malate.

(*b*) Ratio $\dfrac{\text{rate of anaerobic oxidation of malate by fumarate}}{\text{rate of oxidation of malate by molecular oxygen}}$.

The ratio (8) may be measured in two different ways, either in terms of

(9) $\dfrac{\frac{1}{2}\text{ succinic acid formed anaerobically}}{\text{O}_2\text{ consumed aerobically}}$,

or in terms of

(10) $\dfrac{\text{CO}_2\text{ formed anaerobically}}{\text{CO}_2\text{ formed aerobically}}$.

From Table VI we obtain for the oxidation of malate

(9*a*) $\dfrac{\frac{165}{2}\ \mu\text{l. equivalents of succinate formed anaerobically}}{495\ \mu\text{l. O}_2\text{ used aerobically}} = 0{\cdot}17$, and

(10*a*) $\dfrac{38{\cdot}5\ \mu\text{l. CO}_2 + 30\ \mu\text{l. bicarbonate formed anaerobically}}{345\ \mu\text{l. CO}_2 + 434\ \mu\text{l. bicarbonate formed aerobically}} = 0{\cdot}088.$

Thus the reduction of fumarate by malate is −17 % and 8·8 % respectively of the reduction of O_2 under the same conditions. The lower figure of 8·8 % for (1*a*)

indicates that relatively less carbon dioxide is formed if fumarate is the oxidizing agent. The oxidation is thus less complete anaerobically.

The ratios (9*a*) and (10*a*) of which we have made more than 20 measurements vary considerably in different experiments. The figures given were among the lowest which we found. The highest values observed for (9*a*) were 80% and for (10*a*) 50%. Some light on the cause of this variation is thrown by the phenomenon discussed in the following paragraph which shows that the oxidation of fumarate is promoted by, and consequently linked up with, the presence of other oxidizable substances.

(*c*) *Effect of other substances on the oxidation of malate.* It will be seen from Table VII that more bicarbonate is formed from fumarate aerobically in the presence of glucose (542 μl.) than in the absence of glucose (434 μl.). The formation of bicarbonate is proof of an oxidation of fumarate as explained above; glucose thus promotes the oxidation of fumarate. This promotion of the oxidation of fumarate can be brought about by other substances and can be shown aerobically as well as anaerobically. Table VII shows an experiment in which

Table VII. *Promotion of the oxidation of fumarate by lactate and pyruvate*

Substrates added	0·5 ml. fumarate (0·2 *M*)	0·2 ml. *dl*-lactate (0·01 *M*)	0·2 ml. pyruvate (0·01 *M*)	0·5 ml. fumarate (0·2 *M*) + 0·2 ml. *dl*-lactate (0·01 *M*)	0·5 ml. fumarate (0·2 *M*) + 0·2 ml. pyruvate (0·01 *M*)
μl. O_2 taken up after					
40 min.	118	116	89	260	246
80 min.	337	130	99	600	578
160 min.	930	148	119	1325	1225

the aerobic oxidation of fumarate was measured in the presence of small quantities of lactate and pyruvate and it will be seen that the absorption of oxygen in the presence of fumarate + lactate or fumarate + pyruvate is greater than would be expected from the experiments with the single substrates. Thus in the case of lactate the sum of the single experiments is 930 + 148 μl. whilst both substrates together take up 1325 μl. oxygen.

Examples of this effect under anaerobic conditions, i.e. the promotion of the fumarate fermentation by other substances, will be given later (e.g. § III 7). The most remarkable substance exerting such an effect is molecular hydrogen. This effect constitutes another difficulty in the quantitative interpretation of results but since it is comparatively small it is, as a rule, of minor importance.

4. *Fumarate and acetate*

The results obtained with further substrates will be presented in an abridged form. Instead of the full course of the reaction only final values will be given which represent the changes occurring during the period recorded. It should be noted that the oxidation of the "substrate" was not always allowed to go to completion, as in the case of glucose (Table VI). The results obtained with a series of substrates are recorded in Table VIII.

Only points of special interest in respect to the "programme" of this paper will be discussed, namely:

(1) The rate and chemical mechanism of the anaerobic decomposition of the substrate in the absence of fumarate.

(2) The rate and chemical mechanism of the anaerobic decomposition of the substrate in the presence of fumarate, as compared with the aerobic oxidation of the substrate. The ratios of these

rates are represented by the quotients (9) and (10). These quotients were calculated by subtracting the figures obtained in the presence of fumarate alone from the figures obtained in the presence of fumarate + substrate.

Table VIII. *Reactions between fumarate and various substrates*

			No. 1	No. 2	No. 3	No. 4	No. 5
1	Substrate added		Na acetate, $3H_2O$	Na pyruvate	Glycerol	*dl*-Glyceraldehyde	*dl*-Lactate
2	Quantity of substrate		1·36 mg.	0·1 ml. 0·1 *M*	0·1 ml. 0·1 *M*	0·1 ml. 0·1 *M*	0·1 ml. 0·05 *M*
3	Duration of experiment		120 min.	120 min.	120 min.	120 min.	200 min.
4	mg. bacteria per 3 ml solution		3·8 mg.	2·4 mg.	2·5 mg.	2·5 mg.	0·95 mg.
	Oxidations by molecular oxygen:						
5	Blank	O_2	− 38·1	− 34·4	− 47·5	− 47·5	− 29·2
		CO_2	+ 30·4	+ 32·2	+ 35·0	+ 35·0	+ 29·3
		HCO_3^-	+ 6	− 5	+ 5	+ 5	+ 8
6	Substrate	O_2	−387	−434	−484	−266	−287
		CO_2	+169	+342	+390	+264	+197
		HCO_3^-	+184	+189	− 5	− 7	+100
7	Fumarate	O_2	−702	−289	−501	−501	−313
		CO_2	+337	+121	+322	+322	+233
		HCO_3^-	+655	+422	+477	+474	+246
8	Substrate + fumarate	O_2	−998	−806	−778	−674	−469
		CO_2	+522	+635	+566	+516	+339
		HCO_3^-	+871	+629	+518	+472	+302
	Anaerobic oxidation by fumarate:						
9	Fumarate	CO_2	+ 51	+ 42	+ 59	+ 59	+ 33
		HCO_3^-	+ 19	+ 45	+ 47	+ 47	+ 28
		Succinate	+540	+157	+240	+240	+134
10	Substrate	CO_2	+158	+305	+309	+305	+ 85
		HCO_3^-	+162	+ 43	+ 5	+ 64	+ 80
		Succinate	+990	+662	+758	+666	+306
	Anaerobic fermentation:						
11	Blank	CO_2	—	+ 11	+ 8	+ 8	—
		HCO_3^-	—	− 5	− 1	− 1	—
12	Substrate	CO_2	—	+182·5	+ 17	+ 32	—
		HCO_3^-	—	−141	− 12	− 22	—

