THE STUDY OF "RESTING" OR NON-PROLIFERATING BACTERIA.

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If a medium containing either 1 per cent. ammonium succinate or 1 per cent. ammonium fumarate together with certain inorganic salts be inoculated with *B. pyocyaneus* and incubated aerobically at 37° C. a vigorous growth of the organism will ensue, the rates of growth in both cases not being markedly different from each other. If the growth be allowed to proceed for a fortnight, analysis of the media shows that essentially similar products (mixtures of the lower fatty acids) have been produced in both cases. If the growth be allowed to proceed for not longer than three days, analysis shows that in the succinate medium over 80 per cent. of the succinic acid has not been utilised, whilst in the other medium the entire fumaric acid has disappeared, giving rise in its stead to pyruvic acid which is further utilised as growth proceeds (1)[1]. Quantitative data support the view that the breakdown of succinic acid occurs through the intermediate production of fumaric acid, the rate of fermentation of fumaric acid being greater than that of succinic acid, so that fumaric acid is not detected among the products of fermentation of succinic acid. If this view is true, it should be possible to show that *B. pyocyaneus* has the power of oxidising succinic acid to fumaric acid. It is clear that the proliferating organism cannot be used to demonstrate this experimentally, for the fumaric acid is utilised as quickly as it is formed. Hence it is necessary to use the organism in such a condition that proliferation is completely (or almost completely) eliminated. Under such circumstances the fumaric acid will accumulate and its detection will be made possible.

This has been accomplished by the use of "resting" bacteria (2, 3). With the Thunberg methylene blue technique it is possible to employ suspensions of bacteria in much the same way as muscle or other tissues. In presence of washed muscle, which reduces methylene blue only very slowly, sodium succinate brings about a very quick reduction, the process being controlled by a thermolabile enzyme related to succinic acid. Similarly (1, 2) a suspension of *B. pyocyaneus* shows the same phenomenon, demonstrating its possession of an enzyme which has the effect of converting succinic acid into fumaric acid in the presence of methylene blue. The suspension is used under such conditions that in the time of the experiment little or no proliferation can have occurred to mask or interfere with the reaction. Bacteria used under these

[1] References are cited by numbers. See p. 146.

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conditions are termed resting bacteria in order to discriminate their reactions from those due to bacteria used under such conditions that proliferation occurs. The word "resting," though not a good one because of the designation of the sporing stage of certain organisms as a resting stage, need give rise to no confusion and the term resting organism, as defined, is useful for comparison with resting muscle and other tissues. "Resting" bacteria are simply bacteria in a state of non-proliferation and may be investigated in a manner similar to enzyme or catalytic systems. They enable a study to be made of the changes which are induced in a defined substrate by the non-growing cell, and it is important that such knowledge as this should be obtained in order to interpret clearly the results found with proliferating bacteria. For instance it is possible, with the resting organism, to compare and contrast closely related substances such as the sugars, results with which by the ordinary fermentation methods are complicated by the fact that growth is occurring. A growing organism with its complex synthetic machinery introduces factors, at present difficult to analyse, which may obscure the *first* changes that the sugars undergo in the presence of the organism. With the resting organism it is possible to distinguish between closely related substances by reactions uncomplicated by the synthetic operations of growth.

The conditions, that growth shall be small or absent during the experimental time, are easily secured if the experiments are carried out:

1. In the absence of some factor which is known to be essential for growth, *e.g.* in the absence of a nutritive source of nitrogen among the substrates whose reactions are being investigated.
2. Anaerobically.
3. In a short time—usually under thirty minutes.
4. At a relatively high temperature (*i.e.* a temperature at which growth is known either to be inhibited or not to occur but which does not seriously interfere with the reaction under investigation).
5. Using, when possible, substrates which are known not to induce the anaerobic growth of the organism under investigation.

Bacteria used under these conditions lend themselves to investigation in the same manner as enzymes or other catalytic systems, and it has been possible to show that the bacterial suspensions have many factors in common with enzyme systems. The resting organism technique enables important chemical distinctions to be drawn between various bacteria. For instance, in contrast to *B. coli* or *B. pyocyaneus*, *B. alkaligenes* is practically incapable of activating nitrates, chlorates and some of the sugars. Such distinctions become of considerable value when taken in conjunction with the growth characteristics of these organisms. The easy manipulation of resting bacteria, and the fact that conditions can be well controlled, make it possible to obtain reproducible and consistent results.

