LXXVII. DEHYDROGENATIONS PRODUCED BY RESTING BACTERIA. I.

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It has been shown in earlier communications [Quastel and Whetham, 1924; Quastel, Stephenson and Whetham, 1925] that succinic acid readily reduces methylene blue in presence of resting or non-proliferating bacteria (*B. coli* and *B. pyocyaneus*), and that under similar conditions fumaric acid acts as a hydrogen acceptor. It has been shown, also, that these bacteria activate not only succinic, fumaric, and malic acids, but nitrates and chlorates, these substances effecting oxidations in presence of the bacteria which they do not produce at a perceptible rate in their absence.

A systematic study has now been made of the activating powers of *B. coli communis*, and this communication is intended to be the first of a series dealing with the subject. Numerous substances have been found to exert reducing and oxidising powers in presence of this organism.

Harden and Zilva showed some years ago [1915] that washed *B. coli comm.* will not reduce methylene blue and demonstrated, in a qualitative manner, that it acquired the power of reduction on addition to it of various substances incapable by themselves of effecting the reduction. Many of the substances, which they tested, will be dealt with in detail below.

The technique adopted throughout the work has been described fully in the papers quoted above. The rate of reduction of methylene blue was measured under standard conditions of temperature and hydrogen ion concentration in the presence of the organism and the substance under investigation. The conditions were always strictly anaerobic and such that growth of the organism did not occur. Sometimes the method was varied by using nitrate or chlorate as hydrogen acceptors, the amount of reduction in a given time being determined by an iodometric estimation of the nitrite or chlorite produced.

In the preparation of the emulsion of bacteria, the following points were found important. The organism was grown for 2 days on tryptic broth and separated by centrifuging; it was then washed very thoroughly by repeated centrifuging with normal saline to remove all traces of broth which is itself a powerful hydrogen donator in presence of *B. coli*. The bacteria thus washed were suspended in normal saline solution through which first air and then

nitrogen were bubbled vigorously for a short time to produce a homogeneous suspension and to remove traces of easily oxidisable material. The suspension was then diluted with saline until a small volume (say 0·5 cc.) of the suspension did not reduce 1 cc. of 1/5000 methylene blue solution at p_H 7·2 and temperature 45° within several hours. In the presence of a concentration of sodium succinate equal to $M/100$, this quantity of organism was usually found to reduce the same amount of methylene blue in about 10 minutes. If the time of reduction was much shorter than this, the emulsion of bacteria was further diluted. The emulsion was kept surrounded by ice, since at room temperature there occurs a rapid diminution in activating power and a liberation of reducing substances, probably due to an autolysis of the cells. At 0° these changes are hardly noticeable during the first fortnight, and even after that proceed very slowly. A suspension of *B. coli* which had been kept in the ice-chest for 4 months still exhibited strong activating powers, and a sub-culture on agar gave a heavy growth.

It has been previously shown [Quastel and Whetham, 1924] that the reducing activity of the organism is directly proportional to its concentration, and that the reaction between succinate and methylene blue in presence of the organism has a temperature coefficient of 2·1 between 30° and 60°.

The experimental results given below are typical examples of a large number of observations. The work was done with a number of different strains and suspensions of *B. coli*, and these are referred to as *coli* 1, *coli* 2, etc. The results obtained with *coli* 1 can be compared strictly quantitatively with one another, but only qualitatively with the results obtained with *coli* 2, etc.

The substances under investigation were always brought to p_H 7·2 by means of caustic soda or hydrochloric acid.

The fatty acids.

Table I gives the results obtained with the fatty acid series.

Table I.

Each vacuum tube contained 2 cc. of phosphate buffer p_H 7·4, 1 cc. of 1/5000 methylene blue solution, 0·5 cc. of *coli* 1, and *X* cc. of the fatty acid solution. The volume of solution in each tube was made up to 6·5 cc. with distilled water. The reduction was carried out *in vacuo* at 45°. A control tube showed no reduction in 6 hours.

Fatty acid	*X* cc.	Concentration of fatty acid	Reduction time in minutes
Formic *M*/20	1	*M*/130	2·0
„ *M*/2	1	*M*/13	1·5
Acetic *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	2·8
Propionic *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	26·0
Butyric *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	3·0
Isobutyric *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	Not completely reduced in 3 hours
Isovaleric *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	5·0

Formic acid is the most powerful hydrogen donator of the series, and with the exception of some of the sugars is the most active substance we have yet investigated. It has no reducing action *in vitro* on methylene blue, even in strongly alkaline solution.

