XXIV. DEHYDROGENATIONS PRODUCED BY RESTING BACTERIA. IV.

A THEORY OF THE MECHANISM OF OXIDATIONS AND REDUCTIONS *IN VIVO*.

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AN attempt will be made, in this communication¹, to interpret in a consistent manner the mechanism of oxidations and reductions *in vivo*. The scheme which will be advanced has formed the basis of the writer's experimental work during the last year and has been developed solely from results with "resting," or non-proliferating, bacteria. No result contradictory to that anticipated by the scheme has, so far, been obtained and what predictions have been made (*e.g.* with formamide, pyrotartaric acid, etc.) have been verified. The scheme, in a certain sense, embraces both Wieland's and Bach's views.

The need for a logical interpretation of the mechanism of oxidation is apparent when the diversity of the material which forms the effective substrate in animal or bacterial oxidations is examined. No very obvious chemical connection appears to exist between those molecules capable of performing the functions of a hydrogen donator in presence of bacteria or muscle tissue (as for instance lactic acid, formic acid, succinic acid) and it seems necessary, in order to comprehend the activity of these molecules, to resort to a hypothesis which claims that for every such molecule there is, related to it, some specific enzyme. The enzyme, on Wieland's views, "activates" the hydrogen of its substrate which then becomes susceptible to oxidation.

Now 103 substances have been examined as possible donators or acceptors of hydrogen (using the methylene blue technique) in the presence of bacteria (*B. coli*), and of these 56 are activated. That is, of the 56 substances some reduce methylene blue, whilst others oxidise leucomethylene blue under con-

¹ The views expressed in this communication are contained in a dissertation which was laid before the authorities of Trinity College, Cambridge, in August, 1924.

ditions (p_H 7.4 and 45°) when they are apparently quite inactive in the absence of the organism. It would follow that we have to imagine that the cell of *B. coli* contains at least 56 specific "hydrogen transportases" (to use Thunberg's terminology), and, *prima facie*, the actual existence, in one cell, of such a large number of specific enzymes dealing with but one type of phenomenon seems very doubtful; moreover, the fact that no success has attended efforts to separate even one—or a group—of these enzymes makes the conclusion all the more improbable.

Granting however, for the time being, that such enzymes may exist, the more important problem of the mechanism of activation by the enzyme awaits consideration. Wieland's theory gives us no information on the actual mechanism of activation and it is, it seems to me, essentially ignorance of this mechanism, rather than the postulation of numerous specific enzymes, which prevents our anticipating, with any measure of certainty, the susceptibility to oxidation of any given molecule. Considerable experience of oxidations in the animal body enables us to state whether a certain molecule is likely to be oxidised *in vivo*, and, with Knoop's β -oxidation rule, to foretell how the molecule may suffer oxidation. But more than this, with no picture of the chemical mechanism of the event, it is impossible to say.

The problem of determining the mechanism of oxidation resolves itself into three separate problems: (1) determination of the site of activation of a substrate molecule; (2) determination of the mechanism of activation of this molecule; (3) determination of the process of oxidation after activation has occurred. These will be examined but it will be useful first to consider one or two difficulties in connection with the specific enzyme hypothesis.

From our knowledge of the behaviour of succinic acid *in vitro* it appears most unlikely that succinic acid (normal or unactivated) will donate hydrogen to a protoplasmic constituent and that the latter will then reduce methylene blue, that is to say, that a protoplasmic constituent will act as a "hydrogen carrier" between succinic acid (unactivated) and methylene blue. If this were the case, it would be most difficult to explain the relative inertness of malonic or glutaric acids or of substances which are far more prone to oxidation *in vitro* than succinic acid. Succinic acid (or its hydrogen atoms) must be activated prior to reaction, and it is usually assumed that the activation is due to the formation of some unstable complex molecule with the enzyme, this unstable molecule having the effect of rendering the hydrogen atoms of its succinic acid moiety labile. What happens, then, when fumaric acid is activated to become a hydrogen acceptor? (It oxidises leucomethylene blue at p_H 7.4 and 45° in presence of *B. coli* and not in its absence.) If vaguely we comprehend that the lability of hydrogen in succinic acid may be due to combination with an enzyme, it is with much less clearness that we can conceive of a change in fumaric acid, due to combination, which will result in the latter's oxidising power.

Again it is difficult to imagine that there exists a specific enzyme related

to chlorate, especially as this substance, after reduction in presence of *B. coli*, gives rise to chlorite which is extremely toxic to the organism.

Yet if the specific enzyme hypothesis be incorrect or unsatisfactory, what is the explanation of the high reducing power *in vivo* of succinic acid compared with that of glutaric acid, of alanine compared with glycine, of glutamic acid compared with aspartic acid, of acrylic acid with propionic acid, of lactic acid with glycollic acid and so on?

THE SITE OF ACTIVATIONS DUE TO BACTERIA.

The site of reduction of the dyestuff (*e.g.* methylene blue) cannot be outside the organism (*i.e.* in the medium), for if this were so the enzyme responsible for reduction would be extracellular and all observations show that such enzymes are not formed. For instance the filtrates of media on which the organism has been growing, and the solutions obtained after the washing of bacteria with slightly alkaline buffer solution, hypertonic or normal saline solutions, distilled water, etc. give no indication of the presence of a "hydrogen transportase."

If the site of reduction be within the bacterial cell then the cell membrane must be permeable to both the reactants, methylene blue (M.B.) and the hydrogen donator (D.).

Assuming the cell membrane to be composed entirely of lipid material, then from Loewe's work [1912] we know that M.B. will be adsorbed and not dissolved, *i.e.* that M.B. will not proceed through the membrane. On the other hand if the membrane be not entirely of lipid nature diffusibility of the M.B. is possible. We will assume that diffusion of the dyestuff through the membrane occurs. Then the rate of reduction of M.B. will depend on the slower of the two processes, (1) the diffusion of M.B. into the cell, (2) the reaction of M.B. with D. inside the cell.

If the rate of diffusion be slower than the chemical reaction, then the temperature coefficient of the reduction will be that of the diffusion process. Experiment shows that the coefficient is too high; it is 2.1 between 30° and 60° for succinic acid and methylene blue in presence of *B. coli* [Quastel and Whetham, 1924]. It follows that the chemical process must be slower than the rate of diffusion of M.B. into the cell.

Again the rate of diffusion of M.B. will be proportional to the difference between the concentrations of M.B. in the cell and of M.B. in solution. Hence if the reduction of M.B. in the cell be so fast that only a minute quantity of M.B. be present in the cell at any moment then the velocity of its reduction will be approximately proportional to its concentration outside the cell. Experiment shows that the rate of reduction is for the most part linear (see Fig. 1), *i.e.* it is independent of the concentration of M.B. Hence the rate of reduction cannot be governed by the rate of diffusion of M.B. into the cell (even at the low concentration of 1 in 100,000) and must therefore be determined by the slower chemical process.

From this it is possible to estimate the lower limit of the rate of diffusion of M.B. into the cell, under the conditions normally employed. We will take the following figures of a single experiment: in presence of 1 cc. *B. coli* suspension containing approximately 5×10^6 cells, 1 cc. of 1/5000 sol. of M.B. was reduced in 10 minutes at p_H 7.4 and 45° the total volume being 6 cc. and 1 cc. *M/20* formic acid (as neutral salt) being the hydrogen donator.

Fig. 1. Rate of reduction of methylene blue by succinic acid *M/140* in presence of *B. coli*. ($p_H = 7.2$.)

The concentration of M.B. is 1 g. in 30,000 or 1 g. mol. in 9,600,000 cc. (since the molecular weight of methylene blue is approximately 320), *i.e.* $\frac{60.6 \times 10^{22}}{9.6 \times 10^6}$ molecules per cc. (60.6×10^{22} being the Avogadro constant) which is 6.3×10^{16} . Then the number of M.B. molecules reduced in 1 min. per unit cell is approximately $\frac{6 \times 6.3 \times 10^{16}}{5 \times 10^6 \times 10} = 7.5 \times 10^9$. If the reduction therefore occurs within the cell the minimum rate of diffusion of M.B. into the cell will be 7.5×10^9 molecules per min.

Taking a cell of *B. coli* as a cylinder 3μ long and diameter 0.5μ , its volume is $\frac{\pi 3\mu^3}{16} = 0.6\mu^3 = 0.6 \times 10^{-12}$ cc. The total number of M.B. molecules within the cell when the concentration of dyestuff is equal on both sides of the membrane is $0.6 \times 10^{-12} \times 6.3 \times 10^{16} = 3.8 \times 10^4$.

Hence the maximum time taken for M.B. to diffuse into a *B. coli* cell to reach a concentration within the cell equal to that outside is $\frac{3.8 \times 10^4}{7.5 \times 10^9}$ mins. which is approximately $\frac{3}{10,000}$ of a second.