Acetate is not attacked anaerobically in the absence of fumarate. The ratio (9) and (10) calculated from Table VIII yield the following figures:

$$\text{for (9)} = \frac{225}{296} = 0{\cdot}76, \text{ for (10) } \frac{250}{404} = 0{\cdot}62.$$

The rate of the anaerobic oxidation is thus 76 or 62 % of the aerobic oxidation.

It should be remembered that the experiments recorded in Table VIII were designed to measure rates, and not the degrees of completion of the oxidations. In other experiments, carried out in the same way, but continued until the oxidation of acetate was completed, it was found that up to 790 μl. fumarate were reduced by 1·36 mg. sodium acetate. 1 mol. of acetate had thus donated 7 atoms of hydrogen. Since acetic acid contains only 4 atoms of hydrogen, part of the hydrogen required for the reduction of fumarate and part of the oxygen appearing as CO_2 must be derived from water:

$$CH_3.COOH + 2H_2O + 8 \text{ fumarate} = 2CO_2 + 8 \text{ succinate}.$$

This participation of water in oxidations will be discussed in § VIII 4.

5. *Fumarate and pyruvate*

(*a*) *Decomposition of pyruvate in the absence of fumarate.* Pyruvic acid has long been known to be fermented anaerobically by *Bact. coli* and as chief end-products acetic acid, formic acid, hydrogen, CO_2, lactic acid, succinic acid and alcohol have been identified [*v.* Tikka, 1935; Mazza, 1934; for references].

Formation of succinate. In view of the role of succinate as hydrogen carrier, discussed later in this paper, it is of interest to study the origin of succinate in *Bact. coli*. Mazza established the formation of this substance from pyruvate by isolating it; quantitative data about the rate and the yield of succinate formation from pyruvic acid will be given in § III 11 of this paper.

Formation of lactic acid. No anaerobic formation of lactic acid was observed with our strains of *Bact. coli* in the presence of pyruvate if the medium was bicarbonate saline saturated with 5% CO_2, within the range *p*H 6·6–8·0. Considerable quantities of lactate appear, however, if bicarbonate saline is saturated with 100% CO_2 at *p*H 5·5. For instance 246 μl. lactic acid were formed in an experiment in which 1120 μl. pyruvic acid had been fermented [*v.* Tikka, 1935].

Formation of CO_2. All the experiments referred to in this section were carried out with cells grown aerobically and such cells do not split formate into bicarbonate and hydrogen. They form very little (not more than 2–5%) CO_2 from pyruvate, if the medium is bicarbonate saline saturated with 5% CO_2. Much CO_2, however, is liberated from bicarbonate of the saline. For 100 mol. of pyruvate about 80–90 mol. of bicarbonate are decomposed, the main reaction being the splitting of pyruvic acid into formic and acetic acids:

(11) $CH_3.CO.COOH + H_2O = CH_3.COOH + H.COOH.$

A higher pressure of CO_2 associated with higher acidity affects the formation of CO_2 from pyruvate. Instead of 2–5% CO_2 up to 30% CO_2 is formed. These observations suggest that in acid medium, in presence of 1 atmosphere of CO_2 *Bact. coli* ferments pyruvate like cocci and animal tissues, according to the equation

(12) $2CH_3.CO.COOH + H_2O = CH_3.COOH + CO_2 + CH_3.CH(OH).COOH,$

whilst in less acid and neutral medium reaction (11) prevails.

Both (11) and (12) are, of course, only approximate accounts since they do not allow for the formation of succinate or alcohol.

Effect of small amounts of fumarate on the fermentation of pyruvate. The rate of fermentation of pyruvate can be conveniently measured manometrically, if the cells are suspended in bicarbonate saline, by following the course of the CO_2 evolution. This method shows (Table IX) that the fermentation of pyruvate commences slowly but increases with time. Addition of fumarate diminishes the lag period and increases the rate of reaction very considerably. The effect of

Table IX. *Effect of fumarate on the fermentation of pyruvic acid by* Bact. coli

1·4 mg. bacteria per flask; bicarbonate saline; *p*H 6·9; 40°

Time min.	μl. CO_2 formed in the presence of: 0·02 *M* pyruvate	0·02 *M* pyruvate + 0·0006 *M* fumarate	0·02 *M* pyruvate + 0·006 *M* fumarate
10	12·3	20·9	31·4
20	38·5	55·6	79
40	105	136	190
80	257	306	430

fumarate is catalytic, since small quantities, e.g. 1/30 equivalent of the pyruvate present have already a pronounced effect. α-Ketoglutarate acts in a similar manner. We cannot offer any explanation for this effect which we had also observed when studying fermentation of pyruvic acid by *Staphylococcus* [Krebs, 1937].

(*b*) *Oxidation of pyruvate by fumarate.* The ratios (9) and (10) calculated as in the case of acetate are:

$$\text{for (9)} \ \frac{\frac{1}{2} \times 505}{517} = 0{\cdot}49,$$

$$\text{and for (10)} \ \frac{261}{721} = 0{\cdot}36.$$

The rate of the anaerobic oxidation of pyruvate is thus 36–49 % of that of the aerobic oxidation.