Most of the work on resting bacteria has been carried out with *B. coli*. This organism has the power of activating a large number of substances, *i.e.* these

substances perform reactions (*e.g.* reduction of methylene blue) in presence of the cell which they cannot perform (at a perceptible rate) in its absence. Harden and Zilva in 1915 showed that washed *B. coli*, which only reduces methylene blue very slowly, acquires the property of reducing the dyestuff quickly in the presence of various substances. A detailed investigation (3) of this phenomenon by the Thunberg technique shows that *B. coli* has an even wider range of activating action than muscle. For instance, not only is succinic acid activated but formic, lactic, glutamic, aspartic, hydroxybutyric, glycollic, tartaric, glyceric and other acids[1] are also activated. In fact out of 103 substances investigated, 56 were activated.

It is possible to arrange the substances which reduce methylene blue, only in presence of the cell, in order of their reducing powers. Thus it is found that formic acid is seven times, and lactic acid about six times as powerful as succinic acid, whilst succinic acid is about a hundred times as powerful as acetic acid or glycollic acid. Now these figures, representing relative reducing powers of various substrates, do not necessarily indicate anything specific about the substrates themselves. For instance it can be argued that there is in the cell seven times as much enzyme dealing with formic acid as there is enzyme dealing with succinic acid. Nevertheless the figures do represent characteristics of the *cell* under the conditions of experiment.

In any one particular experiment with methylene blue something of the order of 10×10^6 cells are used, so that only the average activating power of the organism is determined. It is unlikely that every cell is identical in its quantitative effects with its neighbour, but it is to be expected that the average activating power will be the same for any strain of the same organism which has been grown and handled in the same way. The fact that reproducible and consistent results are always obtained shows this to be the case. It is clear, however, that a suspension of bacteria, as it grows older, will gradually change its average condition, for disintegration of the cells occurs and a change in the physical condition of the cell produces a change in its activating powers. Hence the figures representing the relative reducing powers of substrates must be referred to a particular condition of the organism. Now it is found that dead organisms, for instance *B. coli* killed by exposure to toluene, have certain activating powers just as powerful as the freshly grown organism, so that in attempting to determine the *condition* of an organism it is useless to do so merely by counting the live and dead cells in the suspension. Had a live cell *always* the same activating powers and a dead cell *no* activating powers, a bacterial count would be very important in ascertaining the variation of activating power with the condition of the suspension. But the reverse of this is actually the case. A dilute solution of potassium permanganate has an entirely different type of effect on *B. coli* from that due to toluene though both substances are lethal, and it is clear that the difference in action cannot be ascribed to a difference in the magnitude of lethal action. In fact the whole

[1] These acids are always used in the form of their neutral salts.

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question as to whether an organism can survive a particular treatment, to which the organism has been submitted, does not enter the problem here. What must be determined is the cause of the change in the relative reducing powers of substrates due to the exposure of the cell to different environments. When a working hypothesis, expressed in physico-chemical terms, has been obtained which will satisfactorily explain this change, then it will be possible to correlate the chemical or physical effects of a treatment with the lethal action of the latter. Until then a consideration of the question of the ability (or inability) of the cell to proliferate subsequent to treatment cannot affect our conclusions nor help to enlighten the problems under investigation.

Before such an hypothesis can be put forward it is necessary to enquire into the nature of the mechanisms which bring about the activations due to the cell.

When succinic acid reacts with methylene blue in the presence of *B. coli*, fumaric acid and leucomethylene blue are formed. Now it is found that in presence of this organism fumaric acid itself is activated so that it becomes capable of oxidising leucomethylene blue back again to methylene blue (2). In other words, an equilibrium between the four substances is established and the equilibrium point can be ascertained. Thunberg, later, showed that a similar equilibrium obtains in presence of muscle, and the equilibrium point with muscle is, within the experimental limits of error, the same as with bacteria. The fact that two such dissimilar biological systems give the same equilibrium point indicates that a true catalytic system is being investigated and incidentally demonstrates that resting bacteria under the conditions specified may be regarded just as much a true catalytic system as any enzyme. Were this not the case, were certain of the products of reaction being utilised for purposes of proliferation, such an equilibrium point could not have been established. Another example of the determination of an equilibrium which could not have been obtained with proliferating bacteria is that existing between *l.* aspartic acid, fumaric acid and ammonia (4).

The fact that *B. coli* was found to activate fumaric acid as a hydrogen acceptor (or oxidising agent) made possible the prediction that anaerobic growth of *B. coli* should occur in a medium where fumaric acid replaced oxygen, as for instance in a mixture of lactate and fumarate, or in glycerol and fumarate. Experiment confirmed this (5). Anaerobic growth of *B. coli* which will not occur in lactate alone, or glycerol alone or fumarate alone, does occur in a mixture of lactate and fumarate or glycerol and fumarate. This is one example in a number of cases which indicate the manner in which it has been possible to relate the reactions of resting bacteria to the problems of growth.

