LXXXIV. SOME PROPERTIES OF THE DEHYDROGENATING ENZYMES OF BACTERIA.

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Introduction.

It has been shown on the activation theory put forward by one of us [Quastel, 1926] that not only are many of the phenomena of biological oxidations and reductions brought into line with one another and made interpretable on a common basis, but that the relative effects of the introduction of various groups into a particular substrate on the hydrogen-donating or -accepting power of the latter can be qualitatively estimated. For instance it can be shown that the introduction of a CH_3 group into glycollic acid should produce a substance (lactic acid) which would be a more powerful hydrogen donator than glycollic acid, whilst the introduction of a COOH group should produce a substance (hydroxymalonic acid) which would have feebler hydrogen-donating powers than glycollic acid towards the same hydrogen acceptor (methylene blue) and in the presence of the same activating system. Again the relative effects of the introduction of CH_3 or OH or $CH_2.COOH$ into succinic acid and other substances can be qualitatively estimated, and the predicted results are found to be in agreement with the experimental.

In this examination, however, of the properties of substances on the activation theory it has been tacitly assumed that all the substances in question are accessible to or adsorbed by the active centres or the enzymes, and the facts that results predicted on the activation theory are borne out by experiment, and that no instance has yet been found which is contrary to the expected result, are in favour of the truth of this assumption. Yet it may be maintained that this agreement between predicted and experimental result is entirely fortuitous. If it can be shown by experiment, however, that the substrates whose properties we are investigating (whether they be activated or not) are actually adsorbed by certain active centres, this will provide strong support of the active centre hypothesis and will render much more remote the possibility that the agreement between the experimental results and those predicted on the theory of activation is simply a coincidence.

The aim of the investigation to be described in this paper has been to ascertain

(1) whether the assumption is correct that a number of substrates, for which results were predicted on the activation theory and were confirmed by experiment, are actually adsorbed at active centres (whether they are activated or not);

(2) whether any particular type of compound is adsorbed at one centre rather than at another.

We should expect *a priori* that a centre possessing certain groups would adsorb a particular type of compound and that another centre with different groups would adsorb a different type of compound, *i.e.* the active centres would evince a *specificity of adsorption*. But of the total number of molecules capable of being adsorbed at a particular centre we expect only a few to be activated. The number of these would depend upon the strength and nature of the polarising field and the structure of the substrate molecules. Thus there are two factors which limit the specificity of action of a centre (or enzyme), (*a*) the specificity of adsorption at the centre, (*b*) the structure of the adsorbed molecules in relation to the nature of the activating field at the centre.

Experimental procedure.

It has been shown [Quastel and Wooldridge, 1927, 1] that the effect of exposing *B. coli* to toluene is to bring about a considerable number of inactivations. The enzymes for lactic, succinic and formic acids are, however, left intact. Hence toluene-treated *B. coli* forms suitable material for determining the action on the lactic, succinic and formic acid enzymes of a number of substances, inert in presence of the treated organism but possibly active with the normal organism. The experimental procedure is to determine the time of reduction of a quantity of methylene blue by, say, lactic acid in presence of the treated organism and to compare this with the time of reduction obtained with a mixture of the lactic acid and the substance under investigation, *e.g.* oxalic acid, under exactly similar conditions. From the two times, the percentage retardation of the velocity of reduction of methylene blue by lactic acid is easily determined. The following is an example of the procedure in one experiment. In a vacuum tube (Thunberg) were placed 2 cc. phosphate buffer solution p_H 7·4, 1 cc. 1/5000 methylene blue solution, 1 cc. $M/50$ lactic acid (neutralised) solution, 2 cc. water and 1 cc. of a suspension of the toluene-treated organism. The tube was then evacuated and placed in the water-bath at 45° and the reduction time to a standard colour corresponding to 95 % reduction of the dye was determined. The reduction time was 10 minutes. In another tube was placed the same mixture of phosphate buffer solution, methylene blue, lactic acid and toluene-treated organism. But instead of water there were added 2 cc. $M/4$ oxalic acid (neutralised). The reduction time to the standard colour was determined as before. The time was 180 minutes. The percentage retardation by this con-

centration of oxalic acid ($M/14$) of the velocity of reduction by lactic acid ($M/350$) was $\frac{180-10}{180}.100 = 95\ \%$ (approx.).