In calculating this figure no account has been taken of the fact that M.B. molecules can pass out of the cell as well as pass in. Correction for this would make the maximum time of diffusion, *i.e.* $\frac{3}{10,000}$ of a second, smaller.

This means, if we accept the view that the whole of the reduction occurs within the cell, that immersion of a suspension of *B. coli* (freshly grown) into a dilute solution of methylene blue (1/30,000) would be followed by an extremely rapid penetration of the dyestuff into the cell. When it is considered that in vital staining dilute solutions of methylene blue are ineffective and strong concentrations must be used [see v. Möllendorf, 1921] and that no penetration of methylene blue from dilute solution into living yeast cells has been observed, although these cells can effect reduction in presence of donators, it appears to be most unlikely that such rapid diffusion of methylene blue into *B. coli* actually takes place.

It is more in harmony with the facts to conclude that the site of reduction of M.B. is not wholly within the cell and is therefore at the outer cell surface.

The question now arises as to the site of activation of the substrate. Assuming for the purposes of argument that the cell is permeable to the substrate it is clear that there exist the following three possibilities: (1) that activation occurs at some structure (or surface) within the cell; (2) that activation is due to combination with some protoplasmic constituent (or enzyme); (3) that activation occurs at the cell surface. Now it is unlikely that the reduction of M.B. occurs within the cell. Hence even if activation of the substrate does occur at some intracellular structure the fact that the molecule will become "normal" out of contact with the structure makes it improbable that such an activation would account for reduction of the dye at the cell surface. If, on the other hand, the activation were due to the formation of an unstable complex molecule (enzyme and substrate) then, since reduction does not occur at the cell's inner surface, the cell membrane must be permeable to the complex molecule and reduction must be effected at the outer surface. But if such permeability were possible we should expect to find the "reducing enzyme" in a broth medium in which bacteria have grown and from which they have been removed. Attempts to find such a soluble reducing enzyme have entirely failed. Again the sterile filtrates of bacteria which have been disintegrated can effect no reduction of methylene blue in presence of such active donators as succinic and formic acids¹.

It is possible to conceive of a reduction occurring through the membrane, the M.B. forming a layer on the outer side of the membrane, the complex molecules (substrate and enzyme) forming a layer on the inner side, and reaction occurring as in the oxidation, through an artificial membrane, of fumaric acid by ferric hydroxide [Girard, 1925]. Now it may be shown (see

¹ I owe much to the kindness of Dr D. Thomson of the Pickett-Thomson Laboratory who has disintegrated suspensions of bacteria for me in his special apparatus. The p_H of *B. coli* during disintegration was not allowed to exceed 8.1.

Table I) that the velocity of reduction of M.B. due to a mixture of two donators (say succinic and lactic acids) is, within the limits of experimental error, the sum of the velocities due to each donator. Thus if we take a concentration of a donator so large that an increase in concentration appears to have little or no effect on the velocity of reduction, the addition of another donator makes

Table I.

Full details of the experimental technique are to be found in previous papers [Quastel and Whetham, 1925, 1, 2]. The following point in technique is of use: when an organism is itself highly reducing, contact for half an hour at room temperature with 2 % saline with subsequent centrifuging and suspension in normal saline is beneficial. The activating powers of the organism are apparently not impaired by this treatment.

Each vacuum tube contained 2 cc. phosphate buffer, 1 cc. 1/5000 M.B., organism and donator, the total volume being made up to 7 cc. with water. Reductions were carried out *in vacuo* at 45°. Calculated times of reduction for mixtures of donators were obtained by considering rate of reduction as linear (Fig. 1); no correction has been made for divergence from linearity towards the end of reduction. *Coli* 1, *coli* 2, etc. are different suspensions and strains of *B. coli*; results with *coli* 1 can be compared strictly quantitatively with one another but only qualitatively with results with *coli* 2, etc. All substances under investigation were brought to p_H 7.2 by sodium hydroxide or hydrochloric acid. Controls with all organisms (absence of donators) did not reduce within two hours. The following are typical results.

Organism	p_H	Donators	Reduction time	
			(observed)	(calc.)
0.5 cc. <i>Coli</i> 1	7.2	Galactose <i>M</i> /280	24' 30"	
" "	"	Acrylic acid <i>M</i> /70	11 25	
" "	"	Galactose <i>M</i> /280 + acrylic acid <i>M</i> /70. ...	8 30	7' 50"
1 cc. "	"	Mannitol <i>M</i> /35	11 50	
" "	"	" <i>M</i> /140	14 30	
" "	"	Allyl alcohol <i>M</i> /46.6	19 5	
" "	"	" <i>M</i> /35	19	
" "	"	Mannitol <i>M</i> /140 + allyl alcohol <i>M</i> /46.6 ...	8 50	8 15
" "	"	Succinic acid <i>M</i> /47	14 30	
" "	"	Mannitol <i>M</i> /140 + succinic acid <i>M</i> /47 ...	7 45	7 15
0.5 cc. <i>Coli</i> 2	7.4	Alanine <i>M</i> /13	52	
" "	"	" <i>M</i> /6.5	45 20	
" "	"	Succinic acid <i>M</i> /130	17 35	
" "	"	Alanine <i>M</i> /13 + succinic acid <i>M</i> /130 ...	11 20	13 5
0.5 cc. <i>Coli</i> 3	7.4	Succinic acid <i>M</i> /130	10 10	
" "	"	Oxalic acid <i>M</i> /6.5	No reduction	
" "	"	Succinic acid <i>M</i> /130 + oxalic acid <i>M</i> /6.5 ...	11	10 10
0.2 cc. <i>Coli</i> 4	7.4	Succinic acid <i>M</i> /120	20	
" "	"	Glycerol <i>M</i> /5.5	26 30	
" "	"	Succinic acid <i>M</i> /120 + glycerol <i>M</i> /5.5 ...	14	11 30
" "	"	Allyl alcohol <i>M</i> /12	3 30	
" "	"	Succinic acid <i>M</i> /120 + allyl alcohol <i>M</i> /12 ...	2 55	3
0.5 cc. <i>Coli</i> 5	"	Succinic acid <i>M</i> /140	19	
" "	"	Lactic acid <i>M</i> /140	7 10	
" "	"	Formamide <i>M</i> /1050	4 20	
" "	"	Succinic acid <i>M</i> /140 + lactic acid <i>M</i> /140 ...	6 20	5 15
" "	"	Succinic acid <i>M</i> /140 + formamide <i>M</i> /1050 ...	3 25	3 30

a definite increase in velocity of reduction. In a word, each donator appears to act as though the other were absent¹. If then we are to imagine the reducing effect of, say, a high concentration of succinic acid to be due to the formation of a layer of the succinic acid-complex molecules at the inner

¹ Whether this is strictly true for all donators or at all concentrations of the donators cannot, at present, be definitely stated. Clearly it cannot be strictly true in the case of two donators where one is powerfully adsorbed by the cell and the other not.

surface of the membrane it is difficult to see how, say, lactic acid-complex molecules effect their reduction independently of the layer of succinic acid molecules. Again, this view entails so many questionable assumptions (*e.g.* that the cell is permeable to every substance which is activated, that the velocity of diffusion of the substrate into the cell is greater than the chemical process of reduction within the cell, that there exist numerous intracellular specific enzymes) and appears to be so inadequate to explain such well-known facts of biological oxidation as asymmetric oxidation of the double bond, and β -oxidation, that it would be pointless at present to go any further with it.

Summing up, it seems justifiable to assume that not only the site of reduction of M.B. but also the site¹ of activation of the substrate molecule is at the cell surface.

SOME CONSIDERATIONS OF CELL SURFACES.

In any growing organism we may assume that there exists in each cell a definite and ordered series of reactions which is characteristic of the organism. If this were not the case there could be none of the fixity or specific nature of organisms which is their characteristic feature. The well-known facts with regard to chromosomes, for instance, clearly indicate the ordered series of events within the cell.

The constitution of the cell membrane will be as characteristic a feature of the organism as any structure within the cell [see also Leathes, 1925]. It is sure, too, from the work of Hardy, Langmuir, Harkins and Adam on the orientation of molecules at an interface, that the membranes will have a definite molecular structure, the molecules composing the membrane being orientated with regard to one another in a particular way dependent upon their chemical nature and upon the character of the phases on either side of the membrane. Now since the chemical constitution of the membrane is, probably, very complex and to some extent fluctuating during growth it follows that its molecular structure cannot be expected to evince a homogeneity or symmetry. We should expect, rather, that certain parts of the surface would be occupied by certain molecules or groups of molecules and other parts by other molecules and so on, so that if it were possible to make instantaneous observations at discrete points of the surface it would be found that there existed an irregular distribution of groups, and that the surface, in fact, exhibited a "geography." Doubtless this "geography" or structure will change its form as the cell grows and divides, but so long as the membrane remains intact and suffers no profound chemical change this structure will remain, as a whole, the same and a characteristic feature of the cell.