The activation of fumaric acid as a hydrogen acceptor formed a difficulty in the Wieland theory of biological oxidations. When the work on resting bacteria commenced, and it was found that bacteria could effect as many dehydrogenations (reductions) as muscle, it seemed that the facts were in accordance with the Wieland theory as developed by Thunberg. But when it

was found that fumaric acid could be activated as an *acceptor* of hydrogen it was apparent that the Wieland theory, which envisaged only the activation of hydrogen atoms and hence the enzymic production of only hydrogen *donators*, was inadequate. The inadequacy consisted in confining the activation only to hydrogen atoms. A more reasonable interpretation of the phenomena was that activation of the substrate molecule *as a whole*(6) occurred, the activated molecule behaving as a hydrogen donator or acceptor, according to the nature of the molecule and the general circumstances under which the reactions occurred. Further investigation with bacteria showed that a variety of substances are activated as hydrogen acceptors, *e.g.* nitrates and chlorates(5).

On the Wieland theory, as extended by Thunberg, it is necessary to believe that every substrate has its specific enzyme, otherwise it is difficult to understand why succinic acid, say, should be so active and its homologues, glutaric acid and malonic acid, so inert. It follows that we have to imagine that there exists in the cell of *B. coli* 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. Granting, however, the existence of such a large number of specific dehydrogenases, it becomes very difficult to understand how the cell possesses enzymes for or is able to cope with material to which hitherto it has not been accustomed if its content of specific enzymes, though large, is yet limited. Such considerations as these led the writer to seek another interpretation of the mechanism of cellular activations.

The view was put forward(6) that the process of activation consists in a polarisation (an internal electrical change) of the substrate molecules at particular regions or centres on the surface structures (interfaces) of the cell. If polarisation is *sufficient*, then the molecule may be regarded as in an activated condition and in this condition it is capable of entering into chemical reaction. Whether a particular molecule is activated or not at a centre depends upon the nature and strength of the polarising field at the centre and upon the nature of the substrate molecule. It was found that this theory brought into line many of the diverse phenomena in biological oxidations and reductions, gave a reasonable and consistent interpretation of the facts and predicted new facts which were verified by experiment.

The phenomenon of specificity is one of the most remarkable characteristics of enzyme action, and the activation theory as developed by Quastel and Wooldridge(8,9) provides a clearer interpretation both of this phenomenon and of the mechanism of enzyme action. Specificity of action is seen to depend upon three factors:

(1) Specificity of adsorption at the activating centre.

(2) The nature and strength of the polarising field at the centre.

(3) The constitution of the adsorbed molecule.

Owing to the fact that different centres located on the colloidal structures of the cell will be differently chemically constituted, a series of substances

adsorbed at one centre will be different chemically from a series adsorbed at another. For instance experiment shows that at the enzyme or active centre related to the activation of lactic acid only substances containing the structure —CO—C(OH)— or —CHOH—C(OH) are adsorbed, *e.g.* oxalic acid, hydroxymalonic acid, glycollic acid, pyruvic acid, etc. are adsorbed, whilst substances such as malonic acid, succinic acid, acetic acid, etc. are not adsorbed (7).

Out of the series of substances adsorbed by a particular centre (or enzyme), and all therefore capable of being polarised, only a limited number are activated, *i.e.* only a number dependent on their molecular constitution and on the nature and strength of the polarising field receive the critical energy required to transform them into "active" molecules.

The reason therefore why an enzyme is so specific in its action is in the first place because only a limited number of substances—containing a certain type of structure—is accessible to or adsorbed by the enzyme, and in the second place because out of this limited number of substances specifically adsorbed only a few are capable of being turned into "active" molecules capable of the reactions under investigation. Thus each enzyme has a limited and definite range of specificity of action.

If the enzymic activity of the cell be considered as due to the active centres which form part of the colloidal aggregates of the cell, there is no necessity to regard the cell as elaborating numerous specific molecules each possessing a specific activating action on a particular substrate. It may be considered that as the cell grows the various synthetic processes culminating in the formation of proteins, nucleotides, etc. are such as to bring about the formation of active centres on these structures. The centres are simply a property of the surface structures of the colloidal materials which make up the cell as a whole, and it is simply due to the conditions which obtain in the living cell that just that arrangement or juxtaposition of cellular material occurs with the formation of active centres. The centres may be regarded as points or regions of instability, places where only a loose fitting occurs of some of the molecules constituting the cellular aggregates. The actual magnitude of the colloidal structure containing the centre is not of great significance. It may be small enough to be classed as "soluble" and to diffuse through a membrane, or large enough to be classed as "insoluble." The distinction is simply one of degree. It would be expected, however, that the smaller the structure the less chance is there of its possessing a number of different centres and hence of its possessing an extensive range of specificity.