In all cases substances under investigation were neutralised (if necessary) with sodium hydroxide to p_H 7·4, and solutions in the vacuum tubes were always buffered at this p_H. Reductions were always carried out at 45°.

The toluene-treated organism was prepared by shaking a suspension of freshly grown *B. coli* with toluene. Previous to treatment with toluene the freshly grown organism was washed three times with physiological saline solution, aerated for one hour, nitrogen was passed through for another hour to help to remove oxygen, and finally the suspension of the organism in saline was evacuated in order to remove gases as much as possible from the suspension. The latter precaution is necessary as it is not easy to remove the last traces of oxygen from the bacterial emulsion when it is mixed with various solutions in the vacuum tubes.

After the suspension of the organism was shaken with toluene it was allowed to stand, still in contact with toluene, at room temperature for 1 hour. The bacterial emulsion was then pipetted clear from the layer of toluene, and the emulsion was used in this state.

Reduction times were taken to a standard 95 % reduction of the methylene blue in order to eliminate the departure from the linearity of reduction velocity during the last 5 % reduction.

The experimental error in obtaining the time of reduction is about 1 to 2 minutes. Hence where retardations in the time of reduction are of this order the effects are not taken as real. Thus if there be a retardation of a normal reduction time of 20 to 22 minutes, by admixture of a substance, the percentage retardation, which is 9 %, is not regarded by us as real. It is necessary, therefore, to adjust the amount of organism so that the normal reduction time (*i.e.* the time without admixture of extraneous substances) is of the order of 15 to 25 minutes, so that the experimental error of 2 minutes gives a relatively small apparent percentage retardation (up to 14 %). Retardations greater than 20 % are real; the longer the time of retarded reduction the greater is the accuracy of observation.

Results with lactic acid (toluene-treated organism).

The percentage retardations of the velocity of reduction of methylene blue due to lactic acid by various substances are given in Table I. The following substances, among those investigated, are strongly adsorbed: glycollic acid, oxalic acid, glyoxylic acid, hydroxymalonic acid, glyceric acid, α-hydroxybutyric acid, mandelic acid, pyruvic acid, mesotartaric acid, *dl*-tartaric acid, and presumably lactic acid.

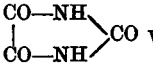
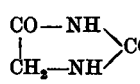
The structure common to these substances, and presumably that necessary for adsorption at the lactic acid enzyme is —CO—COH* or —CHOH—COH* where H* is mobile, *i.e.* the substance is acidic.

Table I.

Percentage retardations by various substances of the velocity of reduction of methylene blue by lactic or succinic acids (neutralised) in the presence of toluene-treated *B. coli* or an extract obtained from autolysed *B. coli*. All substances used were first adjusted to p_H 7·4 and the experiments were made *in vacuo* at 45° as described for Table II. The "toluene-treated *B. coli*" and "extract" were prepared as described in the text.

	Toluene-treated *B. coli*			Extract	
Added substance	Concentration of added substances	% retardation on succinic enzyme *M*/140 succinic acid	% retardation on lactic enzyme *M*/350 lactic acid	Concentration of added substances	% retardation on lactic enzyme *M*/70 lactic acid
α-Hydroxybutyric acid	*M*/70	—	>30	*M*/70	>30
Hydroxy*iso*butyric acid	*M*/14	0	0	—	—
Glyceric acid	*M*/28	0	>80	*M*/14	>80
Pyruvic acid	*M*/14	10	90	*M*/28	68
Alanine	*M*/14	0	25	*M*/14	21
Acetic acid	*M*/14	0	0	*M*/14	0
Glycollic acid	*M*/14	0	>80	*M*/28	>95
Glycine	*M*/14	0	25	*M*/14	21
Glycol	*M*/7	0	0	*M*/7	0
Oxalic acid	*M*/28	14	>95	*M*/28	>95
Glyoxylic acid	*M*/14	0	>82	*M*/28	>95
Malonic acid	*M*/14	>95	15	*M*/28	0
Hydroxymalonic acid	*M*/14	0	90	*M*/28	>95
Glycerol	*M*/7	0	10	*M*/28	18
Phenylacetic acid	*M*/14	77	55	*M*/7	40
Mandelic acid	*M*/14	52	76	*M*/7	>95
Phenylpropionic acid	*M*/35	>95	61	—	—
Tricarballylic acid	*M*/10·5	52	0	*M*/28	18
Citric acid	*M*/10·5	30	0	*M*/28	25
Pyrotartaric acid	*M*/7	90	50	—	—
Tartaric acid	*M*/14	10	47	*M*/14	20
Mesotartaric acid	*M*/14	50	90	*M*/28	>95
Parabanic acid	*M*/140	42	80	*M*/112	>95
Hydantoin	*M*/56	0	0	*M*/56	0
Succinic acid	—	—	—	*M*/14	0
Glutaric acid	*M*/14	43	25	—	—
Succinimide	*M*/10	0	0	—	—
Glutamic acid	*M*/14	5	27	*M*/28	0
Cyanoacetic acid	*M*/7	15	0	—	—
Thiodiglycollic acid	*M*/14	0	0	—	—
Laevulinic acid	*M*/14	0	0	—	—
Glucose	*M*/14	0	0	*M*/14	0
Mannitol	*M*/14	0	0	—	—
Formic acid	—	—	—	*M*/28	0
Fumaric acid	—	—	—	*M*/14	0
Aspartic acid	—	—	—	*M*/28	25
Malic acid	—	—	—	*M*/28	31