Apart from these figures there is another point to which attention should be drawn. The reaction between fumarate and pyruvate, as measured by the formation of succinate, is more rapid than the rate of the analogous reactions between fumarate and acetate, as may be seen from Table V. A mixture of acetate and formate equivalent to pyruvate reduces only as fast as acetate only, formate being inactive (§ III 10). We must conclude from these facts that pyruvate reacts with fumarate as such, and not only after primary splitting into acetate and formate. It is very probable, that the direct reaction between pyruvate and fumarate is:

(14) Pyruvate + fumarate + H_2O = acetate + CO_2 + succinate.

6. *Fumarate and glycerol*

(*a*) *Anaerobic decomposition of glycerol.* Harden [1901], Harden & Norris [1912], Virtanen *et al.* [1930], Tikka [1935], and Braak [1928] studied the anaerobic fermentation of glycerol by *Bact. coli* and similar organisms. All these investigators found that the process is very slow, and confirmed Harden's discovery that succinate occurs among the end-products of the fermentation. Both points, the rate of fermentation and the formation of succinate, are of interest in connexion with the interaction between fumarate and glycerol and we have therefore examined these two points for our special experimental conditions.

If thick washed suspensions of *Bact. coli* are suspended in bicarbonate saline anaerobically, carbon dioxide is evolved on addition of glycerol. The evolution of CO_2 is quantitatively accounted for by a decrease in bicarbonate and it is thus due to formation of acid. 1 mg. of dry cells produces 2–3 μl. acid from glycerol per hour, whilst in the presence of glucose, under similar conditions, 200–300 μl. acid are formed. The fermentation of glycerol as measured by the production of acid is thus 100 times slower than that of glucose, and about 50–100 times slower than the oxidation of glycerol by fumarate.

The yield of succinate is remarkably high. Per 100 mol. of glycerol fermented 40–45 mol. of succinate are formed. The yield of succinate, though not the rate of succinate formation, is higher than the yield obtained from glucose or from pyruvate. Wood & Werkman [1936] observed a similarly high yield of succinate in the fermentation of glycerol by propionic acid bacteria. These high yields are of interest in view of the mechanism of the formation of the succinate. It is difficult to assume that glycerol is first resynthesized to carbohydrate and that succinate is then formed from sugar [see Kluyver, 1935].

If the amount of succinate formed is compared with the carbon dioxide evolved from the medium, it will be seen that the figures are equivalent within the limits of error. Succinic acid is thus practically the only acid formed in the anaerobic breakdown of glycerol in *Bact. coli* under our conditions.

Table X gives an example of an experiment on the fermentation of glycerol.

Table X. *Fermentation of glycerol by* Bact. coli

Each cup contained 60 mg. bacteria in bicarbonate saline; 0·014 *M* $NaHCO_3$; 5% CO_2 in N_2; after the first manometric reading 0·2 ml. 0·1 *M* glycerol (448 μl.) was added in flask 2.

Flask no. ...	1	2
μl. CO_2 evolved after 60 min.	93	180
,, 120 min.	167	325
,, 240 min.	273	552
,, 360 min.	366	732
μl. succinic acid formed	122	304
Extra succinic acid formed in the presence of glycerol	—	182
Extra CO_2 evolved	—	366

Wood & Werkman [1936] found that CO_2 is bound during the glycerol fermentation of propionic acid bacteria and we have therefore carried out experiments to see if CO_2 or bicarbonate is utilized by *Bact. coli*. We were unable, however, to detect a disappearance of CO_2 or bicarbonate.

(*b*) *Oxidation of glycerol by fumarate.* The oxidation of glycerol by fumarate is a very rapid reaction so that the spontaneous decomposition of glycerol can be neglected in the calculations. The ratios yield the following figures calculated from the data in Table VIII:

$$\text{for (9)} \frac{258}{277}=0{\cdot}93$$

$$\text{and for (10)} \frac{208}{285}=0{\cdot}73.$$

7. *Fumarate and glyceraldehyde*

(*a*) *Anaerobic decomposition of glyceraldehyde. dl*-Glyceraldehyde is slowly fermented anaerobically [Virtanen & v. Hausen, 1932] and succinate is found among the end-products of the fermentation. Examples giving the rate of fermentation and the yield of succinate are found in Table XII. The rate of reaction is insignificant as compared with oxidation of glyceraldehyde by fumarate.

(*b*) *Oxidation of glyceraldehyde by fumarate.* The following figures are obtained:

$$\text{for (9)} \frac{213}{173}=1{\cdot}23$$

$$\text{and for (10)} \frac{263}{192}=1{\cdot}36.$$

In this case the anaerobic oxidation appears to be even more rapid than the aerobic reaction. It is however certain that glyceraldehyde promotes the anaerobic breakdown of fumarate as indicated by the increased yield of bicarbonate from fumarate in the presence of glyceraldehyde. Because of this interfering effect it is not possible to calculate the true ratios (9) and (10).

8. *Fumarate and lactate*

There is no anaerobic breakdown of lactate in the absence of fumarate.

From Table VIII we calculate

$$\text{for (9)} \frac{156}{172}=0{\cdot}91$$

$$\text{for (10)} \frac{52+54}{116+56}=0{\cdot}62.$$

The rate of the anaerobic oxidation of lactate by fumarate is thus 91 or 62% of the rate of oxidation by oxygen. We found the same figure for *d*, or *l*, or *dl*-lactate.

9. *Fumarate and hydrogen*

(*a*) *Reactions of hydrogen in the absence of fumarate.* In cells containing "hydrogenlyase" the reactions

(15) $H_2 + CO_2 \rightleftharpoons HCOOH,$

or (15*a*) $H_2 + HCO_3^- \rightleftharpoons HCOO^- + H_2O,$

take place [see Woods, 1936]. In cells grown aerobically these reactions do not occur at a significant rate, and the experimental conditions for studying the reactions between fumarate and hydrogen (16) are therefore simpler in aerobic cells. For most of the following experiments aerobic cells were therefore used. A few tests showed that the rate of (16) is about the same in aerobic and anaerobic cells.

(*b*) *Reduction of fumarate by hydrogen.* From the work of Stephenson and her collaborators [1937] it is known that H_2 reacts in *Bact. coli* with fumarate according to (16).