Associated with certain molecules or groups of molecules orientated in

¹ Or perhaps, better, that a site of activation is at the cell surface; for there is no evidence against an activation occurring at an intracellular surface or structure. Such an activation would, however, as pointed out, be ineffective in the case of reduction of M.B. Disintegration of bacteria besides breaking up the cell surface will also destroy intracellular surfaces.

the membrane, there will exist electric fields whose nature will be dependent on the nature of the groups which give rise to them. Some of these fields will be very powerful, due either to certain active radicles or groups or to the juxtaposition or association of certain molecules or groups; others, on the other hand, will, for the same reasons, be very weak. Hence we may imagine scattered over the cell surface many locally intense electric fields, these being a function of the "geography" of this surface. The fields will be modified by any chemical or physical changes which affect the structure of the membrane. The point to which I desire to give most emphasis is the local nature of these electric fields of varying intensity; at certain parts of the surface there may be most powerful fields, although the average field taken over the whole surface may be very weak¹.

Since the membrane structure will be expected to vary somewhat with functional activity we may expect some alteration of the electrical condition of the surface and hence of the *total* surface charge. Hardy and Harvey [1911] from experiments with *Gonium*, *Vorticella* and *Amoeba* incline to the view that surface charge does vary with variations in the state of activity of the living cell. But even if the total surface charge alters, we may still expect the presence of local fields such as have been pictured above.

It is from a consideration of the effects induced in a molecule by the application of an external electric field that I would suggest that an interpretation of the mechanism of activation is forthcoming.

If we are to ascribe the activating powers of an organism to the presence of local intense electric fields, it would follow that, since the surface structure of an organism will fluctuate to some extent during growth, there will exist differences in activating power between one cell and another. Since, however, in experiments with *B. coli* and M.B., some millions of cells are used for each reduction, it is clear that the average activating power of the organism is observed. This average activating power will be the same for any strain of the same organism which has been grown and "handled" in the same way; it will vary to some extent with different strains and it would not be expected that the average activating power of one organism would be identical with that of a totally different organism.

¹ It is important to realise the difference between a surface structure such as has been pictured and, let us say, that of a protein such as caseinogen or albumin. In the former the molecules—or conglomerates of molecules—are in a state of very great complexity due to the juxtaposition of substances which are continually being produced by an actively growing cell. Certain associations of such substances will result in the production of unstable structures—structures of high potential energy—which only exist while the conditions, which make such associations possible, obtain. Such structures as these, I imagine, are the sources of the most powerful electric fields. Molecules such as albumin or caseinogen are relatively stable units compared with these structures, and in a surface composed entirely of albumin or caseinogen a distribution of intensely active centres, which is characteristic of cellular (and intracellular) surfaces, does not exist.

THE MECHANISM OF ACTIVATION.

Let us consider the effect of an external electric field upon the unsaturated linkage. In Sir J. J. Thomson's words [1923, 1] we will "suppose that there is a double bond between two carbon atoms $C_2=C_1$, the octets of electrons round C_1 and C_2 having four electrons in common situated between C_1 and C_2 . If an external electric field acts on the molecule, tending to make electrons move from left to right, some of the electrons held in common may be so far displaced from C_2 and towards C_1 that they can no longer be regarded as shared with C_2 . If two of these are displaced far enough for this to happen, the octet round C_1 will be intact and C_1 will be saturated, whilst the octet round C_2 will be reduced to a sextet, so that C_2 will be unsaturated and chemically active: there are still two electrons left between C_1 and C_2 to form a single bond binding C_1 and C_2 together." The effect of the field, then, is to produce a shift of the electrons from their normal paths to new orbits, and of course, when out of contact with the field, the orbits will revert to normal. The same force, however, which produces a shift in the electrons will also alter the relative positions of the protons (hydrogen ions). Hence, interpreting in chemical terms the effect of an external or polarising field upon the double bond $-C_1H=C_2H-$, there will be a shift of a proton from C_1 towards C_2 or *vice versa*, the extent of the shift depending upon the nature and strength of the external field. The greater the shift the more unsaturated and chemically active will one end of the bond become. We shall identify a molecule in such a polarised condition as one in an activated state, the polarising field being the activating source.

The following equations represent the change after maximum activation:

Now the actual shift of the proton will be determined, not only by the external electric field but by the electrical condition of the molecule itself, *i.e.* by the nature of the groups composing the molecule. Thomson [1923, 2] has shown that the "electrical effect of replacing an atom of hydrogen by another atom or radicle may be represented by the introduction of an electric doublet at the hydrogen atom." If the radicle forms a system requiring an electron to complete its octet the positive end of the doublet will be towards the molecule with which the radicle is combined. Thus if such a radicle replaces a hydrogen combined to a carbon atom the positive end of the doublet will be towards the carbon and the electrical effect at the carbon atom will be to drive away positive electricity and attract negative. The reverse effect holds with a radicle in which there is one electron over after providing for

complete octets. Table II summarises the electrical effects of various radicles at the C atom situated in the α -position to the radicle.

Table II.

Radicle	No. of electrons	Electrons necessary to complete octet	Effect at the α -C atom	Polarity
$-\text{CH}_3$	7	+1	Positive electricity repelled	Non-polar
$-\text{OH}$	7	+1	"	Polar
$-\text{NH}_2$	7	+1	"	"
$-\text{CH}_2\text{COOH}$	23	+1	"	"
$-\text{COOH}$	17	-1	Positive electricity attracted	"
$-\text{CONH}_2$	17	-1	"	"
$-\text{COCH}_3$	17	-1	"	"

The aldehyde group [Thomson, 1923, 2] and the group $-\text{CO}\cdot\text{COOH}$ act in the same sense as the carboxyl.

Let us now consider the effect of activating the structure $\text{A}\cdot\text{CH}=\text{CH}\cdot\text{X}$ where A is assumed to have no directive influence on the movement of the proton. Assuming maximum activation to have occurred, three typical results are shown in Table III.

Table III.

X	Normal form of molecule	Activated form of molecule
$-\text{COOH}$	$\text{A}-\text{CH}=\text{CH}-\text{COOH}$	$\text{A}-\check{\text{C}}-\text{CH}_2\cdot\text{COOH}$
$-\text{CH}_3$	$\text{A}-\text{CH}=\text{CH}-\text{CH}_3$	$\text{A}-\text{CH}_2-\check{\text{C}}-\text{CH}_3$
$-\text{CH}_2\text{COOH}$	$\text{A}-\text{CH}=\text{CH}-\text{CH}_2\text{COOH}$	$\text{A}-\text{CH}_2-\check{\text{C}}-\text{CH}_2\text{COOH}$

The effect of a carboxyl group at X is to produce an electric field at the α -carbon atom which attracts positive electricity. The same effect will to some extent affect the β -carbon atom also and possibly render this more liable to attract positive electricity than it was before the introduction of the carboxyl group. The field, however, is far stronger at the α -C atom and hence the shift of the proton will be from the β -C to the α . This shift will render the β -C chemically unsaturated, whilst the α -C becomes relatively saturated. Hence when oxidation occurs *after* activation, a symmetrical oxidation (or addition of negative electricity at the α - and β -C atoms) will not take place; only a β -oxidation is possible. On the other hand if a $-\text{CH}_3$ group is present at X the movement of the proton ($\text{H}^\bullet$) after activation will be from the C in the α -position to that in the β , and the α -C will now attract negative electricity. (The repelling effect of the CH_3 group on hydrogen at the adjacent C is seen in acetoacetic ester, which exists normally to the extent of 98 % in the keto form [Knorr, 1911].)

Consider, now, the cases of fumaric acid and crotonic acid. The normal acids $\text{COOH}\cdot\text{CH}=\text{CH}\cdot\text{COOH}$ and $\text{CH}_3\cdot\text{CH}=\text{CH}\cdot\text{COOH}$ will, after activation, become $\text{COOH}\cdot\check{\text{C}}\cdot\text{CH}_2\cdot\text{COOH}$ and $\text{CH}_3\cdot\check{\text{C}}\cdot\text{CH}_2\cdot\text{COOH}$ respectively. In the case of activated fumaric acid there is a field acting at the unsaturated

carbon which attracts positive electricity (H^+) and is due to the adjoining carboxyl group. Hence activated fumaric acid will tend to attract hydrogen. In the case of crotonic acid the field acting at the unsaturated C due to $-CH_3$ will result in a strong attractive effect for negative electricity. Hence we should not expect either of the crotonic acids to be such powerful hydrogen acceptors as fumaric acid, and they should be easily oxidised, after activation, to acetoacetic acid. Experiment shows this to be true (see Table IV), *isocrotonic* acid acting as a fair hydrogen donator in contrast to fumaric acid. The ease with which the crotonic acids are oxidised to acetoacetic acid is shown by the following experiment. In tubes are placed 2 cc. phosphate buffer p_H 7.4, 2 cc. $M/2$ crotonic or *isocrotonic* acid (as the neutral sodium salt) and 3 cc. of a fairly thick suspension of *B. coli*. The tubes are allowed to stand 24 hours at 37° after which they are tested for the presence of acetoacetic acid by the usual nitroprusside reaction. A very good positive test results in the cases of both acids. Better yields of acetoacetic acid are obtained by adding to the mixture of buffer, crotonic acid and *B. coli*, 2 cc. $M/20$ sodium nitrate solution. In the presence of nitrate, an intense acetoacetic acid test may be obtained, even in the absence of oxygen.