It has been found from work on resting *B. coli* that lactate is activated as a hydrogen donator and nitrate as a hydrogen acceptor. It would be expected, therefore, that when anaerobic growth occurs in a mixture of lactate and nitrate there will be transference of hydrogen with the production of pyruvate and nitrite. Experiment (5) shows that pyruvic acid does not appear in the medium until nitrites have been formed in such a concentration (0·4 per cent.) that they become toxic to the cell, *i.e.* when they prevent further pro-

liferation. The pyruvic acid is utilised by the organism until the nitrites have reached their toxic concentration; above this concentration, the pyruvic acid accumulates in the medium. This means that *B. coli* which cannot grow in presence of the toxic concentration of nitrite is still capable of activating lactate and nitrate (otherwise pyruvic acid would not appear). Further investigation has confirmed this. The next step in the enquiry was to determine what occurred when a suspension of *B. coli* was submitted to the action of nitrites under defined conditions, and it was found(8) that whereas certain activating mechanisms of the organism were very sensitive to the action of nitrites others were much more resistant.

This effect is an example of a general phenomenon. If a suspension of *B. coli* be submitted to a variety of different treatments, *e.g.* to different temperatures, or to different hydrogen-ion concentrations or to a number of different substances, the activating mechanisms disappear step by step as exposure to the treatment is prolonged(8,9). Each mechanism behaves as though it were a different unit with its own particular properties—each in fact behaves as though it were a separate enzyme.

The view has been put forward that a living, though not proliferating, cell may possess some property expressed by the reactions which have been investigated and which would not be possessed by the cell were it disorganised or dead. This criticism is groundless as shown by the following evidence:

(1) If a suspension of *B. coli* be shaken with toluene or ether, the organism is no longer capable of reproduction in the usual nutrient media and therefore, so far as can be ascertained, is dead. But it can still give rise to a large number of activations, *e.g.* with formic, lactic, and succinic acids, at rates not markedly different from those due to the normal or untreated organism.

(2) The effect of exposure of *B. coli* to some abnormal treatment (resulting usually in the death of the organism) is to produce, as described above, a step by step elimination of the various activating mechanisms. Now were the activations of the cell due to the latter acting as an organised whole, it would be expected that at some particular point the cell would become disorganised and its activation would cease. Each mechanism would be eliminated at the same time and there would be no step by step degradation. Since this is contrary to what actually occurs, it cannot be supposed that the activations of the cell depend upon the survival of the latter as an intact organised system.

Indeed there is no evidence as yet to support a contention that the reactions of the cell are *dependent* on its survival as a living unit. There is no doubt, however, that the power of the cell to proliferate is dependent upon the intactness of (among other things) many of the activating mechanisms which have been described. This is to be anticipated, for it cannot be expected that a cell will survive whose cellular or intracellular structures have been so changed by exposure to abnormal conditions that they can no longer perform activations.

Quantitative work(9) showed that the activating mechanisms or enzymes

did not all act independently of one another. For instance lactic and α-hydroxybutyric acids behave as though one enzyme is activating both, whereas lactic and succinic acid behave as though there is a distinct enzyme for each. The fact was also ascertained that treatment of the cell had the effect of altering quantitatively the relation of an enzyme to its substrates. For instance, the evidence shows that the lactic acid enzyme of *B. coli* also activates glycollic acid. But if *B. coli* be treated with toluene, the lactic acid enzyme which is still as active as before to lactic acid has no effect on glycollic acid although the latter is still specifically adsorbed at the enzyme (7). The same phenomenon is exhibited in a number of other cases. It is inexplicable on permeability considerations and it is quite useless to account for it in terms of the lethal action of toluene, for, as indicated earlier, it is not known yet in physico-chemical terms what is meant by killing a cell. The facts, however, are interpretable on the active centre theory. For here, as shown above, a centre has definite range of specificity and if it be attacked by chemical or physical means two events may happen: (*a*) a particular grouping in the centre may be destroyed with the result that a particular type of compound made accessible to the centre by this grouping can no longer be activated, (*b*) the surface structure may become modified with a consequent alteration in the activating power of the centre.

On such lines as these, this study of bacteria is now progressing. A knowledge of the chemical and physical nature of the cell is fundamental to any enquiry concerning the reactions which cells can effect, and this study of bacteria, on somewhat new lines, has led to the elucidation of facts which not only throw more light on problems of general biochemical interest but which make clearer the relationship of the cell to its environment.

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