(16) $H_2 + \text{fumarate} = \text{succinate}.$

We made some unexpected observations when studying reaction (16) with quantitative methods. In the following experiment we compared the quantity of fumarate added and metabolized with the quantities of hydrogen absorbed and succinate formed and it will be seen that much more succinate is formed than can be accounted for by the absorption of hydrogen. Two flasks were used for the experiment and they contained in their main chamber 3 ml. bacterial suspension in phosphate saline (*p*H 6·8; 2·37 mg. bacteria), 0·1 ml. fumarate solution (0·198 *M*), equivalent to 442 μl., in the side-arm, and alkaline hydrosulphite in the centre chamber for the absorption of CO_2 and O_2. The gas in the first flask was nitrogen, in the second hydrogen. In nitrogen no change of pressure occurred, in hydrogen an absorption began when the fumarate was added to the bacteria:

Hydrogen absorbed after	20	min.	62 μl.
,,	40	,,	130 ,,
,,	60	,,	184 ,,
,,	80	,,	224 ,,
,,	100	,,	234 ,,

The amount of succinate found after the absorption of hydrogen had finished was 362 μl. whilst the control in nitrogen (through fermentation of fumarate) yielded 58 μl. succinate. Thus $234 + 58 = 292$ μl. succinate can be accounted for by the action of hydrogen and by the fermentation of fumarate whilst in actual fact considerably more, namely 362 μl. succinate were found. Previous observations discussed in § III 3*c* suggested that the excess succinate may be due to a promoting effect of hydrogen on the fermentation of fumarate and we therefore investigated whether there is an increased formation of bicarbonate from fumarate in the presence of hydrogen. If bicarbonate appears under our experimental conditions it must necessarily be due to the oxidative breakdown of fumarate. The following experiment confirms the expectation. A suspension of *Bact. coli* in bicarbonate saline was used, 3 ml. of which contained 2·37 mg. bacteria and 313 μl. bicarbonate. 3 ml. suspension were measured into each of two flasks provided with two side-arms, the first side-arm containing 0·5 ml.

0·2 *M* fumarate, the second 0·2 ml. 2 *N* H_2SO_4. The gas space of the one flask was filled with 5 % CO_2 in H_2 that of the other with 5 % CO_2 in N_2. After thermal equilibration fumarate was added to the suspension and after 2 hr. incubation the acid was added and the amount of CO_2 evolved determined. In nitrogen 347 μl. bicarbonate were found, in H_2 406 μl. The increase in bicarbonate was thus 34 μl. in N_2 and 93 μl. in H_2. The rate of fermentation as measured by the production of bicarbonate from fumarate was almost trebled by hydrogen. This effect, although unexplained in its mechanism, accounts for the apparent discrepancy between uptake of hydrogen and yield of succinate. Molecular hydrogen like the atomic hydrogen in organic compounds promotes the fermentation of fumarate and causes thus a greater yield of succinate than can be accounted for by its stoichiometric effect.

We have examined a number of other substances as to their capacity of absorbing hydrogen. No absorption occurred in cells grown aerobically in the presence of pyruvate, glucose, glycerol or glyceraldehyde, whilst oxaloacetate reacts. For instance 0·1 ml. of 0·2 *M* oxaloacetate (equivalent to 448 μl.) induced an absorption of 100 μl. H_2 if added anaerobically to *Bact. coli* in an atmosphere of H_2. Since, however, oxaloacetate is readily reduced in *Bact. coli*, it is very likely that not oxaloacetic acid as such, but fumarate formed by reduction from it, reacts with hydrogen.

This point is of interest in view of various schemes of fermentation in which reactions of molecular hydrogen with various substances play a part.

(*c*) *Aerobic experiments.* In the presence of oxygen, succinate undergoes re-oxidation:

(17) $$\text{Succinate} + \tfrac{1}{2}O_2 = \text{fumarate} + H_2O,$$

and it might be therefore expected that in the presence of fumarate the balance sheet of (16) and (17) is experimentally verified, i.e.

(18) $$H_2 + \tfrac{1}{2}O_2 = H_2O.$$

Reaction (18) can in fact be experimentally realized if the two stages (16) and (17) are allowed to take place successively. If, however, H_2 and O_2 and fumarate are present together there is no absorption of hydrogen by fumarate. The gas absorption is only equivalent to the oxidation of fumarate and we must conclude, therefore, that the reaction between hydrogen and fumarate does not take place in the presence of oxygen. Oxygen inhibits "hydrogenase". It may be added that O_2 inhibits also (15).

10. *Fumarate and formate*

(*a*) *Reactions of formate in the absence of fumarate.* It is of interest to consider the splitting of formate into bicarbonate and hydrogen as an oxido-reduction analogous to other processes described in this paper. Previous workers, according to Stephenson [1937], considered the evolution of hydrogen as a simple cleavage, according to

(19) $$*H\!:\!COO\!:\!H = H_2 + CO_2,$$

or (19*a*) $$*H\!:\!COO^- = H_2 + HCO_3^-.$$
$$H\!:\!OH$$

A different view of the mechanism is presented by the following equation:

(20) $$H_2\!:\!O + HCOO^- = H_2 + HCO_3^-.$$

According to (20) formate is oxidized by the oxygen of water in the same way as acetate in (24), or pyruvate in (12) or (14). The hydrogen atoms which become available unite in pairs to form molecular hydrogen in (20) whilst they are accepted by fumarate or pyruvate in (24), (12) or (14). Reaction (20) is thus analogous

to other oxido-reductions in *Bact. coli*. This view is amenable to experimental investigation, by studying (20) in the presence of heavy water, D_2O. According to the new view, the hydrogen evolved in the presence of formate is derived from the water of the medium; therefore the gas formed in heavy water should be D_2; in a mixture of H_2O, HDO and D_2O, D_2, H_2 and DH should be formed. According to the old view at least one hydrogen atom of the gas (marked with an asterisk in (19) and (19*a*)) is derived from the formate molecule, and therefore D_2 cannot be formed in the presence of heavy water, but only DH and H_2. The decisive experiments have already been carried out and published by Farkas *et al.* [1934] and they confirm our view. The authors showed that D_2 is indeed formed, and they concluded rightly that (19) and (19*a*) do not represent the mechanism of the evolution of hydrogen. They did not suggest, however, a definite alternative explanation.