METHYLENE BLUE AS A HYDROGEN ACCEPTOR.

The value of methylene blue as a hydrogen acceptor lies not so much in the fact that it is "easily" reduced or that it gives a colourless leuco-compound, but that it does not require activation by an enzyme to exert its oxidising power. It is typical of the substances which give oxidation-reduction potentials and may be regarded as "active" *in vitro*. Thus we can use methylene blue as a standard for comparing the activities of various molecules in presence of an activating source (*e.g.* *B. coli*). But if a substance in presence of *B. coli* does not perceptibly reduce methylene blue or oxidise its leuco-compound, it does not necessarily follow that the substance has not been activated. We may say that it is inert compared with another substance but not necessarily as inert as it is in absence of the activating source. It is, moreover, clear that a substance apparently inert towards methylene blue may reduce some other hydrogen acceptor. It should be possible, therefore, with a series of substances of known oxidation-reduction potentials to estimate the activation of a substance by an organism by determining at what potential hydrogen donation is just observable. A reliable potentiometric method would, of course, accomplish the same end.

In the activated structure $-\overset{\vee}{C}-CH_2-COOH$, it is necessary to compare the relative affinities of this structure and of, say, methylene blue for hydrogen. The latter will go to the molecule in which the affinity is stronger and if this be methylene blue, reduction of the dyestuff and oxidation of the structure will occur. Thus

If the affinity be stronger with the activated structure, this will act as an oxidising agent towards leuco-methylene blue. Thus

With "active" oxygen as an acceptor, it is possible that the affinity of this for hydrogen is greater than that of the activated structure; so that finally we may have a structure which is oxidised by "active" oxygen and yet itself acts as an oxidising agent towards leuco-methylene blue. Hence the dual nature of the activated double bond. Fumaric acid is a good instance [cf. Quastel and Whetham, 1924; Quastel, Stephenson and Whetham, 1925].

THE ACTIVATION OF THE SATURATED BOND.

Consider the structure $-\text{CH}_2-\text{CH}_2-\text{COOH}$. It is clear that in this case somewhat different considerations must be taken into account from those in the case of the corresponding unsaturated structure. It is necessary, with both structures, to consider in more detail the condition of the carboxyl group. We may assume that we have to deal normally with the ionised carboxyl (in feeding experiments or experiments with bacteria an acid is given as the neutral salt). The charged carboxyl group has an effect on the hydrogen attached to the α -C and it is reasonable to suppose that such a hydrogen will be rendered mobile and will oscillate between the α -C and one of the oxygen atoms in the carboxyl group. Thus the normal forms of the structure $-\text{CH}_2\cdot\text{COOH}$ will be represented by the equilibria:

Now the movement of the α -H (proton) is much more likely to occur in a saturated structure (I) than in the corresponding unsaturated structure $=\text{CH}-\text{COOH}$, for in the latter the attractive effect of the α -C for positive electricity will oppose the attractive effect of the oxygen in the carboxyl. If, however, there be attached to the α -C atom a grouping which has the effect of repelling positive electricity at this atom, a shift or movement of the hydrogen will take place. In a saturated structure (I) movement of an α -H occurs since the demand of the α -C for positive electricity is, at any rate partially, satisfied by the presence of the second hydrogen, which remains more or less fixed.

Now the complete picture of activation of $-\text{CH}_2-\text{CH}_2\text{COOH}$ is represented by the following equilibria:

On activating III a proton will move in the direction of the atom where the attraction for positive electricity is strongest, *i.e.* from the β - to the α -C; IV will undergo the same process as I (*i.e.* $I \rightleftharpoons II \rightleftharpoons III$) and the final activated form will be V. But V, as we have already seen, is the activated form of VI, so that if there be present in the original system an activating source, the structure I and a hydrogen acceptor whose affinity for hydrogen is greater than that of V, reaction will occur to produce VI and the reduced form of the hydrogen acceptor. This scheme $I \rightarrow VI + 2H$ represents, in the clearest way, Wieland's theory of activation of hydrogen.

FURTHER CONSIDERATIONS ON THE INFLUENCE OF RADICLES.

It is clear that not only the electrical effects due to radicles, but the effects of the polarities of radicles must be taken into account—polarity being used, here, in the sense employed by Hardy and Langmuir. Examples of polar groups, *i.e.* groups polar to or “dissolved” by water are the carboxyl, hydroxyl and amino groups. Examples of non-polar groups, *i.e.* groups directed to non-aqueous solvents, are methyl, or ethyl groups, or fatty chains generally.

If the cell membrane be considered as a non-aqueous phase it follows that the presence of non-polar groups in a substrate molecule will tend to produce an adsorption of the molecule at the cell interface. A blocking—or partial blocking—of the membrane will ensue, the extent of which will depend upon the nature of the non-polar and polar moieties of the molecule. This will result in retardation of the velocity of a process which depends upon the activation, by the cell, of one or both of the reacting molecules. Thus methyl, ethyl and propyl alcohols retard and inhibit reductions dependent on the presence of *B. coli*, the inhibition being greatest, for equivalent concentrations, with propyl alcohol and least with methyl alcohol, and total inhibition of the reducing effect of different donators by a given alcohol requiring approximately, as we should expect, the same concentration of the alcohol [Quastel and Whetham, 1925, 1]¹.

We should expect a hydrogen donator possessing non-polar groups to inhibit its own reductions—the effect being observed as the concentration of the donator is changed. This is true in the cases of acetaldehyde (Table IV), and of ethyl and propyl alcohols and of *isobutyric*, *isovaleric* and *caproic* acids [Quastel and Whetham, 1925, 1].

The effect, then, of a non-polar group, whatever its electrical effect, is to produce an apparent diminution in the activity of the molecule of which it is a constituent.

The double bond is interesting in this connection. Langmuir [1917] has shown that it is polar in character. With this is consistent the fact that allyl

¹ An error was made in the curve Fig. 6 [Quastel and Whetham, 1925, 1]. The reduction times for 0.25 *Coli* should read 8', for 0.5 *Coli* 4' 15" and 1.0 *Coli* 2' 15", instead of 8' for the three amounts of organism.

alcohol, which is a good donator, does not apparently retard either its own reduction or those of other donators (Table IV).

Again phenol, with three double bonds, has but a slight inhibitory effect on reductions due to succinate, whereas the corresponding saturated compound, cyclohexanol, has a most powerful inhibitory effect. Toluene, in which the polar OH of phenol has been replaced by the non-polar CH₃, inhibits more vigorously than phenol [Quastel and Whetham, 1925, 1]. Generally speaking, were it desired to find a vigorous inhibitor of reactions dependent on the activity of, or taking place at the surface of, a cell, I should seek a molecule possessing powerful non-polar groups, and yet sufficient polarity to make it possible for it to go into aqueous solution. Such an inhibitor would, also, act as an antiseptic.

The polar effects of various radicles are summed up in Table II (p. 175). It is necessary also to bear in mind (1) the basic nature of the amino-group and the acidic nature of the carboxyl; (2) the ability of such radicles as CH₃, OH, NH₂, to part with hydrogen and hence to change their polar and electrical characters.

Let us, now, apply these considerations to substances for which experimental details have been obtained.

Table IV.

Conditions as in Table I.

The following are typical of a large number of results:

Organism	p _H	Donator	Reduction time
5 cc. <i>coli</i> 6	7.4	Succinic acid <i>M</i> /140	27'
" "	"	Thiodiglycollic acid <i>M</i> /52.6	Not reduced in 2 hrs.
" "	"	" <i>M</i> /26.3	"
1 cc. <i>coli</i> 7	7.2	Acrylic acid <i>M</i> /140	"
" "	"	" <i>M</i> /80	8"
" "	"	" <i>M</i> /70	5
" "	"	" <i>M</i> /35	4' 20"
1 cc. <i>coli</i> 8	7.2	Allyl alcohol <i>M</i> /140	37 45
" "	"	" <i>M</i> /70	26 30
" "	"	" <i>M</i> /35	19
1 cc. <i>coli</i> 9	7.2	β-Hydroxybutyric acid <i>M</i> /140	Not fully reduced in 2 hrs., appears to reach an equilibrium after 3' which remains stationary for 1 hr.
" "	"	" <i>M</i> /112	Indication of slight reversal after 4', 75 % of the methylene blue having been reduced. Complete reduction in 87'.
" "	"	" <i>M</i> /93	Complete reduction in 5', followed by slight reversal after 21'.
1 cc. <i>coli</i> 10	7.4	Succinic acid <i>M</i> /140	11' 30"
" "	"	Pyrotartaric acid <i>M</i> /70	77
" "	"	" <i>M</i> /14	71
" "	"	Tartaric acid <i>M</i> /14	65
" "	"	" <i>M</i> /70	Not reduced in 2 hrs.
" "	"	Tricarballic acid <i>M</i> /105	65'
" "	"	" <i>M</i> /42	64
" "	"	" <i>M</i> /21	62
" "	"	Pyromucic acid <i>M</i> /70	23
" "	"	" <i>M</i> /28	21' 30"
" "	"	" <i>M</i> /14	18 30
" "	"	Oxalic acid <i>M</i> /14	Not reduced in 2 hrs.
1 cc. <i>coli</i> 11	7.4	Succinic acid <i>M</i> /140	7'

Table IV (continued).