Acetic acid, malonic acid, glycol, glycerol, glycine, succinic acid, formic acid, hydroxy*iso*butyric acid, citric acid, etc. are either not adsorbed or only very feebly so, illustrating the specificity of adsorption for a particular *type* of structure by the lactic acid enzyme. It was interesting to determine the effect of substitution of the two OH groups of oxalic acid.

Parabanic acid  was found to be powerfully adsorbed (% retardation = 80), but hydantoin  , as we would anticipate from the previous results, was not adsorbed under our experimental conditions.

The affinity of oxalic acid for the lactic acid enzyme (which does not activate oxalic acid) is very striking. A concentration of $M/7000$ will bring about a 50 % retardation of the velocity of reduction of methylene blue due to $M/140$ lactic acid.

Pyruvic acid is strongly adsorbed. Its retardation of the normal velocity of reduction is not due to its acting as a hydrogen acceptor (*i.e.* to its oxidising leucomethylene blue), for it does not appreciably retard the velocity of reduction due to succinic acid. Were it a hydrogen acceptor in presence of the toluene-treated organism, like fumaric acid, it would retard reductions both by lactic and succinic acids.

Since fumaric, malic, and aspartic acids act as hydrogen acceptors in presence of the organism they cannot be used with this organism to determine whether their adsorption occurs at the lactic acid enzyme. From other evidence we have found that neither succinic nor fumaric acid is adsorbed by this enzyme, that malic acid is adsorbed, but only to a slight extent and not nearly so powerfully as glycollic acid.

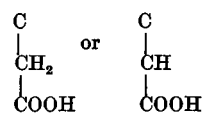
Mesotartaric and *dl*-tartaric acids are interesting in demonstrating the effect of a change in internal structure on adsorption. Mesotartaric acid is much more powerfully adsorbed than tartaric acid.

Results with succinic acid (toluene-treated organism).

The results are given in Table I. The following substances, among those investigated, are strongly adsorbed: succinic acid, pyrotartaric acid, tricarballylic acid, phenylpropionic acid, malonic acid, glutaric acid, mesotartaric acid.

The substances adsorbed by the lactic acid enzyme (with the exception of mesotartaric acid), acetic acid, glycine, glycerol, glycol or the sugars are either not adsorbed by the succinic acid enzyme or only feebly so. Phenylacetic and phenylpropionic acids are more adsorbed at the succinic acid enzyme than at the lactic acid enzyme; but parabanic acid, which has a definite effect at the succinic acid enzyme is less powerful here than with the lactic acid enzyme. The same applies to mesotartaric acid.

The structure necessary for adsorption at the succinic acid enzyme seems to be



associated possibly with another carboxyl group. Malonic acid has a very powerful affinity for the succinic acid enzyme (though it is neither a hydrogen acceptor nor donator), its action being similar to that of oxalic acid for the lactic acid enzyme. The phenyl group may also play a part in facilitating adsorption at the succinic acid enzyme. It is interesting that mandelic and hydroxymalonic acids should offer such clear contrasts to phenylacetic acid

and malonic acid respectively, in their relative effects on the succinic acid and lactic acid enzymes.