Such a type of reaction is not so surprising as appears at first sight since analogous reactions are common in inorganic chemistry. For example:

$$2Na^+ + 2H_2O = 2NaOH + H_2,$$

or

$$2CrO + H_2O = Cr_2O_3 + H_2.$$

(*b*) *Reduction of fumarate by formate.* The behaviour of formate towards fumarate depends on the conditions under which the cells have been grown. There is no interaction between formate and fumarate if the cells have been grown aerobically on broth agar, whilst cells grown anaerobically on glucose broth bring about the reaction:

(21) Formate + fumarate = succinate + bicarbonate.

In studying (21) we determined the bicarbonate and the succinate formed and no change in the concentrations of these two substances was found if cells grown aerobically were used. An instance of an experiment with cells grown anaerobically is recorded in Table XI.

Table XI. *Reaction between fumarate and formate in* Bact. coli

Cells grown anaerobically. Manometric experiment. Each flask contained 3 ml. bacterial suspension with 1 mg. bacteria; flasks with two side-arms and centre chamber were used; 2 series were carried out, one with phosphate saline (alkaline hydrosulphite in centre chamber) and the other in bicarbonate saline. The first yielded figures for H_2, the second for $H_2 + CO_2$.

Substrates added to 3 ml. suspension	1	2 0·1 ml. formate (0·1 *M*)	3 0·5 ml. fumarate (0·2 *M*)	4 0·1 ml. formate (0·1 *M*) 0·5 ml. fumarate (0·2 *M*)
H_2 formed in 100 min.	9	175	0	0
CO_2 formed in 100 min.	2	2	76	121
Bicarbonate formed in 100 min.	−7	170	38	226
Succinate formed in 100 min.	—	—	157	467

The experiment shows (second column) that formate, as is well known, is split into bicarbonate and hydrogen, both products being equivalent within the limits of error (170 and 175 μl.). If fumarate is present, however, no hydrogen is formed from formate but instead bicarbonate and succinate appear. Whilst the increase in bicarbonate is about the same as in the absence of fumarate, the increase in succinate is considerably above the expected amount (310 μl. instead of 188 μl.). Furthermore, there is an additional formation of free carbon dioxide, if formate is added to fumarate.

Since it is known that the anaerobic cells, unlike aerobic cells, split formate into bicarbonate and hydrogen it is interesting to enquire whether the splitting

of formate into bicarbonate and hydrogen is essential for the interaction between formate and fumarate. Experiments show that there is no reaction (21) unless the cells contain active hydrogenlyase; the parallelism between the occurrence of active hydrogenlyase and of reaction (21) in *Bact. coli* is strict and this leads to the conclusion that reaction (21) is not a direct reaction, but the result of the two reactions (20) and (16). In addition to the parallelism mentioned there are the following facts which indicate that (21) is the balance sheet of (20) and (16):

(1) Small quantities of hydrogen are actually formed in the course of reaction (21). In the experiment recorded in Table XI, for instance, 12 μl. hydrogen were set free in flask 4 after 10 min. but were reabsorbed subsequently.

(2) The rate of (16) is at least as great as that of (20) as the theory requires.

(3) Reaction (16) induces according to § III 9 an increased fumarate fermentation. Formate has the same effect as indicated by the excess of CO_2 and succinate mentioned above. The effects of formate and of hydrogen are of the same magnitude.

11. *Occurrence and formation of succinate in* Bact. coli

The previous sections have shown that fumarate functions as hydrogen acceptor for a number of oxidizable substances in *Bact. coli*. The demonstration of the carrier function, as explained before, has to include the proof of the occurrence of the carrier. It is immaterial obviously whether the occurrence of the reduced or of the oxidized carrier is shown.

Since Grimbert [1896] discovered succinate among the products of fermentation of sugar in *Bact. coli*, the formation of this substance has often been demonstrated qualitatively. Our own experiments which are recorded in Table XII supplement the existing literature with quantitative data about the rate of formation of succinate. First it will be seen that considerable quantities

Table XII. *Formation of succinate in* Bact. coli

Bicarbonate saline; anaerobic conditions; the experiments were continued until the fermentation, as measured by gas production, was finished; cells grown aerobically; blanks deducted.

mg. cells used	Time required for decomposition of the substrate (min.)	Substrate added μl.*	Initial *p*H	Succinate formed μl.	$Q_{succinate}$	$\frac{\text{Succinate formed}}{\text{Substrate fermented}} \times 100$	Strain used
62	360	—	7·0	122	0·33	—	1318
62	360	Glycerol (448)	7·0	182	0·49	40·6	1318
3	180	Glucose (628)	7·5	93	10·3	14·8	1318
3	240	Laevulose (639)	7·5	161	13·4	25·2	1318
3	220	Mannose (640)	7·5	157	14·1	24·5	1318
3	380	Mannitol (636)	7·5	191	10·0	30·0	1318
18	380	Galactose (380)	7·5	290	2·5	76·3	1318
18·7	360	*dl*-Glyceraldehyde (224)	7·0	64	0·57	28·6	1318
4·75	140	Pyruvate (1346)	7·3	56·2	5·1	4·2	1318
6·22	200	Pyruvate (2700)	7·5	266	11·9	9·9	1318
18	40	Pyruvate (1344)	7·5	143	11·9	10·6	1318
18	40	Pyruvate (1344)	6·2	146	12·2	10·8	1318
8·2	60	Pyruvate (1344)	7·5	96	11·7	7·1	1
7·4	180	Pyruvate (1344)	7·5	65	2·9	4·8	2
9·4	60	Pyruvate (1344)	7·5	57	6·1	4·2	3072
53	280	—	7·5	92	0·58	—	3072
53	160	Galactose (251)	7·5	253	1·8	101	3072
48	990	—	7·1	348	0·43	—	1
75	990	—	7·1	300	0·24	—	2

* 1 millimol = 22400 μl.

of succinate appear in washed cells to which no substrate has been added. These cells show also a small formation of acid if suspended in bicarbonate saline and the comparison of acid formation and succinate formation indicates that 80–100% of the acid formed by washed cells is succinic acid. This holds for the 4 strains examined.

