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Recent Researches on Biological Oxidation.

ATTEMPTS have been made to formulate the vital process of combustion according to a single scheme. It is thought to be a catalytic oxidation occurring on a surface, activated by a heavy metal (iron or copper). Of late years this universal catalyst is believed to have been met with in the so-called " respiratory ferment " which, as far as concerns its chemical nature, is considered by Warburg to be related to hæmin. Liebig put forward the hypothesis that iron accelerates vital oxidations. If we adopt this view, we are faced with the difficulty of understanding why the oxygen, after activation by the metal, does not indiscriminately oxidise every substance which the cell presents to it. We fail to understand, for instance, why the organism has so much difficulty in oxidising formic and oxalic acids, whilst experience shows that the degradation of acetic acid and of the higher fatty acids proceeds with ease and rapidity.

Wherever during metabolism large molecules are broken up, in some way or other, enzymes are called into play, and the latter likewise show a definite mode of action. The process of biological hydrolysis, of outstanding importance, is not carried out by any single ferment system, for each kind of substrate, whether carbohydrate, protein or fat, is split by its own specific ferment. Indeed, the selective action demanded by the nature of the substrate extends much further. Substances which are chemically so closely related as the bioses (saccharose, maltose, and lactose) are dependent each on its own ferment.

If such a simple chemical reaction as the hydrolysis of ethers, esters, and amides requires the intervention of several aids, it is hardly likely that a single catalyst will suffice for the much more complicated process of the oxidative destruction of organic molecules. In any case, this idea obtrudes itself when we consider the extraordinarily delicate reaction which regulates the chemical changes of living matter.

The ferment systems which serve to hydrolyse food materials occur in colloidal solution, whether in the fluid of the cell or in the secretion of definite organs. In general these ferments can easily be separated from the living organism and it is further easy to preserve for a shorter or longer time the catalytic activity, which has been assigned to them in vital processes. Indeed, there are methods,

worked out very thoroughly by Willstätter and his school, which enable us to separate the enzyme material from inactive admixtures and thus raise its activity to a value many times that which it possessed in its original vital condition.

No fundamental difference in the nature of enzymes is encountered throughout the vast range of living organisms, ascending from unicellular fungi to man. The invertase of yeast splits sucrose in the same way as does the ferment of the pancreas, and pancreatic amylase does not differ from the amylase of malt in its action in converting starch into sugar. Likewise the hydrolytic degradation of the fats and the proteins presents no important differences throughout the whole range of living matter. It is specially remarkable that the most recent study of the proteases of yeast has revealed almost all the enzyme systems which were known to be produced by mammalian organs.

In contradistinction to these factors which facilitate investigation, the separation of biological oxidation systems from living matter is a problem which has so far only been solved in special cases. Vital oxidation, in its most important reactions, is intimately connected with life. Death is accompanied by the spontaneous decay of this enzyme function. As yet we lack the means with which to accomplish the complete degradation, by dead material, of a substrate familiar to the cell; we cannot imitate the vital process. At least in so far as concerns the whole complex of respiration, it would appear that the available enzymatic activity is closely connected with that regulating principle which, for want of more exact knowledge, we can but describe by a circumlocution called life.

Thus the essential stage of vital oxidation in the higher organisms presents to research difficulties which have hitherto proved insuperable. Fortunately, however, this enzymatic process, unlike the hydrolytic, reveals a simpler chemistry and less labile enzyme systems when we pass from warm-blooded animals to unicellular organisms. We can indeed clearly recognise how the capacity of living matter for developing energy-yielding processes increases with phylogenetic development. The lowest stage is doubtless that of life without oxygen, in which the energy requirements are met by hydrolytic processes, or by dismutative redistribution of hydrogen. These are the processes which may be conveniently grouped together as fermentation. We may perhaps suggest that it is the greater energy requirement of morphologically and physiologically complex organisms which has introduced atmospheric oxygen into the metabolism of living matter.