The effects of malonic acid and substituted malonic acids.

Table II shows reduction times due to lactic, succinic and formic acids in presence of toluene-treated organism and the effects on these times of the admixture, with the normal substrate, of either malonic acid, hydroxymalonic acid, or ethylmalonic acid. Results with oxalic acid are also inserted for comparison. It will be seen how specifically effective malonic acid is on the succinic acid enzyme, and hydroxymalonic and oxalic acids are on the lactic acid enzyme. The formic acid enzyme is not affected. Ethylmalonic acid has little or no effect on the three enzymes.

Table II.

Effects of the addition of certain substances upon the times of reduction of methylene blue by succinic, lactic and formic acids in the presence of toluene-treated *B. coli*. Each vacuum tube contained 2 cc. phosphate buffer p_H 7·4, 1 cc. 1/5000 methylene blue solution, 1 cc. toluene-treated *B. coli*, 1 cc. of the donator at the head of each vertical column, 1 cc. of the substances, in a concentration of $M/2$, given horizontally and 1 cc. water. All the acids were brought to p_H 7·4 with sodium hydroxide. The reductions were carried out *in vacuo* at 45°. ∞ indicates that reduction was not complete in 3 hours.

Added substance, $M/14$	$t_{succinate}$, $M/140$	$t_{lactate}$, $M/350$	$t_{formate}$, $M/700$
NaCl	23′	10·7′	12′
Malonic acid	∞	14·5′	11·7′
Hydroxymalonic acid	21·3′	∞	10′
Ethylmalonic acid	20·5′	18′	10·5′
Oxalic acid	32·5′	∞	11·3′

Activations by toluene-treated B. coli.

Among the substances adsorbed by the lactic acid enzyme the following are activated as hydrogen donators by the normal organism: tartaric, glycollic, mandelic, hydroxymalonic, glyceric, α-hydroxybutyric and lactic acids. After treatment of *B. coli* with toluene (see Table III for results) the following only are activated: lactic, α-hydroxybutyric and glyceric acids. The activations of lactic and α-hydroxybutyric acids are either not at all or only relatively slightly affected by the toluene treatment whilst that for glyceric acid ($M/28$) seems to be decidedly affected. Glyceric acid is interesting in that its activation does not appear to be inhibited to the same extent by the presence of oxalic acid ($M/14$) as are those of α-hydroxybutyric and lactic acids. It is possible that the explanation will be found in a consideration of the relative adsorption coefficients of these substances at the lactic acid enzyme. As we have shown in earlier papers toluene effects the elimination of a number of dehydrogenating enzymes, including those for the sugars and glutamic acid.

The lower fatty acids.

In earlier papers we considered that the activation of acetic acid ran parallel with that for formic acid. This appears to be an error. Normal *B. coli* activates formic, acetic and propionic acids [Quastel and Whetham, 1925] but after its treatment with toluene the activations of acetic and pro-

pionic acids no longer occur although that for formic acid is left intact. The fact that we obtained positive results for acetic acid with toluene-treated organism we suspect to be due to our sample of acetic acid (*Kahlbaum 100 %*) probably containing formic acid to the extent of 1 in 5000 (which would be sufficient to account for the result) rather than to the fact that we are now working with a different strain of organism. Our present sample of acetic acid effects no reduction of methylene blue in presence of the toluene-treated organism although it will do so in presence of the normal organism[1] (Table III). Acetic acid will not retard the time of reduction due to formic acid on admixture with the latter. As stated earlier the formic acid enzyme seems to be independent of the substances we have yet investigated.

Table III.

Effects of toluene upon the activation of certain donators by *B. coli*. The toluene-treated *B. coli* was prepared from the normal *B. coli*. Conditions as in Table II.