All the hexoses form succinate rapidly, the rate being highest with glucose, mannose and fructose, whilst the yield is highest with galactose. Two different strains yielded 76 and 100 mol. of succinate for each 100 mol. of galactose fermented.

Pyruvate and mannitol yield succinate about as rapidly as the hexoses. Glycerol is very slowly fermented but gives a high yield of succinate.

12. *Replacing fumarate by malate*

In all the experiments described in this paper fumaric acid may be replaced by *l*(+)malic acid, since *Bact. coli* contains a very potent fumarase [Quastel & Whetham, 1924] which catalyses the reaction fumarate + $H_2O \rightleftharpoons$ malate. The speed with which the equilibrium is established is more rapid than that of all the other reactions which fumarate and malate undergo. It is therefore in accordance with expectation that we find no significant differences if we add fumarate or malate to the cells.

d(−)Malate reacts slowly in *Bact. coli* aerobically as well as anaerobically. The rate of the aerobic oxidation and of the anaerobic fermentation is about 1/5 of that of *l*(+)malate. *l*(−)Aspartate which is known to yield fumarate in *Bact. coli* can replace fumarate qualitatively [*v.* Harden, 1901] but in an anaerobic experiment in which glycerol was the substrate for the oxidation, the rate of oxidation by aspartate was 48% only of that observed in the presence of fumarate. It appears thus that the production of fumarate from aspartate was slower than the oxidation of glycerol by fumarate.

It may be mentioned that methylene blue may also replace fumarate in many of the reactions described, and since it can act as hydrogen donator towards O_2 it can also function as hydrogen carrier. One may be tempted to assume that methylene blue acts through the intermediation of fumarate and succinate, e.g. that the reduction of methylene blue by hydrogen is the result of the following two reactions:

H_2 + fumarate = succinate

Succinate + methylene blue = fumarate + leucomethylene blue.

However, H_2 reacts with methylene blue about twice as fast as with fumarate, and fumarate therefore cannot be the intermediate in the reaction between H_2 and the dye.

13. *Reoxidation of succinic acid*

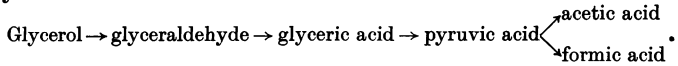
It was shown in the preceding sections that fumarate acts as hydrogen acceptor. If it is a hydrogen carrier, its reduced form, succinate, must act as hydrogen donator. It is well known that succinate is in fact readily oxidized in *Bact. coli* in the presence of molecular oxygen. An instance of the rate of this reaction under our conditions is given in Table VI, and it will be seen that the rate of reoxidation of succinate is faster than that of the reduction of fumarate by glucose. We find that the same applies to other substrates (except lactate, see § VIII 1); the rate of hydrogen transport by fumarate is therefore determined by the rate of its reduction.

The oxidizing agent for succinate is molecular oxygen, activated through the intermediation of specific enzymes and possibly specific carriers of the cyto-

chrome type. This settles point 4 of the programme in the case of fumarate, the question of the nature of the chemical grouping accepting the hydrogen from succinate.

14. *Source of the hydrogen transported by fumarate*

Now point 3 of the programme, the nature of the grouping donating hydrogen to fumarate, remains to be discussed. This question can be studied in a more general way by examining the reduction of fumarate by different organic molecules (see Table V), but this method does not exactly define the reacting hydrogen atoms. More detailed information can be obtained by the following kind of considerations: the chief pathway of the oxidative breakdown of glycerol is probably



Fumarate has been shown to oxidize glycerol, glyceraldehyde, pyruvic and acetic acids and the rates of oxidation are in the case of the first two substances almost the same as that of the aerobic oxidation. We may conclude from this that fumarate is concerned with the hydrogen transport which brings about the above series of chemical changes. We shall see in § VIII 4 that in many cases the hydrogen transported by fumarate is not the hydrogen of organic molecules, but the hydrogen of water.

§ IV. Pyruvate as hydrogen carrier

Since the occurrence of pyruvate and the re-oxidation of lactate in *Bact. coli* is sufficiently well established by previous workers, the evidence for the carrier action of pyruvate will be complete if it can be shown that the reduction of pyruvate to lactate is brought about by normally occurring substrates. So far only one reaction is known in which pyruvate is reduced, namely (12). This reaction is important in the respiration of certain cocci [Krebs, 1937], whilst in *Bact. coli* it seems to occur in acid medium only (see § III 5*a*).

§ V. Oxaloacetate as hydrogen carrier

If oxaloacetate is added to *Bact. coli* a rapid decomposition sets in during which bicarbonate, free CO_2 and succinate are formed. As shown in Table XIII

Table XIII. *Fermentation of oxaloacetate in* Bact. coli

Strain no.	mg. bacteria used	Oxaloacetate added	°C.	Time required for decomposition of oxaloacetate min.	μl. succinate formed	μl. CO_2 liberated
1318	7·7	672	40	45	320	470
1	10·3	672	40	110	346	354
1	14·4	504	18	300	256	251

about 50 % of the added substrate is reduced to succinate and about the same quantity of free CO_2 is formed. The initial rate of this "fermentation" of oxaloacetate is very rapid. About 100 μl. CO_2 per mg. and per hour are formed at 40° if excess of oxaloacetate is present.

The fermentation indicates that oxaloacetate can be reduced, either by another molecule of oxaloacetate, or more probably by one of the anaerobic split products of oxaloacetate, pyruvate, acetate or formate. Addition of one of

the latter three substances has no effect on the yield of succinate from added oxaloacetate and we have not been able to define in detail the oxidative equivalent for reduction of oxaloacetate. Since the products of the reduction, fumarate and succinate, are readily re-oxidized, and since oxaloacetate can be formed from succinate, oxaloacetate may be considered as a hydrogen carrier. As long as the oxidative equivalent of the "fermentation" of oxaloacetate is unknown, it is not possible to define the exact role of oxaloacetate as hydrogen carrier. It may well be that it takes part in some of the dismutations observed in the presence of fumarate. The organic substrate may oxidize fumarate to oxaloacetate and a secondary reduction may regenerate fumarate. This would not affect the balance sheet which appears to be a reduction of fumarate by the substrate.