Organism	pH	Donator	Reduction time
1 cc. <i>coli</i> 11	7.4	<i>n</i> -Crotonic acid <i>M</i> /14	Not reduced in 2 hrs.
" "	"	" <i>M</i> /7	"
" "	"	<i>iso</i> Crotonic acid <i>M</i> /28	"
" "	"	" <i>M</i> /14	22'
" "	"	" <i>M</i> /7	14
" "	"	Succinic acid <i>M</i> /140 + <i>n</i> -Crotonic acid <i>M</i> /14	7' 30"
" "	"	Allylamine <i>M</i> /14	Partial reduction only in 2 hrs.
" "	"	" <i>M</i> /7	"
1 cc. <i>coli</i> 12	7.4	Formamide <i>M</i> /5650	58' 10"
" "	"	" <i>M</i> /2825	56 15
" "	"	" <i>M</i> /1883	33
" "	"	" <i>M</i> /1412	11 30
" "	"	" <i>M</i> /1130	5 10
" "	"	" <i>M</i> /565	4 10
" "	"	Laevulinic acid <i>M</i> /35	34
" "	"	" <i>M</i> /70	36
" "	"	Succinic acid <i>M</i> /140	12 30
" "	"	Acetic acid <i>M</i> /14	25
" "	"	Acetamide <i>M</i> /10.5	No reduction in 2 hrs.
" "	"	Succinimide <i>M</i> /140	90'
" "	"	" <i>M</i> /35	60
" "	"	Methylamine <i>M</i> /22	Not reduced in 2 hrs.
" "	"	Ethylamine <i>M</i> /28	"
" "	"	Acetaldehyde <i>M</i> /105	48' 30"
" "	"	" <i>M</i> /42	60 20
" "	"	" <i>M</i> /21	72 20
" "	"	" <i>M</i> /10.5	Not reduced in 2 hrs.
2 cc. <i>coli</i> 13	7.4	None (control)	22' (a highly reducing organism was chosen)
" "	"	Glutaconic acid <i>M</i> /70	17'
" "	"	" <i>M</i> /14	15
" "	"	Mesaconic acid <i>M</i> /21	17' 30"
" "	"	Citraconic acid <i>M</i> /14	18
" "	"	<i>iso</i> Crotonic acid <i>M</i> /14	8
" "	"	Fumaric acid <i>M</i> /70	Not completely reduced in 2 hrs.
1 cc. <i>coli</i> 14	7.4	0.1 cc. acetyl acetone	"
" "	"	0.5 cc. "	"
" "	"	1.0 cc. "	"
" "	"	Succinic acid <i>M</i> /140	25"
" "	"	Glutaconic acid <i>M</i> /28	Partial reduction in 2 hrs.
" "	"	Mesaconic acid <i>M</i> /28	Not reduced in 2 hrs.
" "	"	Citraconic acid <i>M</i> /28	"
0.5 cc. <i>coli</i> 15	7.4	Laevulinic acid <i>M</i> /14	45'
" "	"	Acetyl acetone <i>M</i> /4	Partial reduction in 2 hrs.
" "	"	Sodium chloride <i>M</i> /14 (salt control)	No reduction in 2 hrs.

THE ACTIVATION OF UNSATURATED ACIDS.

Acrylic acid¹ is a good hydrogen donator in presence of *B. coli* (Table IV). Both *n*-crotonic (m.p. 72°) and *isocrotonic* acids are activated by the organism, the latter acting as a fair hydrogen donator (Table IV).

The question of the effect of *cis-trans*-isomerism now arises. It must be immediately clear that the electrical conditions within a *cis*-molecule cannot be identical with those of the *trans*-isomer. Hence the effects of the same activating source on the two molecules will differ; the difference in activation depending upon the extent to which the various groups in the molecules

¹ The reduction experiments with allyl alcohol, acrylic acid, and β -hydroxybutyric acid, mentioned in this paper, were performed in collaboration with Miss M. D. Whetham.

enhance or diminish each other's electrical effects according to their relative positions. Thus the stereo-structure of a molecule plays a most important part in determining its activity in presence of an activating source.

n-Crotonic and *isocrotonic* acids show, very clearly, a difference in hydrogen donating powers. Fumaric and maleic acids exhibit a similar and striking difference (Table IV) [see also Quastel and Whetham, 1924].

In mesaconic and citraconic acids we have our first examples of the antagonistic action of the methyl upon the carboxyl group. Consider the molecule

The effect at C_1 of the adjoining carboxyl group is to attract, and of the methyl group to repel positive electricity. Hence, on activation, it is unlikely that a proton at C_2 will move to C_1 . In fumaric acid, where there is symmetry of the molecule and no CH_3 effect, the proton can move from C_1 to C_2 or *vice versa*, the positions being equivalent. With mesaconic or citraconic acid there is no proton at C_1 to move towards C_2 , and hence, although activation will have made a shift in the electrons of the double bond, we cannot expect these acids, in presence of the same activating source, to exhibit the same reactivity as fumaric acid. At C_1 , however, the methyl group is affected by the carboxyl, and the demand of C_1 for positive electricity, which cannot be satisfied by a proton from C_2 owing to the repellent effect of CH_3 , will occasion the shift of a proton, after powerful activation, from this group. The following scheme represents the mode of activation of mesaconic acid.

III represents the final activated form following on ionisation of the carboxyl and movement of the β -H in accordance with the representations given earlier. Itaconic acid represents a possible stage after II, in the absence of a hydrogen acceptor. III will become oxidised, in presence of a suitable acceptor, say active oxygen, to the aldehyde, suffer decarboxylation and subsequent oxidation and then proceed through fumaric acid to pyruvic and acetic acids. Thus mesaconic and citraconic acids are liable to attack, the reactive point being the methyl group. Clearly, however, the activation necessary to produce III from I will be much greater than that necessary to produce the active from the normal form of fumaric acid, and only a powerful hydrogen acceptor such as oxygen would be expected to exhibit reactivity with mesaconic acid.

Experiment shows mesaconic and citraconic acids to be relatively inert towards methylene blue in presence of *B. coli* (Table IV). The difference between the two acids is demonstrated by the fact that bacterial growth occurs abundantly on the *trans* and not so well on the *cis* form [Liot, 1923] just as in the case of fumaric and maleic acids¹.

It would be expected from what has been said above that all substances of the type

would, on activation, exhibit a reactivity, at any rate with oxygen, the point of attack being the methyl group. After oxidation, decarboxylation, etc., the final form would be $-\text{CH}=\text{CH}.\text{COOH}$ which would then, on activation, behave in the same way as crotonic or fumaric acid. This is in agreement with general experience on the oxidation *in vivo* of acids with branched chains [Raper, 1914].

We turn, now, to the homologue of fumaric acid—glutaconic acid, I. The activated form is II.

Since the effect of the $-\text{CH}_2.\text{COOH}$ moiety is to produce a field at the β -C to repel positive electricity, it would not be expected that II would be a good H acceptor. Experiment (Table IV) demonstrates this. But in considering the production of II another fact must be taken into account. It has been shown by Thorpe and his co-workers that in the three carbon system there is a tendency for a mobile H atom to take up a position on the central C atom of the system, and that the system so produced is relatively stable. Glutaconic acid is, therefore, represented as $\text{COOH}.\text{C}'\text{H}.\text{CH}_2.\text{C}'\text{H}.\text{COOH}$ (III), a "semi-aromatic" structure which possesses a considerable measure of stability. It cannot be assumed, therefore, that glutaconic acid will react towards an activating source as readily as, say, fumaric acid. Since III, however, will give rise to a certain amount of I, a slight activation (production of II) will be expected. Dakin [1922], in fact, has found that glutaconic acid gives rise, in presence of muscle, to a small quantity of β -hydroxyglutaric acid under conditions in which fumaric acid is transformed into malic acid to the extent of 70 %. In presence of *B. coli* glutaconic acid appears to be relatively inert (Table IV), a slight hydrogen donating power being observable at high concentration.