Table II gives results with the fatty acids obtained with a different strain of *B. coli* (*coli* 2).

Table II.

Procedure exactly as described in Table I. *Coli* 2 used instead of *coli* 1. 2 cc. of an *M*/2 sol. of fatty acid were used in each case yielding a final concentration of *M*/6·5.

Fatty acid	Reduction time in minutes
Formic	1·2
Acetic	9
Propionic	11
Butyric	30
Valeric	23
Isovaleric	25
Caproic	Not reduced
Heptylic	,,
Caprylic	,,
Nonylic	,,

Apart from formic acid, the lower fatty acids are active donators of hydrogen only at high concentrations. Caproic acid exerts a slight activity (see Table III) but higher members than this are apparently inert. A change in the activity of the organism has apparently a far greater effect on the reduction velocities due to the fatty acids than on those due to donators such as formic or succinic acids, which are active at low concentrations.

Fig. 1 illustrates the variation in times of reduction of 1 cc. of 1/5000 methylene blue solution with varying concentrations of acetic, propionic and butyric acids at p_H 7·4 and 45°, always using the same amount of organism (*coli* 1). The propionic acid curve is of a different type from those of acetic and butyric acids. In view of the oxidation of these fatty acids in the body, it is interesting that propionic acid should offer so clear a contrast to acetic and butyric acids in presence of resting *B. coli*.

The reduction time-concentration curves of acetic, butyric and formic acids (Fig. 1) exhibit a feature common to many of the hydrogen donators we have investigated, namely, a rapid decrease of reduction time up to a definite concentration, above which increase in concentration of the hydrogen donator appears to have little or no effect on reduction velocity. At first sight this phenomenon might be explained on the usual grounds by assuming that the enzyme becomes "saturated" with regard to the donator, so that increase in concentration of substrate above this saturation point does not produce greater reaction velocity. There are, however, difficulties in the way of accepting this interpretation. For example, in some cases, *e.g.* propionic acid (Fig. 1) and succinic acid (Fig. 2), such a "critical" concentration is hardly apparent, and the curves do not exhibit the discontinuity which would be expected if the enzyme surface became truly saturated at some particular concentration of the substrate. In a number of cases a definite relationship appears to hold

between the velocity of reduction and the concentration of the hydrogen donator. This is

$$v = k + k' \log c,$$

where v = velocity of reduction (reciprocal of time of reduction),
c = concentration of the hydrogen donator,
and k and k' are constants.

Beyond stating that in a number of cases we have found such a logarithmic relationship to accord with the experimental results, we do not wish to pursue the subject further at present, and hope to deal with it in detail in a later communication.

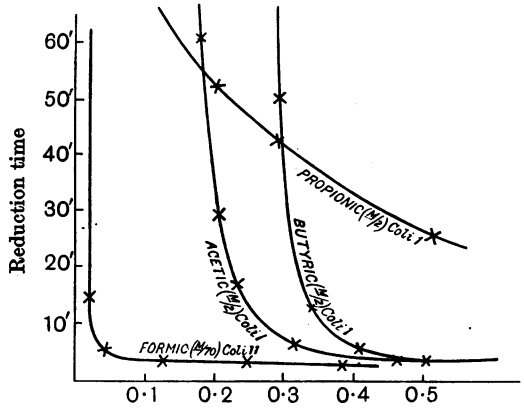

Fig. 1. Concentration of fatty acid

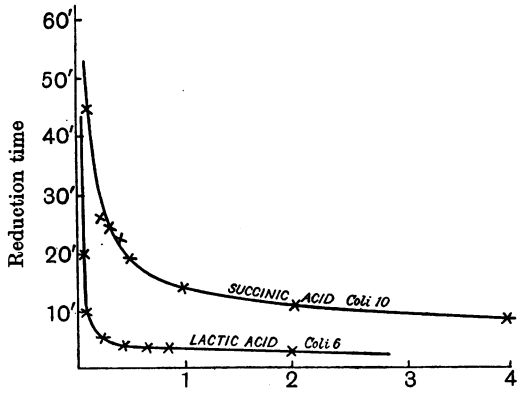

Fig. 2. Concentration (× M/140)

The products of the anaerobic oxidation of the fatty acids in presence of resting *B. coli* have not yet been investigated, but it is clear that in the case of formic acid there is a complete oxidation to carbon dioxide. There is a great increase in p_H during the anaerobic oxidation of formic acid, and carbon dioxide is evolved on treatment with acid.