Let us adopt the hypothesis that the higher we go up the phylogenetic scale, the more delicate and complex becomes the nature of

biological oxidation. We then see that it will be good research tactics to begin by investigating aerobic fungi. Here the enzymic oxidation of alcohols and aldehydes to acids in the cells of acetic acid bacteria presents an ideal subject for investigation, which cannot be improved upon. Under conditions which can easily be reproduced, the reacting oxygen is used exclusively for bringing the substrate to a higher level of oxidation, resulting finally in an acid. This elementary biological oxidation process can be followed with analytical precision in all its details.

The investigation of this very simple example of enzymic oxidation shows at once that the theory of the activation of molecular oxygen does not supply an adequate explanation of the process. A different conception, which connects the enzyme with the substrate rather than with the oxygen, seems more promising. If we assume that it is not the strength of the oxidising agent which is increased, but rather the reactivity of the hydrogen involved in the process, then the molecule of oxygen appears as that component which combines with the activated hydrogen to form water, after an intermediate stage of hydrogen peroxide.

$$\begin{aligned} CH_3{\cdot}CH_2{\cdot}OH + O_2 &\longrightarrow CH_3{\cdot}CHO + HO{\cdot}OH \\ CH_3{\cdot}CH_2{\cdot}OH + HO{\cdot}OH &\longrightarrow CH_3{\cdot}CHO + 2H_2O \\ \hline 2CH_3{\cdot}CH_2{\cdot}OH + O_2 &\longrightarrow 2CH_3{\cdot}CHO + 2H_2O. \end{aligned}$$

Since according to this conception the enzymic attack is not on the oxygen, but on the hydrogen, the transformation may suitably be described as dehydrogenation. This process leads from the aldehyde stage, *via* its hydrate, to the acid, in the manner formulated above.

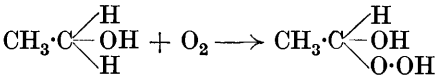
The crucial evidence for this view lies in the fact that acetic acid fermentation can dispense with oxygen. At an equal concentration the bacteria are able to utilise quinone instead of oxygen, and the rate of transformation of alcohol and aldehyde into acetic acid is even increased. The kinetics of the two processes have the closest similarity. In the same way methylene-blue may replace oxygen in the biological transformation of alcohol into acid; in this case however, the velocity is smaller.

There are a large number of substances which inhibit biological oxidation processes. Among them hydrocyanic acid has been most fully investigated. Now it is very remarkable that moderate concentrations of hydrocyanic acid do not hinder the reactions with quinone and with methylene-blue; it has been inferred that the oxygen also requires a stimulus, indeed one from a metallic catalyst, and this stimulus is lost when hydrocyanic acid acts on the ferment. The following experiment indicates a simpler explanation and makes

the assumption of oxygen activation unnecessary. When we examine the dehydrogenation of alcohol by acetic acid bacteria in the presence of both oxygen and quinone, we find that the aerobic process is inhibited by quinone as well as by hydrocyanic acid, and that at equal concentration the degree of inhibition is about the same. In so far as the quinone is hydrogenated to hydroquinone the oxygen again comes into play. The difference between quinone and hydrocyanic acid as inhibitors of the aerobic reaction evidently depends only on the circumstance that quinone does, and hydrocyanic acid does not, participate in the enzymic reaction. Common to both substances there is a great affinity for active surfaces which results in an inhibition of the competing oxygen.

If we regard the function of cellular oxidation catalysts as consisting in an activation of hydrogen in the substances to be oxidised, then this hydrogen must be considered to react with molecular oxygen to form hydrogen peroxide as a first stage of the reaction. Hence it is of the greatest importance to demonstrate the formation of this primary product of biological oxidations. But the demonstration is not easy, for wherever a cell uses up oxygen in its metabolism, we find that cell provided with ferments, the so-called catalases, whose function is to decompose hydrogen peroxide into water and oxygen. These catalases, which as a rule cannot be separated from the system of the dehydrases, are, like the latter, also sensitive to hydrocyanic acid. A method of detecting hydrogen peroxide might be based on an attempt to cut out the catalase action by careful dosage of the inhibiting agent, without entirely stopping the action of the dehydrases. In the case of the acetic acid bacteria the relative susceptibility of the two enzyme systems to hydrocyanic acid is so unfavourable that it has hitherto been impossible to demonstrate hydrogen peroxide as an intermediate product in the conversion of alcohol into acid.