§ VI. Carbon dioxide as hydrogen carrier

Reaction (12), which occurs in cocci, and in *Bact. coli* if the medium is acid, is an intermolecular oxidoreduction in which two molecules react with the elements of water, one being reduced, the other being oxidized. Reaction (11) may be considered in an analogous manner as an intermediate oxidoreduction in which CO_2 (or H_2CO_3) acts as hydrogen acceptor for the hydrogen of the water:

(22) $CH_3.CO.COOH + H_2O + CO_2 = CH_3.COOH + CO_2 + HCOOH.$

In this view, formate arises from the CO_2 and the H_2O of the medium, and not from the carboxylic group of the pyruvic acid. Although there does not appear to be a possibility of conclusive experimental proof for the mechanism in (22), various considerations can be put forward which favour the scheme. First, (22) brings into line the fermentation of pyruvate with a number of other "dismutations" in *Bact. coli* in which the elements of water are taken up by two reactants (§ VIII 4). Secondly, the view offers a possibility of explaining the necessity of carbon dioxide for the growth of bacteria. It has been known for some time that CO_2 is essential for the growth of certain bacteria including *Bact. coli* [*v.* Gladstone *et al.* 1935]. The fact that CO_2 is required suggests that it takes part in metabolism, but no metabolic reaction of general importance in which CO_2 may play a role has hitherto been shown. Reaction (11) is an intermediate stage in the breakdown of many substrates (glucose, glycerol, lactate, succinate) and (22) suggests that CO_2 is required as hydrogen acceptor for this reaction. Since the formate produced by the reaction is oxidizable in the presence of oxygen in *Bact. coli*, CO_2 may act not only as hydrogen acceptor but catalytically as hydrogen carrier.

§ VII. Effect of "activators" on the metabolism of *Bact. coli*

In a previous paper on *Staphylococcus* we showed that the rates of fermentations and of oxidations in this organism can be accelerated by boiled extracts from yeast. We find similar effects in *Bact. coli* although the degree of acceleration is here generally less marked. With our strain 2 increases of 200–300 % of the rate of the following reactions were observed: oxidation of glucose, succinate, lactate, glycerol, fermentation of pyruvate, fermentation of glycerol, hydrogenation of fumarate by molecular hydrogen. The oxidation of formate was not affected by yeast extract.

This strain gave comparatively low metabolic quotients without yeast extracts ($Q_{O_2}^{\text{glucose}} = 80$). Two other strains of *Bact. coli* which gave higher quotients ($Q_{O_2}^{\text{glucose}} = 150$) showed relatively smaller accelerations on addition of

yeast extract. Yeast extract thus tends to level the differences in metabolic rates of different strains. Boiled extracts from cytolysed mammalian blood cells act in a similar way to yeast extract as, for example, boiled and filtered solutions of "carbonic anhydrase" prepared according to the directions of Roughton [1934].

§ VIII. Discussion

(1) *Role of hydrogen carriers in respiration.* We have seen that fumarate, pyruvate, oxaloacetate and probably carbon dioxide can act as hydrogen carriers in *Bact. coli*. The majority of substrates, for example glucose, glycerol, glyceraldehyde and acetate, do not react directly with the activated oxygen but only through one or several of the hydrogen carriers. Among these carriers fumarate is the most important one.

The figures obtained for the ratios (9) and (10) indicate that succinate is not the only substance with which molecular oxygen reacts "directly". The observed facts concerning the rates of oxidation of carbohydrates and their biological derivatives can be accounted for if we assume that activated oxygen reacts with 4 substances: succinate, formate, lactate and malate. This view is supported by the following facts and considerations:

(*a*) Formate is oxidized in the presence of 0·01 *M* formaldehyde which inhibits the oxidation of most substrates including succinate. The cells must therefore possess a separate system for the aerobic oxidation of formate.

(*b*) Some strains of *Bact. coli* oxidize lactate more rapidly than succinate (see e.g. Table II). In the case of our strain 2 lactate is twice as rapidly oxidized. Although lactate can be oxidized by fumarate (see § III 8) there must be a second mechanism of oxidation of lactate in which fumarate is not required.

(*c*) The discrepancy between the rates of the aerobic and anaerobic oxidations of fumarate (see § III 3) makes it necessary to assume that oxygen reacts with fumarate not only through succinate but also in a more direct way.

(*d*) It was shown in § II 4 that the oxidations of succinate, lactate and formate tend to summate if the substrates are present together. This is further evidence in favour of separate systems concerned with the oxidation of these substrates.

(*e*) Lactate and formate are oxidized in toluene-treated cells [Quastel, 1924]. We find that these cells do not bring about dismutations with fumarate. This again supports the view that fumarate is not necessarily required for the oxidation of lactate and formate.

The picture which thus emerges from the present investigation indicates that oxygen, in respiration, reacts with only a very limited number of substances. Most of the oxidations are brought about by intermolecular dismutations which are essentially the same type of reactions as fermentations.

(2) *Mechanism of dismutations.* It should be recalled that all our reaction schemes are simplified and neglect the role of enzymes, coenzymes and other catalysts. Many of the reactions are "carrier-linked reactions" described by Quastel & Wooldridge [1929] and Green *et al.* [1934]. The recent work of Dewan & Green [1937] suggests that coenzyme I is the natural "carrier" in the cells.

(3) *Previous work on intermolecular oxidoreductions in* Bact. coli. Several of the reactions studied in this paper have been described or discussed by previous workers. Thus Quastel *et al.* [1925] found that *Bact. coli* grows anaerobically if fumarate plus glycerol or fumarate plus lactate are present in the broth and they concluded that reduction of fumarate by the second substrate provides the

energy for the growth; they proved the reduction of fumarate by the isolation of succinate. Harden [1901] has shown that *Bact. coli* reduces aspartate in the presence of glucose to ammonium succinate. The data published previously, however, do not allow us to decide whether the dismutations play a role in the normal metabolism of the cell. It is the main object of the present paper to co-ordinate the dismutations to the major metabolic processes, in the first place to respiration.