Pakes and Jollyman [1901] have shown that *B. coli comm.* will decompose formic acid into equal volumes of carbon dioxide and hydrogen. If nitrates are also present in the nutrient medium, nitrites but not hydrogen are produced.

There is some evidence that the fatty acids have a retarding effect on reduction velocity at high concentrations, possibly due to adsorption of the acid on the organism. For instance, isobutyric acid has an optimum concentration as a hydrogen donator, being less active at higher concentrations. The same applies to isovaleric and caproic acids (see Table III).

Table III.

Conditions as in Table I.

Fatty acid	*X* cc.	Organism	Concentration of fatty acid	Reduction time in minutes
Isobutyric *M*/20	1·0	0·5 *coli* 1	*M*/130	No reduction
„ *M*/2	2·0	„	*M*/6·5	145
„ *M*/2	4·0	„	*M*/3·25	Not completely reduced in 6 hours
Isovaleric *M*/2	0·5	0·5 *coli* 3	*M*/26	57
„ *M*/2	1·0	„	*M*/13	49
„ *M*/2	2·0	„	*M*/6·5	Not completely reduced in 2 hours
Caproic *M*/2	1·0	0·5 *coli* 4	*M*/13	74
„ *M*/2	2·0	„	*M*/6·5	Not reduced in 2 hours
„ *M*/2	3·0	„	*M*/4·3	„ „

It is possible that in the case of the higher homologues of caproic acid the reducing activity is masked by greater adsorptive effects which increase with the length of the fatty chain.

The saturated dibasic acids.

Oxalic acid is inert as a hydrogen donator, and malonic, glutaric, and adipic acids may also be regarded as inert, for under the conditions of our experiments these substances have never been found to bring about a complete reduction of methylene blue though they give indications of a slight activity (see Table IV).

Table IV.

Conditions as in Table I.

Acid	*X* cc. of neutralised acid sol.	Concentration of the acid	Reduction time in minutes
Oxalic *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	„
Malonic *M*/20	1	*M*/130	„
„ *M*/2	3	*M*/4·3	Partial reduction not complete in 4 hours
Succinic *M*/20	1	*M*/130	11·0
„ *M*/2	3	*M*/4·3	4·0
Glutaric *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	Partial reduction not complete in 4 hours
Adipic *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	Partial reduction not complete in 4 hours

Oxalic, glutaric, and adipic acids (when mixed with succinic acid) do not retard the reduction due to the succinic acid, but malonic acid has a definite

retarding effect. It is difficult to explain the anomalous behaviour of malonic acid, but there is no doubt as to the reality of the effect (see Table V).

Table V.

Each vacuum tube contained 2 cc. of phosphate buffer p_H 7·4, 1 cc. of 1/5000 methylene blue solution, 0·5 cc. of *coli* 1, 1 cc. of sodium succinate solution *M*/20, 1 cc. of the neutralised dibasic acid (*M*/2), and 1 cc. of water.

Acid	Reduction time in minutes
None (control)	11·0
Malonic	34·5
Glutaric	10·5
Adipic	10·2

The hydroxy acids.

Glycollic acid donates hydrogen only at relatively high concentrations, its behaviour being similar to that of acetic acid. Lactic acid, on the other hand, is an extremely powerful hydrogen donator even at low concentrations. Glyceric acid is active at lower concentrations than glycollic acid but is not as powerful as succinic acid. Tartaric acid is a very feeble donator, whilst citric acid is almost inert (see Table VI).

Table VI.

Conditions as before. *Coli* 5.

Acid	*X* cc. of neutralised acid sol.	Concentration of acid	Reduction time in minutes
Glycollic *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	3·0
Lactic *M*/20	1	*M*/130	3·0
„ *M*/2	3	*M*/4·3	1·5
Glyceric *M*/20	1	*M*/130	45·0
„ *M*/2	3	*M*/4·3	3·5
Tartaric *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	21·0
Citric *M*/20	1	*M*/130	No reduction
„ *M*/2	3	*M*/4·3	Not completely reduced in 1 hour

Fig. 3 gives the reduction time-concentration curves for glyceric and glycollic acids (in presence of 0·5 cc. of *coli* 5) and Fig. 2 for lactic acid (in presence of 0·5 cc. of *coli* 6).