Substance	Reduction times with normal *B. coli*	Reduction times with toluene-treated *B. coli*
None (control)	100′	∞
Succinic acid, *M*/140	14′	12′
Lactic acid, *M*/350	7′	7′
α-Hydroxybutyric acid, *M*/35	6·5′	9·7′
Glyceric acid, *M*/14	15·5′	71′
Glycollic acid, *M*/14	24′	∞
Mandelic acid, *M*/14	73′	∞
Hydroxymalonic acid, *M*/7	40′	∞
Tartaric acid, *M*/7	21′	∞
Glutamic acid, *M*/14	17′	∞
Acetic acid, *M*/7	24′	∞
Glucose, *M*/140	7′	∞

The action of malonic acid and oxalic acid on the activation of succinic acid and lactic acid respectively (toluene-treated B. coli).

Figs. 1 and 2 show quantitatively the effect of malonic acid at various concentrations on the reduction of methylene blue in presence of a constant quantity (*M*/140) of succinic acid, and of succinic acid at various concentrations in presence of a constant quantity of malonic acid.

Figs. 3 and 4 are similar for lactic and oxalic acids. The succinic and lactic acids are at such concentrations (in Figs. 1 and 3) that increase of these does not appreciably increase the velocity of reduction.

The curves show (*a*) that the effects of malonic and oxalic acids are reversible; (*b*) that malonic and oxalic acids do not inhibit at rates directly proportional to their concentrations but at rates which we would anticipate if they were adsorbed at the enzymes. Sufficient quantitative work has not yet been done to be certain of the exact form of the curves. They appear to follow approximately the logarithmic function found by Quastel and Whetham [1925] to hold for the relationship between velocity of reduction and concentration of substrate.

[1] To perceive the effect of acetic acid it is necessary to use relatively large quantities of the *freshly grown* organism.

Results with normal B. coli.

Owing to the activity of many substances which are rendered inert by treating *B. coli* with toluene, results obtained by using mixtures of substrates with the normal organism are not so clearly defined as those with the treated organism. It has been possible to show, however [Quastel and Wooldridge, 1927, 2], that the substrates do not all act independently of one another—as they should if entirely distinct enzymes (one for each substrate) were

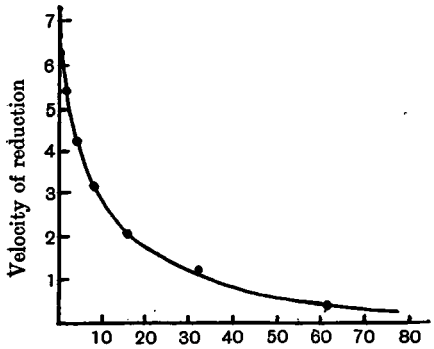


Fig. 1. The effect of variation in malonic acid concentration on the velocity of reduction due to a constant quantity (M/140) of succinic acid.

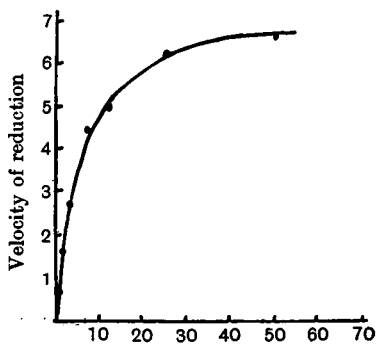


Fig. 2. The effect of variation in succinic acid concentration on the velocity of reduction in presence of a constant quantity (M/700) of malonic acid.

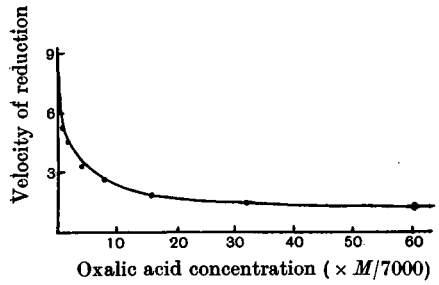


Fig. 3. The effect of variation in oxalic acid concentration on the velocity of reduction due to a constant quantity (M/140) of lactic acid.