(4) *Participation of water in hydrogen transport.* Whilst a number of oxidations by fumarate can be described as a transfer of hydrogen from the organic molecule to fumarate, there are many other cases of oxidation in which water participates as hydrogen donator. Examples are the oxidation of acetate (§ III 4), the oxidation of pyruvate to acetate and the oxidation of formate to bicarbonate.

The idea that it is the oxygen of water that oxidizes organic molecules in respiration has been discussed by Traube, Bach, Batelli & Stern and Wieland. Wieland, e.g. [1932], assumed that a hydrate of the substrate (aldehyde or xanthine) is formed first and that the oxidized product arises from dehydrogenation of the hydrate. It follows from experiments reported in this paper that water takes part even in cases in which there is no chemical evidence for the formation of a hydrate. As long as only the balance sheet of the oxidations is studied, e.g. the reaction

(23) $$CH_3.COOH + 2O_2 = 2CO_2 + 2H_2O,$$

it must remain open whether the oxygen atoms appearing as CO_2 are those contained in the O_2, but when reaction (23) is resolved into

(24) $$CH_3.COOH + 2H_2O + 4 \text{ fumarate} = 2CO_2 + 4 \text{ succinate}$$

and $$4 \text{ succinate} + 2O_2 = 4 \text{ fumarate} + 2H_2O,$$

it becomes clear that molecular oxygen reacts with acetate to form CO_2 only through the intermediation of water. The same considerations apply to analogous cases: e.g. the oxidation of lactate to acetate; reaction (1) has to be formulated in these cases as

$$\text{Substrate} + H_2O + \text{carrier} = \text{oxidized substrate} + \text{reduced carrier}.$$

Summary

1. Fumarate, pyruvate, oxaloacetate and probably carbon dioxide act as respiratory catalysts (hydrogen carriers) in *Bact. coli*. Their roles have been studied.

2. Methods are developed for studying the intermediary transport of hydrogen by carriers. The role of the carrier can be described by the two equations:

(1) Substrate + carrier = oxidized substrate + reduced carrier.
(2) O_2 + reduced carrier = carrier + H_2O.

The partial reaction (1) was studied independently of (2) by adding an excess of the carrier and excluding oxygen.

3. A modification of the method of Szent-Györgyi & Gözsy for the determination of succinic acid is described.

4. An analysis of additive and competitive oxidations which is of interest for the calculations and for the deductions of blanks has been made.

5. Fumarate oxidizes anaerobically glucose, malate, lactate, acetate, glycerol, *dl*-glyceraldehyde, pyruvate, butyrate, acetoacetate, *l*(+)glutamate and molecular hydrogen. Data concerning the rates of the oxidations are given. Formate is not oxidized by fumarate unless the cells contain active hydrogenlyase and it is concluded that there is no direct interaction between fumarate and formate, but only a reduction of fumarate by the hydrogen liberated from formate.

6. The rates of the anaerobic oxidation by fumarate of glucose, malate, acetate, glycerol, glyceraldehyde, pyruvate and lactate were compared with the rates of oxidation by molecular oxygen of the same substrates. The data indicate that fumarate plays an important role as hydrogen carrier in the respiration of *Bact. coli* if the substrates mentioned are present.

7. Pyruvate appears to act as hydrogen carrier only in acid medium, being concerned with the oxidation of pyruvate to acetate according to the equation: 2 pyruvate + H_2O = acetate + CO_2 + lactate.

8. Oxaloacetate is rapidly fermented by *Bact. coli*, about 50 % being reduced to succinate and the remaining 50 % being oxidized. The significance of this reaction for hydrogen transport in respiration is discussed.

9. It is suggested that CO_2 acts as hydrogen acceptor in the case of the oxidation of pyruvate to acetate according to the equation:

$$\text{pyruvate} + H_2O + CO_2 = \text{acetate} + CO_2 + \text{formate}.$$

10. It is shown that many oxidations are primarily dismutations in which the elements of water react, the hydrogen being accepted by the carrier, the oxygen by the organic molecule. The oxygen atoms appearing in the form of carbon dioxide are consequently derived from water, and not from molecular oxygen. Examples of such a participation of water are the oxidations of acetate and of pyruvate.

11. The splitting of formate into hydrogen and bicarbonate is also a dismutation in which water takes part: $HCOO^- + H_2O = H_2 + HCO_3^-$. Experiments with heavy water carried out by Farkas *et al.* [1934] are adduced as evidence.

12. The hydrogen carriers effect the hydrogen transport between organic molecules and molecular oxygen. The observed facts can be explained on the assumption that "activated" molecular oxygen reacts only with succinate, formate, lactate and malate when carbohydrates or related substances such as glycerol, glyceraldehyde, pyruvate and acetate are oxidized. The rest of the oxidations are brought about by dismutations of the substrates with fumarate, pyruvate and oxaloacetate.

13. Fumarate is fermented according to the equation:

$$7 \text{ fumarate} = 6 \text{ succinate} + 2CO_2 + 2 \text{ bicarbonate}.$$

14. Fumarate promotes catalytically the fermentation of pyruvate.

15. Pyruvate yields no lactate and little or no free CO_2 if the reaction is near the neutral point, but yields considerable amounts of lactate and CO_2 if the medium is saturated with 1 atm. CO_2 and if the *p*H is 5·4.

16. Molecular hydrogen is absorbed in the presence of oxaloacetate, but probably only after primary reduction of oxaloacetate to fumarate. Pyruvate does not react with molecular hydrogen. The reaction between hydrogen and fumarate is inhibited by molecular oxygen.

17. The rates of formation of succinic acid from various hexoses, glycerol, *dl*-glyceraldehyde and pyruvate, have been studied. The rate is highest in the presence of glucose and mannose, whilst the yield is higher in the presence of galactose and glycerol.

18. The acid formed anaerobically by washed cells was found to be chiefly succinic acid.

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