The fact that a double bond in a ring can be activated is demonstrated by the activity of pyromucic acid in presence of *B. coli* (Table IV). In a ring, however, which is rendered comparatively stable by tautomerism of hydrogen only a most powerful activation will produce a reactive structure. This fact,

¹ I have not been successful in obtaining any growth of *B. pyocyaneus* on citraconic or maleic acid, although good growth occurs on mesaconic or fumaric acid.

it seems to me, accounts for the relative stability of the benzene ring in the body. Clearly in a compound containing both the benzene ring and a fatty acid chain, the latter will be the susceptible structure.

ACTIVATION OF THE SATURATED DIBASIC ACIDS.

The following scheme represents the activation of succinic acid:

The repelling effect of $\text{COOH}-\text{CH}_2-$ on positive electricity will, after activation of II, result in the production of III, the latter being the activated form of fumaric acid. Now since in succinic acid (1) each of the α -C atoms is equally susceptible to the influence of the neighbouring $-\text{COOH}$ and $-\text{CH}_2-\text{COOH}$ groups, (2) the activated form III readily falls into normal fumaric acid, (3) there are no non-polar groups, we expect the molecule to be a vigorous hydrogen donator—as is the case.

Turning to glutaric acid we expect the activation to proceed on the following lines:

In II the repelling influence due to $\text{COOH}-\text{CH}_2-$ is at the β -C atom; hence the single H at the α -C will not be made mobile, as in succinic acid, and on activation a proton will move from the β -C to the α -C which is attracting positive electricity. The final activated form is IV, which is the activated form of glutaconic acid. The difference between glutaric and succinic acids is in this way made clear; in the latter either of the α -C atoms becomes unsaturated and reactive, in the former only the β -C. As already pointed out, however, in a three carbon system, the tendency is for mobile hydrogen to take up a position at the central C. Hence it is unlikely that IV will be produced from III except by most powerful activation. We should anticipate, therefore, that glutaric acid would not be a good donator of hydrogen to methylene blue. Experiment shows this to be the case [Thunberg, 1920; Quastel and Whetham, 1925, 1].

We should also expect that oxalic acid and thiodiglycollic acid would be inert as H donators in presence of *B. coli*. This is the case (Table IV) [Quastel and Whetham, 1925, 1].

THE EFFECTS OF SUBSTITUENTS IN SUCCINIC AND GLUTARIC ACIDS.

Consider the structure $\text{COOH}.\overset{2}{\text{CH}_2}-\overset{1}{\text{CHX}}-\text{COOH}$ where X is a radicle which produces at C_1 a field repelling positive electricity. After ionisation the structure becomes either

In (A) the attractive field for H at C_1 due to the carboxyl is neutralised (or partially so) by the repelling field due to X. Hence after activation, it is unlikely that a proton will move from C_2 to C_1 . In (B) after activation, a proton should move from C_1 towards C_2 , the activated structure being $\text{COOH}.\text{C}'\text{H}=\text{C}'\text{X}.\text{COOH}$ (I) in equilibrium with 2H^+ .

Now I is the activated form of $\text{COOH}.\text{CH}=\text{CX}.\text{COOH}$ (II) and if the affinity of I for hydrogen is less than that of methylene blue, II will be formed and reduction of methylene blue will occur. The formation of II would be facilitated if this were rendered comparatively stable by the presence of a tautomeric hydrogen atom, as when X is $-\text{OH}$ (and probably $-\text{NH}_2$). When X is $-\text{CH}_3$ mesaconic or citraconic acid is formed; these acids have already been discussed. It is important to note, however, the difference between $-\text{OH}$ (or $-\text{NH}_2$) and $-\text{CH}_3$. In the case of the former the production of a tautomeric keto-enol body stabilises the molecule II; in the case of the latter, oxidation of $-\text{CH}_3$ to $-\text{CHO}$ (as with mesaconic acid) occurs with subsequent oxidation and decarboxylation and the final substitution of a H for the $-\text{CH}_3$. We should expect, then, the structure

to be a hydrogen donator, but not so powerful as succinic acid because the activity depends mainly upon a shift of a proton from C_1 to C_2 , the reverse shift from C_2 to C_1 not occurring.

Experiment shows that where X is $-\text{CH}_3$, $-\text{OH}$, $-\text{NH}_2$ and $-\text{CH}_2\text{COOH}$ hydrogen donation occurs generally at fairly high concentration. (For pyrotartaric and tricarballic acids see Table IV; for details on malic and aspartic acids see Quastel and Wooldridge [1925], Thunberg [1920], Fleisch [1924].)

Malic and aspartic acids need special consideration. Both substances act as weak hydrogen acceptors in presence of *B. coli* [Quastel and Whetham, 1924; 1925, 2]. In the activated structure $\text{COOH}.\overset{\vee}{\text{C}}.\text{CH}_2.\text{COOH}$, two fields act at the unsaturated C, one to attract positive electricity, one to attract

negative. Thus the addition of the elements of water is probable, so that at the activating surface the following equilibrium will ensue:

The effect of the activating field on malic acid, therefore, is to produce a certain amount of activated fumaric acid which acts as a hydrogen acceptor. Hence we have malic acid, after activation, acting both as a hydrogen acceptor and donator. (Probably the donating properties of the acid are obscured in the experiments with *B. coli* by the accepting powers.) The same effect may hold with aspartic acid and the possibility of an equilibrium existing in presence of bacteria between fumaric acid, ammonia and aspartic acid is now under investigation¹.

It is useful to consider the various reactions to which the activated structure $\text{—}\overset{\vee}{\text{C}}\text{—CH}_2\text{—}$ can give rise. These are:

All these reactions², once activation has occurred, may proceed at the same time, the rates of the reactions being dependent upon (1) the extent of activation, (2) the physical and chemical conditions of the environment.

In viewing, then, the oxidation of the structure $\text{—CH}_2\text{—CH}_2\text{—COOH}$, it is only necessary to picture the activated form $\text{—}\overset{\vee}{\text{C}}\text{—CH}_2\text{—COOH}$ as the first step prior to reaction and not necessarily to picture either the β -keto-acid, the β -hydroxy-acid or unsaturated acid as the *first* product of oxidation. These substances may all be regarded, in a sense, as the first products of oxidation.

The difference between aspartic and glutaminic acids, *in vivo*, is also made clear. The latter cannot act as hydrogen acceptor since the corresponding unsaturated acid, glutaconic acid—even assuming that an equilibrium does exist between these two acids—is relatively inert as a hydrogen acceptor. Thus, whereas aspartic acid will enable *B. coli* to grow anaerobically upon glycerol, glutaminic acid has no such effect [Quastel and Stephenson, 1925]. Glutaminic acid, on the other hand, should be a fair hydrogen donator, the exceptional condition obtaining in glutaric acid not holding in this case. The amino-acid should react thus:

¹ The work on this problem, which is being pursued by Mr B. Woolf and the writer, shows, so far, that production of aspartic acid does occur from fumaric acid and ammonia in presence of resting *B. coli*. It is hoped to publish details soon.

² Reactions (2) and (3) may proceed simultaneously to produce a Cannizzaro reaction.

II may react subsequently in the following ways:

Experiment shows glutaminic acid to be a good donator [Thunberg, 1920; Quastel and Whetham, 1925, 2]. For similar reasons α -hydroxyglutaric acid should also be a good donator. Thunberg has shown this to be true in the presence of muscle.

The compound $\text{COOH} \cdot \text{CHX} \cdot \text{CHX} \cdot \text{COOH}$ where X is of the type CH_3 , OH, NH_2 should be relatively feeble or inert as a H donator¹. Tartaric acid has been investigated and this substance is only active at high concentrations [Quastel and Whetham, 1925, 1].

THE EFFECT OF MODIFYING THE CARBOXYL GROUP.

Any modification of the carboxyl group which prevents ionisation of the latter should diminish or entirely remove hydrogen donating power.

Laevulinic acid $\text{CH}_3 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$ should be a fair donator of hydrogen for similar reasons to those put forward for succinic acid ($\text{CH}_3 \cdot \text{CO} \cdot \text{CH}_2$ — acts in the same sense as $\text{OH} \cdot \text{CO} \cdot \text{CH}_2$ —but should not be as powerful as succinic acid owing to (1) the asymmetry of the molecule, (2) greater non-polarity. This is the case (Table IV).

Acetylacetone $\text{CH}_3 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{CO} \cdot \text{CH}_3$ in which all ionisation of the carboxyl has been eliminated should be relatively inert. This is found to be true (Table IV), the feeble reductions which are observed with this diketone probably being due to the unsaturated structures to which acetylacetone easily gives rise.

Succinimide $\begin{array}{c} \text{CH}_2-\text{CO} \\ | \\ \text{CH}_2-\text{CO} \end{array} \rangle \text{NH}$ should also be inert. Experiment shows that only a slight activation occurs at high concentrations; this activity may, indeed, be due to traces of succinic acid produced by hydrolysis (Table IV).