The powerful activity of lactic acid compared with the other α-hydroxy acids is very striking. The product of anaerobic oxidation of lactic acid is pyruvic acid; the oxidation of lactic acid in presence of resting bacteria has been fully discussed in a previous communication [Quastel, Stephenson and Whetham, 1925]. The products of anaerobic oxidation of glycollic and glyceric acids have not yet been investigated.

The polyhydric alcohols.

Glycol donates hydrogen only at high concentrations. Glycerol is less active than glycol. Erythritol has no reducing effect (see Table VII).

Table VII.

Conditions as before. *Coli* 5.

Alcohol	*X* cc. of the *M*/2 alcohol solution	Concentration of alcohol	Reduction time in minutes
Glycol	0·5	*M*/26	Not reduced in 2 hours
„	1·0	*M*/13	71
„	3·0	*M*/4·3	8·25
Glycerol	1·0	*M*/13	53
„	3·0	*M*/4·3	30
Erythritol	1·0	*M*/13	No reduction
„	3·0	*M*/4·3	„

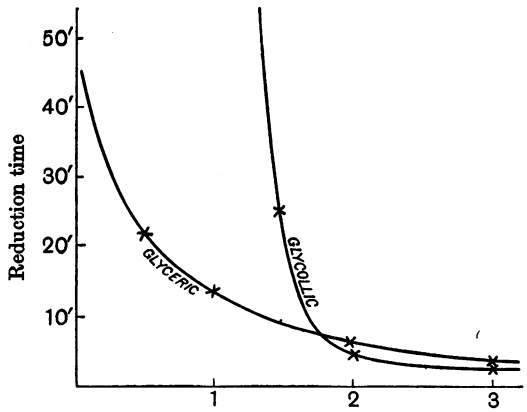

Fig. 3. Concentration of hydroxy acid (× *M*/13)

Glycol, glycerol and erythritol appear to have no retarding effect on reductions due to other substances. The penta- and hexa-hydric alcohols will be discussed later in connection with the sugars.

The glycerophosphoric acids.

α-Glycerophosphoric acid is a much more vigorous donator of hydrogen in presence of resting *B. coli* than glycerol itself, whilst β-glycerophosphoric acid is about as active as glycerol (see Table VIII).

Table VIII.

Conditions as before. *Coli* 5.

Hydrogen donator	*X* cc. of the *M*/2 H donator solution	Concentration hydrogen donator	Reduction time in minutes
Glycerol	1	*M*/13	53·0
„	3	*M*/4·3	30·0
α-Glycerophosphoric acid	1	*M*/13	2·5
„	3	*M*/4·3	2·0
β-Glycerophosphoric acid	1	*M*/13	70·0
„	3	*M*/4·3	20·0

The monohydric alcohols.

The members of this series from methyl to octyl alcohol have been investigated. From butyl alcohol upwards they do not appear to donate hydrogen and all inhibit or retard reductions by other substances. Since these higher alcohols are immiscible with water at ordinary temperatures, they do not lend themselves to quantitative investigation.

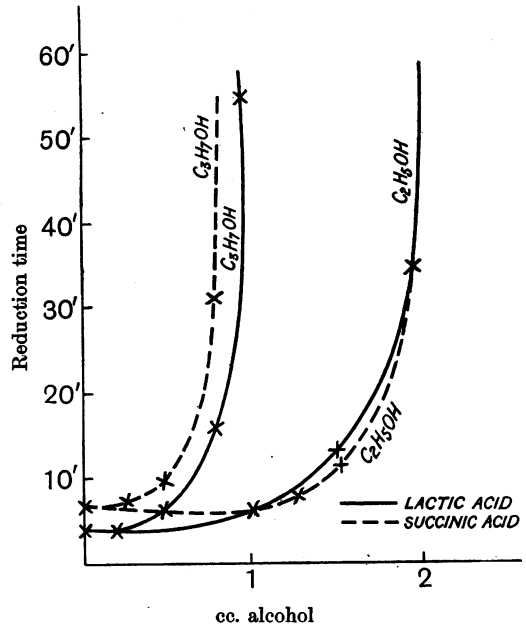

Fig. 4. Effect of alcohols on reduction times of 1 cc. 1/5000 methylene blue by 1 cc. *M*/20 lactic acid and 1 cc. *M*/20 succinic acid.