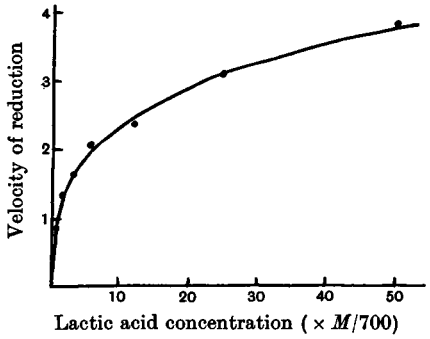


Fig. 4. The effect of variation in lactic acid concentration on the velocity of reduction in presence of a constant quantity (M/700) of oxalic acid.

involved. This was clearly shown in the case of the sugars, where a mixture of two different sugars at their saturation concentrations reduced at a rate either intermediate between the rates of the sugars taken separately or at a rate equal to that of one of them. The enzymes for glucose and for formic acid were clearly independent of one another, but in most other instances

"overlapping" occurred which could only be interpreted by assuming that adsorptions of substrates occurred at enzymes not concerned with their activations. The effects of treating the organism in various ways were examined and it was found that a treated organism did not necessarily have the same power of adsorbing a substrate at an enzyme as it had previous to treatment—a result which we would expect on the view of active centres but which is difficult to reconcile with the view that enzymic activity is due to the effects of specifically active molecules adsorbed at a surface. Results with the normal organism (Table IV) show that the effect of oxalic acid on the lactic

Table IV.

Rates of reduction due to donators A, and mixtures of the donators with other substances B. t_A, t_B and t_{A+B} = times of reduction in mins. respectively due to A, B or $A+B$ in the presence of "resting" *B. coli*. Conditions as in Table II.

A	B (M/14)	t_A	t_B	t_{A+B}
Lactic acid, M/350	Malonic acid	12·3	∞	16
„	Hydroxymalonic acid	„	53	18
„	Ethylmalonic acid	„	∞	13·7
„	Oxalic acid	„	„	25
„	Mandelic acid	10	„	18
Succinic acid, M/140	Malonic acid	21	„	58·3
„	Hydroxymalonic acid	„	53	15
„	Ethylmalonic acid	„	∞	22
„	Oxalic acid	„	„	25
Glucose, M/140	Malonic acid	7·7	„	9
„	Hydroxymalonic acid	„	53	9·3
„	Oxalic acid	„	∞	8·5
„	Mannitol	„	10	8
Glutamic acid, M/14	Malonic acid	18·3	∞	23·3
„	Hydroxymalonic acid	„	53	17
„	Oxalic acid	„	∞	17·3
Formic acid, M/700	Malonic acid	7	„	7
„	Hydroxymalonic acid	„	53	7
„	Oxalic acid	„	∞	6·3
α-Hydroxybutyric acid, M/35	Malonic acid	7·7	„	7
„	Hydroxymalonic acid	„	„	10·5
„	Oxalic acid	„	„	12
„	Mandelic acid	„	„	12
Glyceric acid, M/14	Malonic acid	15·5	„	17
„	Hydroxymalonic acid	„	53	13·7
„	Oxalic acid	„	∞	14·3
„	Mandelic acid	„	„	18·3

acid enzyme is less for equivalent concentrations than with the toluene-treated organism. How general this phenomenon is and what the correct interpretation of the action of toluene is, have yet to be determined. But it would appear that toluene can not only eliminate the activations of a number of substrates which are adsorbed at a particular enzyme or centre (and which are still adsorbed after the eliminations have been effected) but can also change the affinity or adsorbing power of the enzyme for substrates.

Considering further the results with the normal organism it is interesting (Table IV) that neither oxalic acid nor hydroxymalonic acid affects perceptibly the activation of glucose. This shows quite conclusively that the reduction of methylene blue by glucose in presence of *B. coli* does not occur through the

intermediate formation of lactic acid. This acid is undoubtedly formed from glucose in the presence of *B. coli* but the reducing power of glucose on methylene blue is independent of the production of lactic acid.

Malonic acid has apparently an inhibitive effect on the activation of glutamic acid.

The effects of toluene.

The fact that not only toluene but many other capillary-active substances (*e.g.* benzene, ether, acetone, chloroform, ethyl and propyl alcohols) bring about similar inactivations leads us to consider the possibility that the inactivations are due to the association of these substances with certain groups, probably non-polar groups, in the active centres. We have been able to show that at the lactic acid enzyme a number of substrates, *e.g.* glycollic, mandelic, tartaric acids, which are hydrogen donators with normal *B. coli* but are inactive after treatment of the organism with toluene, are adsorbed, whether the organism has been treated with toluene (Table I) or not (Table IV). We have also been able to show that the adsorption of a substrate, *e.g.* oxalic acid, at the lactic acid enzyme differs quantitatively in the case of toluene-treated *B. coli* and in that of the normal organism (see Table V). This is explicable on the active centre hypothesis, for the association of certain groups of the lactic acid centre with toluene will not only result in a change of the adsorption coefficients of the centre for the various substrates capable of being adsorbed at it, but also alter the nature and strength of the polarising field, so that substrates previously activated after being adsorbed may no longer be activated although they are still adsorbed.