Whilst acetic acid is a fairly good donator, acetamide is inert (Table IV).

α -Ketoglutaric acid should be a good H donator (since $\text{COOH} \cdot \text{CO}$ — acts in the same sense as COOH —) and that this is so has been shown by Thunberg [1921].

The esterification of the carboxyl group and hence the loss of the power of ionisation of the latter will also “protect” the molecule from oxidation. The stability of glutathione in presence of muscle [Hopkins and Dixon, 1922] is probably to be attributed to this effect: it will be noted that in glutathione the “effective” carboxyl of glutaminic acid is linked with cystine.

¹ Generally speaking when X is OH we should expect the enfeebling effect to be less owing to the relative ease with which H is dissociated from O.

THE ACTIVATION OF BUTYRIC ACID AND THE EFFECTS OF SUBSTITUENTS.

The activation of butyric acid will be represented by the following scheme:

Butyric acid should be a good H donator, but owing to the non-polar nature of the propyl group the activation is, apparently, diminished considerably. Experiment shows it to be a donator at high concentration in presence of *B. coli* [Quastel and Whetham, 1925, 1]. If, now, we substitute the hydroxyl (polar) group in butyric acid, a considerable increase in activity should result.

On substituting OH in the β -position the effects may be summarised:

- (1) removal of the non-polar influence of the propyl group;
- (2) enhanced repulsion of a proton, on activation, from the β - to the α -C, since both CH₃ and OH act in the same sense. Hence we expect β -hydroxybutyric acid to be a good H donator, and to be much more powerful than butyric acid. Experiment shows this to be the case¹ [Thunberg, 1920]; see also Table IV. The arguments which apply to malic acid also apply to β -hydroxybutyric acid.

On substituting OH in the α -position the effects are:

- (1) removal of the propyl but substitution of an ethyl group;
- (2) partial neutralisation of the attractive effect of the carboxyl group for H at the α -C atom. Hence we should expect this acid to be a H donator but a much poorer one than β -hydroxybutyric acid. It should be more powerful than malic acid. Experiment confirms this [Quastel and Wooldridge, 1925].

If a hydroxyl be substituted in both α - and β -positions it would be expected that the resulting acid would be a H donator intermediate in activity between α - and β -hydroxybutyric acids and more powerful than tartaric acid. This acid has not yet been investigated.

THE ACTIVATION OF PROPIONIC ACID AND THE EFFECTS OF SUBSTITUENTS.

The activation of propionic acid will be represented thus:

The important fact concerning the activation of propionic acid is the repulsive effect by the methyl group on H at the α -C atom (compare succinic acid). Hence the result of activation of propionic acid differs from that of butyric acid where β -oxidation alone can occur. Propionic acid therefore may be expected to be a H donator, its activity being diminished, apparently,

¹ In working with β -hydroxybutyric acid, it is necessary to use a freshly prepared solution. Solutions which have stood for some time, or those which contain bacteria, and exhibit an aceto-acetic acid reaction, are not as active as the freshly prepared solutions.

by the non-polar ethyl group. Experiment shows that it denotes H at fairly high concentration [Quastel and Whetham, 1925, 1]. The structure III may, conceivably, produce acrylic acid, but it is much more likely, since the α -C atom attracts (1) negative electricity because of the methyl group, (2) positive electricity because of the carboxyl group, that the elements of water will be added and lactic acid produced (after the 2H have been taken up by a suitable hydrogen acceptor). These views make it clear why butyric acid, in the body, gives rise to acetoacetic acid and propionic acid to lactic acid or sugar.

On substituting OH in the α -position we find (1) enhanced repulsion of the α -H and (2) removal of the non-polar ethyl and substitution of the methyl group. Lactic acid, therefore, will be a good donator of H and more powerful than propionic acid. Experiment shows that this is true [Quastel and Whetham, 1925, 1]. Its activation may be represented:

If $-\text{COOH}$ be substituted for $-\text{CH}_3$ in lactic acid there should be a very great diminution in hydrogen donating power. Thunberg [1920] has found that tartronic acid is only a very weak donator in presence of muscle.

On substituting $-\text{NH}_2$ in the α -position we expect a similar effect to that following $-\text{OH}$ substitution. Owing, however, to the influence of the basic amino-group on the carboxyl it is difficult to estimate the nett effect of amino-substitution. Probably the internal ring formation between amino and carboxyl has a considerable diminishing influence on the activity of the compound. Alanine is a fair donator [Quastel and Whetham, 1925, 2; Thunberg, 1920].

Substitution of a $-\text{CH}_3$ group in the α -position, whilst enhancing the repulsion of a proton from the α -C, considerably increases the non-polar effect of the ethyl group. Hence we expect *isobutyric* acid to be a H donator of about the same order as propionic acid. This is the case [Quastel and Whetham, 1925, 2]. The activation will proceed on the following lines:

Further activation of III results in:

IV will oxidise to the aldehyde, decarboxylate and finally proceed to propionic acid.

Substitution of OH in the α - and β -positions results in:

(1) removal of non-polar C_2H_5 and CH_3 groups; (2) removal of the vigorous

repulsive effect on the α -H due to $-\text{CH}_3$; (3) partial neutralisation of the attraction for positive electricity at the α -C due to the carboxyl; (4) repulsion of a proton from a β -C to the α -C due to the OH in the β -position.

We expect, then, glyceric acid to be a H donator, not as powerful as lactic acid but more powerful than tartaric acid. This is the case [Quastel and Whetham, 1925, 1].

THE ACTIVATION OF FORMIC ACID AND THE EFFECTS OF SUBSTITUENTS.

The activation of formic acid is very simply represented:

II is the active form and possesses, because of the hydroxyl groups, a powerful attraction for negative electricity. Hence on activation formic acid should be a powerful H donator. This is the case [Thunberg, 1920; Quastel and Whetham, 1925, 1]. On substituting for OH in I a radicle acting in the same sense, *i.e.* $-\text{NH}_2$ or $-\text{CH}_3$, the resulting compounds should also be H donators.

The activation of acetaldehyde is represented thus:

II having a powerful attraction for negative electricity (OH). Owing to the non-polar nature of $-\text{CH}_3$ there should be an apparent diminution in the reducing power. Experiment shows acetaldehyde to be a H donator in presence of *B. coli*, its effect being diminished with increase of concentration of the aldehyde (Table IV).

The activation of formamide is represented thus:

If we accept the formula of formamide as H.C(OH)=NH [Plimmer, 1925] the same activated structure II would be produced. Since the amino-group is polar we expect formamide to be a donator of the same order as formic acid itself. This proves to be the case (Table IV); formamide is active at a concentration of 1/100,000. It may be objected that the activity of formamide may be due to hydrolysis of the latter to formic acid, but from Plimmer's results it seems scarcely likely that the entire activity can be attributed to this cause.

It is interesting to note in this connection the work of Ingold [1924] who has confirmed Nef's view that a hydrogen of acetylene is mobile.

This was proved by isolation of the product of oxidation, keten, $\text{CH}_2=\text{CO}$. Ingold suggests that the normal course of oxidation of formaldehyde and formic acid is not that suggested by Bone and Andrew [1905], but one dependent on the active forms of the aldehyde and of formic acid [Baly, Heilbron and Hudson, 1922]. Thus:

These oxidations are uniform with the oxidation of *iso*acetylenes to ketens, of *isonitriles* to carbimides and of carbon monoxide to carbon dioxide.

ACTIVATION OF ACETIC ACID AND THE EFFECTS OF SUBSTITUENTS.

Just as butyric, propionic and formic acids, after activation, exhibit striking differences from one another, so does acetic acid behave in a unique manner. We may represent acetic acid as follows:

Now in II there is no particular demand at the α -C for positive electricity as this C already possesses two hydrogen atoms. We will examine what can occur after activation of II. A proton may move from the α -C to an oxygen of the carboxyl and the α -C will be left in a reactive unsaturated state. But in this condition owing now to the demand for positive electricity at the α -C due to the carboxyl, the affinity of the activated structure for hydrogen will be very considerable. It would be unlikely, therefore, that oxidation to glyoxylic acid would occur; it is conceivable that the elements of water would be taken up to produce glycollic acid. This would depend on the relative affinities of the activated structure and, say, methylene blue for hydrogen. But even then it would be doubtful whether $-\text{OH}$ would be "accepted" by the structure. Since II is a two carbon group, and, on activation, the movement of a proton can be only in the direction of the carboxyl, it is possible to conceive of a combination of two molecules of II to succinic acid, but this, again, only if the affinity of II for hydrogen is less than that of the methylene blue (or other acceptor in the system). In a three, or more, carbon structure such a synthesis would be unlikely since, on activation, the demand at the α -C for positive electricity would be satisfied by the movement of protons from other parts of the molecule. Hence, on the facts, (1) that acetic acid is a two carbon compound and (2) that two hydrogen atoms are unlikely to be displaced from the α -C owing to the attractive field for positive electricity due to the carboxyl, a synthesis to succinic acid is conceivable. In the case of glycollic acid an oxidation to glyoxylic acid is more likely to occur than one of acetic acid to glycollic acid, but even here the possibility of a synthesis to tartaric acid is not to be ruled out. Experiment shows glycollic and acetic

acids to be H donors of about the same order [Quastel and Whetham, 1925, 1].