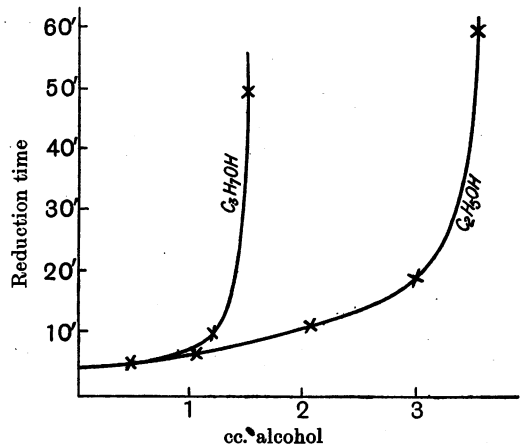

Fig. 5 Effect of alcohols on reduction times of 1 cc. 1/5000 methylene blue by 1 cc. *M*/20 formic acid.

The lowest members—methyl, ethyl and propyl alcohols—all possess the characteristic property of inhibiting the reducing action of hydrogen donators. Total inhibition occurs at definite or critical concentrations of the alcohols, and these concentrations vary with the nature of the hydrogen donator (see Figs. 4, 5, which show the action of varying quantities of the alcohols on the reduction times of a definite quantity of donator, in the presence of 1 cc. of *coli* 7). The critical concentrations of alcohol vary very slightly with the quantity of resting organism used (see Fig. 6) and are independent of the concentration of the donator (see Table IX).

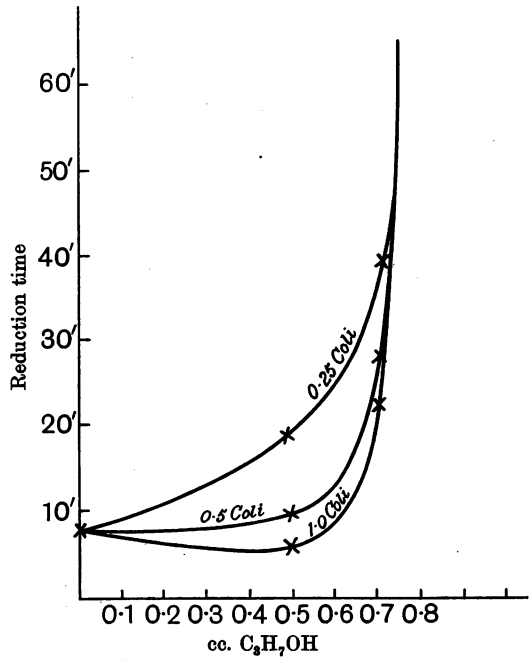

Fig. 6. Effect of propyl alcohol on reduction times of 1 cc. 1/5000 methylene blue by succinic acid.

Table IX.

Each vacuum tube contained 1 cc. of phosphate buffer p_H 7·4, 1 cc. of 1/5000 methylene blue solution, *X* cc. of 1 % sodium succinate, 0·7 cc. of propyl alcohol and 1 cc. of *coli* 7. The volume was made up to 7 cc. with water.

X cc.	Reduction time
0·5	Not reduced
1·0	,,
2·0	,,
3·0	,,

These critical concentrations are least with propyl alcohol and greatest with methyl alcohol, *i.e.* methyl alcohol inhibits least vigorously.

Besides their property of inhibition, ethyl and propyl alcohols possess the power of donating hydrogen to methylene blue (see Table X). Methyl alcohol does not act as a hydrogen donator.

Table X.

Each vacuum tube contained 2 cc. of *coli* 8*, 1 cc. of phosphate buffer p_H 7·4, 1 cc. of 1/5000 methylene blue solution and *X* cc. of the absolute alcohol. The volume was made up to 7 cc. with water.

Alcohol	*X* cc.	Reduction time in minutes
None (control)	—	23·25
Methyl	0·05	23·0
,,	0·1	23·0
,,	0·5	30·0
Ethyl	0·05	11·0
,,	0·10	10·4
,,	1·5	Not reduced
Propyl	0·05	10·25
,,	0·10	9·75
,,	0·50	10·9
,,	1·00	Not reduced

* *Coli* 8 was a highly reducing organism and is included here to demonstrate the alcohol inhibitions.