Table V.

Reduction times of lactic acid with various concentrations of oxalic acid both in the presence of normal "resting" *B. coli* and with toluene-treated *B. coli*. Conditions as in Table II.

Mixture	Normal *B. coli*	Toluene-treated *B. coli*
Lactic acid, *M*/140 (alone)	10·5′	8·5′
„ + oxalic acid, *M*/114688	11·3′	10′
„ + „ *M*/28672	12·5′	13·3′
„ + „ *M*/7168	15′	23·7′
„ + „ *M*/1792	15·5′	48′
„ + „ *M*/448	18·5′	72′
„ + „ *M*/112	19·3′	95′
„ + „ *M*/28	17′	141′

A possible argument for the mode of action of toluene is that this substance not only inactivates a number of enzymes, but also renders the cell more permeable to substrates such as, for instance, oxalic acid. Thus the argument would be that the *entire* lactic acid enzyme is inside the cell which is normally only slightly permeable to oxalic acid and that the effect of toluene is to enter the cell, inactivate a large number of enzymes and render the cell more permeable to oxalic acid, which now, because of its greater concentration within the cell, has a greater effect on the lactic acid activation. We have

already shown in previous publications that permeability considerations will not account for our results, and that indeed [Quastel, 1926] the main site of reduction of methylene blue is at the cell surface. But it is possible finally to dismiss the above argument by the following experiment. Small concentrations of oxalic acid are nearly as effective quantitatively as large concentrations on the rate of reduction of methylene blue by lactic acid with the normal organism (Table VI). Now we would expect, since the velocity of diffusion of a substance into the cell is dependent on its concentration outside, that large quantities of oxalic acid would be much more effective than small concentrations in bringing about a given retardation of the velocity of reduction due to lactic acid. Hence since this is not the case we must conclude that the velocity of diffusion of oxalic acid into the normal cell is comparatively quick and the lactic acid enzyme soon becomes "saturated" with regard to oxalic acid whether the concentration of the latter outside the cell be large or small. On leaving the normal bacteria in contact with oxalic acid (neutralised) at various concentrations for 2 hours at room temperature in the absence of lactic acid and methylene blue, we expect that, at the end of this time, the concentration of oxalic acid inside the cell will have increased, and hence that if the lactic acid enzyme was not quickly "saturated" such saturation, or at any rate a much greater adsorption of oxalic acid at the enzyme, will have been achieved during the 2 hours' interval. Experiment (Table VI) shows that at the end of the 2 hours the various concentrations of oxalic acid show no greater retardations of the velocity of reduction due to lactic acid than in the former case where there had been no special interval allowed for adsorption to occur. Our only conclusion must be that the lactic acid enzyme is quickly fully "saturated" with regard to oxalic acid. The retardation of velocity of reduction effected is of the order of 70 %. Yet when the organism is treated with toluene the retardation effected, under precisely similar conditions, is well over 90 %. Thus permeability considerations do not explain the phenomena and the only satisfactory explanation seems to us at present to be that based on active centres.

Table VI.

Reduction times of lactic acid with various concentrations of oxalic acid in the presence of "resting" *B. coli*. In column *A* the times given were obtained as described in Table II. Column *B* gives results obtained after first allowing the organism to stand in the presence of the neutralised oxalic acid and buffer for 2 hours at room temperature before adding the lactic acid and methylene blue.

	A	*B*
Lactic acid, *M*/350 (alone)	18·5′	15′
,, + oxalic acid, *M*/840	49·6′	42′
,, + ,, *M*/420	61·7′	43′
,, + ,, *M*/210	56·3′	52·5′
,, + ,, *M*/105	55·5′	56·5′

A CONSEQUENCE OF THE ACTIVE CENTRE HYPOTHESIS.

We should be able to demonstrate on the active centre theory that if enzymes drawn from different sources can activate a particular substrate they should all have the power of adsorbing a particular type of compound of which the active substrate in question is an example.