THE ACTIVATION OF THE ALCOHOLS AND AMINES.

The reducing power of these substances will depend on the ease of removal of hydrogen from the structures $-\text{CH}_2-\text{CH}_2-$, $-\text{CH}_2-\text{OH}$, $-\text{CH}_2-\text{NH}_2$, and the presence of certain radicles will markedly influence this removal. Methyl alcohol is relatively inert as a donator, demonstrating the stability of the structure $\text{H}-\text{CH}_2\text{OH}$. Ethyl alcohol, as would be expected, is more active and propyl alcohol (which possesses the $-\text{CH}_2-\text{CH}_2-$ as well as the $-\text{CH}_2\text{OH}$ structures) still more so. Methylamine and ethylamine are both inactive (in presence of *B. coli*), and indicate the greater difficulty of removal of H from $-\text{CH}_2\text{NH}_2$ than from $-\text{CH}_2\text{OH}$. Allylamine is inactive whereas allyl alcohol is a good donator (Table IV).

In the polyhydric alcohols where the non-polar influence of fatty radicles is removed we find glycol to be active and glycerol rather less active [Quastel and Whetham, 1925, 1]. The greater activity of glycol is probably to be attributed to the nearness of approach of the two $-\text{CH}_2$ elements. As these become further removed, as in glycerol and erythritol, there is a greater diminution in activity. It is significant that only when the $-\text{CH}_2$ elements approach nearest, namely, in the five or six carbon compounds where ring formation is possible, a high degree of activity is obtained. In these latter cases, however, so much complexity is introduced by the stereo-structure of the molecules that it would be fruitless, at the moment, to deal with these in any detail. The same applies to the sugars.

CONSIDERATION OF ENERGY TRANSFERENCE.

Let us consider, again, the structure $-\text{CH}_2-\text{CH}_2-\text{COOH}$ (I) which in presence of, or in contact with, the activating source (muscle tissue or bacteria) produces the system $-\overset{\vee}{\text{C}}-\text{CH}_2\cdot\text{COOH}$ (II) + $2\text{H}^\cdot$. If the two protons are taken up by a suitable acceptor, II, as has been shown, has three procedures. The energy derived from the reaction

may be entirely dissipated, but it is more likely that, at any rate, part of the energy may go into the structure II and preserve the latter in an activated condition. The molecule then becomes liable to a further reaction.

Thus the energy derived from the first reaction may prevent the accumulation of the normal form of II, *i.e.* the corresponding unsaturated acid (II *a*), and

carry the action on to III. It would appear, then, that I would be oxidised to III, none of II *a* necessarily accumulating. Now if for I we substituted II *a* we should get, after activation, the same product III but it would not necessarily follow that the yield of III from II *a* would be equal to or even be of the same order as that from I. This simply arises from the fact that when II *a* is presented to the activating source it does not contain the extra energy present in II after the first oxidation has occurred.

It is this which, it seems to me, makes intelligible the fact that α -methyl-phenylacetic acid is fully oxidised in the body whilst phenylacetic acid is not oxidised [Kay and Raper, 1924]. The methyl group in the former, after activation, becomes the reactive point, is oxidised, and the energy, or some part of it, goes into the ring and renders the latter, which is normally fairly stable, susceptible to attack. Phenylacetic acid presents no such "handle" for attack and is moreover rendered inert by removal of the carboxyl by union with glycine.

It is possible also that the energy of an oxidation $A \rightarrow AO$ may be transferred to a molecule of B and render this susceptible to oxidation—an induced oxidation, in effect, occurring.

AEROBIC OXIDATIONS.

Hydrogen acceptors fall into two classes—those that require activation by the cell (or those whose oxidising power is increased by the cell), and those whose oxidising power is independent of the cell. To the former belong nitrates, chlorates, fumarates; to the latter, substances of the type of methylene blue. Apart from the possibility that molecular oxygen, unactivated, may act as a H acceptor, there appear to be at least the three following ways in which activation of oxygen can occur:

- (1) by iron (Warburg);
- (2) by the sulphhydryl group. Hopkins [1925], for instance, has shown that cyanide does not prevent the oxidation of linseed oil under the influence of glutathione at p_H 7.6.
- (3) by substances capable of peroxide formation. Now when the structures $-\text{CH}=\text{CH}-$, $-\text{CH}=\text{O}$, $-\text{CH}=\text{N}-$, become activated to form $-\overset{\vee}{\text{C}}-\text{CH}_2$, etc, it seems possible that oxygen may form a temporary link with the activated structure at the unsaturated carbon, thus:

this compound having high oxidising power and being in effect "active" oxygen. Hence, the very process which activates the hydrogen donator may result also in activating the oxygen. Possibly the predominating activating influence in a solution containing iron is the iron, but it does not necessarily follow on the above view that if the iron be removed aerobic oxidation will entirely cease.

THE SPECIFIC BEHAVIOUR OF ENZYMES.

It does not follow from the view of the mechanism of oxidation, which has just been discussed, that there do not exist, in the cells, certain specific enzymes related to oxidation-reduction processes. It appears to me that their presence may be possible, for if a certain structure possesses chemical or physical characteristics closely related to some particular substrate then only the latter may become "accessible," as it were, to the activating power of this structure. Such a structure would be regarded as a specific enzyme. The mechanism of activation, however, would still be explained on the lines indicated in this paper. The cell surface—or indeed an intracellular surface—can, in a sense, be regarded as an assemblage of enzymes, enzymes which are inseparable because they are actually a function of the surface structure.

Perhaps the main aim of this paper has been to indicate not so much that it is unnecessary to postulate the existence of numerous specific enzymes dealing with oxidations and reductions, but that it is possible to regard specificity of behaviour as belonging rather to the molecules themselves than to the enzymes.

SUMMARY.

An attempt has been made, based upon the electrical and polar properties of molecules, to interpret in a consistent way the mechanism of oxidations and reductions *in vivo*. On the basis of the theory which is advanced it is possible to "explain":

1. β -Oxidation.
2. Asymmetric oxidation of the double bond.
3. Difference between the activations of *cis* and *trans* isomers.
4. The α -oxidation of propionic acid.
5. The mode of oxidation of branched chains, and various other oxidation-reduction phenomena *in vivo*.

Predictions have been made and verified.

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REFERENCES.

- Baly, Heilbron and Hudson (1922). *J. Chem. Soc.* **121**, 1078.
Bone and Andrew (1905). *J. Chem. Soc.* **87**, 1232.
Dakin (1922). *J. Biol. Chem.* **52**, 184.
Fleisch (1924). *Biochem. J.* **18**, 294.
Girard (1925). *Bull. Soc. Chim. Biol.* **7**, 75.
Hardy and Harvey (1911). *Proc. Roy. Soc. Lond. B.* **84**, 220.
Hopkins (1925). *Biochem. J.* **19**, 798.
Hopkins and Dixon (1922). *J. Biol. Chem.* **54**, 527.

- Ingold (1924). *J. Chem. Soc.* **125**, 1528.
Kay and Raper (1924). *Biochem. J.* **18**, 153.
Knorr (1911). *Ber. deutsch. chem. Ges.* **44**, 1141.
Langmuir (1917). *J. Amer. Chem. Soc.* **39**, 1848.
Leathes (1925). Croonian Lectures. *Lancet*, i, 803, 853, 957, 1019.
Liot (1923). *Ann. Inst. Pasteur*, **37**, 234.
Loewe (1912). *Biochem. Z.* **42**, 190.
v. Möllendorf (1921). *Handb. biol. Arbeitsmeth.*, Abt. V, Teil 2, Heft 2, p. 148
Plimmer (1925). *J. Chem. Soc.* **127**, 2657.
Quastel and Stephenson (1925). *Biochem. J.* **19**, 660.
Quastel, Stephenson and Whetham (1925). *Biochem. J.* **19**, 304.
Quastel and Whetham (1924). *Biochem. J.* **18**, 519.
—— (1925, 1). *Biochem. J.* **19**, 520.
—— (1925, 2). *Biochem. J.* **19**, 645.
Quastel and Wooldridge (1925). *Biochem. J.* **19**, 652.
Raper (1914). *Biochem. J.* **8**, 320.
Thomson (1923, 1). *Nature*, **112**, 826.
—— (1923, 2). *Phil. Mag.* **46**, 506.
Thunberg (1920). *Skand. Arch. Physiol.* **40**, 1.
—— (1921). *Arch. Internat. Physiol.* **18**, 601.