The effect of increasing the concentration of alcohol is to produce at first an increasing velocity of reduction. This reaches a maximum and remains constant until a certain concentration of alcohol is reached, when the velocity falls off sharply to zero.

Experiments with the vacuum U-tube (see previous papers) show that the inhibitant effects of the alcohols are not due to oxidation of leuco-methylene blue (as in the case of fumaric acid) but to some physical process, such as adsorption at the surface of the organism. The degree of inhibition is associated with the length and nature of the fatty chain: as shown above, high concentrations of some of the higher fatty acids also bring about a retardation of reducing velocity. The polyhydric alcohols, on the other hand, have no retarding effect.

Inhibitants.

An examination of the inhibiting action of substances other than the monohydric alcohols showed that:

1. Small quantities of cyclohexanol or cyclohexene completely inhibit all reductions by hydrogen donators.

2. Benzene, toluene, phenol, and acetone have definite but relatively slight inhibitory effects (see Table XI).

Table XI.

Each vacuum tube contained 1 cc. phosphate buffer p_H 7·4, 1 cc. 1/5000 methylene blue, 0·2 cc. of 5 % sodium succinate, 0·5 cc. *coli* 9, and *X* cc. of inhibitant. The total volume was made up to 6·5 cc. with water.

Inhibitant	*X* cc.	Reduction time in minutes
Control	—	8
Benzene	0·5	9
Toluene	0·5	14
Phenol (10 % neutral)	0·5	10
Cyclohexanol	0·5	Not reduced in 6 hours
,,	0·2	,,
Cyclohexene	0·5	,,
Acetone	0·5	10

Reducing coefficient.

It is desirable to provide some means for the quantitative comparison of the reducing capacity of the large number of substances which can donate hydrogen to methylene blue in presence of resting *B. coli*. This may be done by comparing one donator with a standard donator—say succinic acid—under similar conditions. The most satisfactory method of comparison is to determine what concentration of a donator will reduce a given quantity of methylene blue in the same time as a definite quantity of the standard donator.

We propose, therefore, to define the *reducing coefficient* of a hydrogen donator with respect to a certain organism as the reciprocal of that (molar) concentration which will reduce 1 cc. of 1/5000 methylene blue solution in presence of a standard amount of the organism in half an hour, succinic acid being taken as a standard and its reducing coefficient being taken as 100. For the determination of the coefficient an amount of freshly prepared organism is taken which will bring about the reduction of 1 cc. 1/5000 methylene blue at p_H 7·2 and 45° in the presence of $M/700$ succinic acid in half an hour.

Table XII gives the reducing coefficients of the substances discussed in this communication. Ethyl and propyl alcohols have not been included, owing to their inhibitory action. The coefficients must only be regarded as approximate and as expressing the correct order of magnitude.

Table XII.

Hydrogen donator	Reducing coefficient (with respect to *B. coli comm.*)
Formic acid	... 700
Acetic acid	... 0·95
Propionic acid	... 0·46
Butyric acid	... 0·64
Valeric acid	... 0·5
Caproic acid	... Less than 0·4
Caprylic acid	,, 0·4
Heptylic acid	,, 0·4
Nonylic acid	,, 0·4
Oxalic acid	,, 0·4
Malonic acid	,, 0·4
Succinic acid	... 100
Glutaric acid ...	... Less than 0·4
Adipic acid	,, 0·4
Glycollic acid	... 0·93
Lactic acid	... 583
Glyceric acid	... 5·2
Tartaric acid	... 0·46
Citric acid	... Less than 0·4
Glycol	... 0·86
Glycerol	... 0·46
Erythritol	... Less than 0·4
α-Glycerophosphoric acid	... ... 4·31
β-Glycerophosphoric acid	... ... 0·4

SUMMARY.

An account is given of the behaviour of a number of substances as hydrogen donators in presence of resting *B. coli* as the activating source and of methylene blue as the hydrogen acceptor. The following groups of substances are dealt with:

1. Fatty acids.
2. Saturated dibasic acids.
3. Hydroxy-acids.
4. Polyhydric alcohols.
5. Monohydric alcohols.

The inhibiting action of the monohydric alcohols and of other substances is discussed. A table is given of the relative reducing powers or reducing coefficients of the hydrogen donators.

We wish to express our indebtedness to Sir F. G. Hopkins for the interest he has taken in this work and the thanks of one of us (M. D. W.) are due to the Medical Research Council for a grant held during this work.

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