Stephenson [1928] has reported that if a suspension of *B. coli* be allowed to autolyse at an alkaline reaction (in the presence of phosphates as buffer) for several days at 37°, and the suspension be then centrifuged, the clear supernatant liquid contains materials which can activate lactic acid as a hydrogen donator.

The enzymes extracted in this way can bring about only the activation of lactic and α-hydroxybutyric acids and also to a very slight extent of glyceric acid.

Table I gives the effects of admixture of various substances on the velocity of reduction due to lactic acid in presence of the extract, and it will be noted that precisely the same type of substances effects large retardations of the velocity of reduction with the extracted lactic acid enzyme as that which is effective with the toluene-treated organism. Thus in spite of the fact that there is apparently a considerable difference in physical condition of the lactic acid enzyme in the toluene-treated organism from that of the extracted "soluble" lactic acid enzyme (which can be shown, however, to be heterogeneous), the properties of the two enzymes with reference to the specific adsorption of substances of the lactic acid type are the same.

It seems reasonable to suspect that the process of extraction of the enzyme (carried out by autolysis at an alkaline reaction for several days) has effected an alteration of the lactic acid centre of the normal organism in a manner similar perhaps to that effected by toluene, and it is only this altered structure which can be dissociated from the remainder of the organism and be examined in a "soluble" (though heterogeneous) condition. Of course there may be a variety of centres to which the lactic acid type of compound is accessible and these differ in their activating powers. The structure which is extractable is the one which can only effect very few activations. Whether this view is right or not it seems to us worth pointing out that treatment of a cell may affect in some way, perhaps only a minor one, the properties of the enzymes of the cell, so that although an examination of the properties of the enzyme extracted from the cell by certain means must be invaluable in indicating its nature and structure, yet the means by which the enzyme was obtained must be considered in determining the properties of the enzyme in its initial condition in the cell.

So far as we can ascertain from our investigation there seem to exist centres which adsorb specifically substances of the succinic acid type, others which adsorb specifically those of the lactic acid type, those which adsorb specifically those of the glucose-mannitol type and those which adsorb only

formic acid. Yet with the normal or untreated organism many of the centres may contain groupings common to each other which result in "overlapping" [Quastel and Wooldridge, 1927, 1, 2]; the effect of treatment is to render the centres more distinct from each other so that less of this "overlapping" occurs and it is easier to determine what type of structure is accessible to one centre and what to another.

Just as has been shown by Coombs [1927] in the case of the xanthine oxidase, there is a considerable specificity of adsorption by the dehydrogenating enzymes of bacteria for substrates. Out of the number of compounds adsorbed specifically at each enzyme or active centre, only a number can be activated and these are just those whose relative activities can be predicted on the basis of the activation theory.

Summary.

1. The enzyme (or active centre) of *B. coli*, which activates lactic acid as a hydrogen donator has the property of specifically adsorbing compounds characterised by the possession of a particular structure which seems to be —CO—COH*— or —CHOH—COH* where H* is mobile, the compound having acidic properties. The specificity of adsorption is very marked.

2. The enzyme (or active centre) which activates succinic acid has also the property of adsorbing compounds characterised by the possession of a particular structure. This seems to be —C—CH—COOH or —C—CH_2—COOH.

3. The formic acid enzyme is independent of the lower fatty acids (other than formic acid itself) and of all the substances we have so far investigated.

4. The activity of glucose as a hydrogen donator is not perceptibly inhibited by the presence of oxalic acid, or of hydroxymalonic acid. The reduction of methylene blue by glucose in presence of bacteria is independent, therefore, of the intermediate production of lactic acid.

5. The action of toluene on *B. coli* is to bring about an elimination of the activity of the organism to many substances, which can still be shown to be adsorbed at an active centre. Toluene appears also to affect the adsorption coefficient of the active centre towards substrates. The effect of toluene is interpretable on the basis of the active centre hypothesis, but difficult to understand on other views.

6. Using a "soluble" preparation of the lactic acid enzyme, it has been shown that this preparation has precisely the same specific adsorbing power on a particular type of compound, as has the lactic acid enzyme of *B. coli*. The activations of the "soluble" preparation are the same as those of the lactic acid enzyme of *B. coli* after treatment with toluene, and the significance of this is discussed.

These observations support the active centre hypothesis of enzyme action.

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