Perhaps it will not be amiss to discuss briefly the possibility of following the reaction in greater detail, for which there are some indications. The activation of the hydrogen atoms, which we imagine to take place on the surface of the enzyme, need not be connected with their immediate transference in pairs to the oxygen molecule. The occurrence of a primary addition compound is quite within the compass of the theory; for instance, in the case of alcohol according to the equation



The breakdown of this compound could take place in two ways,

furnishing *either* aldehyde and hydrogen peroxide, *or* acetic acid and water, thus :

$$CH_3\cdot C\begin{cases} H \\ OH \\ O\cdot OH \end{cases} \begin{cases} \nearrow CH_3\cdot CHO + H_2O_2 \\ \searrow CH_3\cdot CO_2H + H_2O \end{cases}$$

Since Rieche at Erlangen has recently prepared compounds of the same type as these hypothetical intermediates, it will be possible to test these alternatives experimentally.

Let us, after this slight digression, return to the question of the formation of hydrogen peroxide. A British investigator, McLeod of Leeds, discovered some years ago that anaerobic bacteria, which are devoid of catalase, produce hydrogen peroxide when grown under aerobic conditions, and recently the quantitative yield of hydrogen peroxide in the aerobic metabolism of lactic acid bacteria has been determined (Bertho).

Before the above view of the nature of biological oxidations was formulated, special reducing ferments had been postulated, opposed to the oxidative ones, since reducing actions had frequently been observed in the cell. Reduction is, however, simply the counterpart of dehydrogenation. If, for instance, quinone is used as hydrogen acceptor, the resulting hydroquinone appears as the product of a reducing process. Here the hydrogenation of the hydrogen acceptor stands out much more impressively than the conversion of molecular oxygen into water, which is fundamentally a reaction of the same nature. It follows, therefore, that in the metabolism of the cell reduction products will be formed by the intervention of various hydrogen acceptors in the process of dehydrogenation. Conversely we may conclude that, wherever vital reduction takes place, it will be possible to demonstrate the process of aerobic dehydrogenation. The pre-eminence of oxygen is based not only on its high hydrogenation potential, but also on the chemical and physical inertness of its hydrogenation product—water.

Nevertheless it is a remarkable fact that dehydrogenating systems exist, which are rapidly destroyed by oxygen. They have been specially observed in fruits, and it would seem of great interest to vegetable physiology to obtain a deeper insight into these relationships.

The best-known reducing ferment, which was the first to be recognised experimentally as a dehydrogenase, occurs in sterile cow's milk. Schardinger found that formaldehyde, added to milk, decolorises methylene-blue, a change which does not occur in boiled milk. In this reaction acid is formed from an aldehyde, and if the reaction is investigated in the absence of the dye and in the presence of air, the same enzyme is found to accelerate the dehydrogen-

ation of the aldehyde to the acid; in the latter case the hydrogen is taken up by the molecular oxygen. In addition to this aldehydrase, milk contains an enzyme which converts the purine bases xanthine and hypoxanthine into uric acid by a process of dehydrogenation; methylene-blue acts as hydrogen acceptor. This interesting ferment action was discovered by Hopkins and his pupils in the Cambridge Biochemical Laboratory and they also succeeded in isolating a more or less concentrated mixture of the two enzymes from milk. Since catalase is removed during the purification, there is here an opportunity of investigating quantitatively the question of the formation of hydrogen peroxide.

It has thus been possible in the case of the dehydrogenating enzymes of milk, both with xanthine dehydrase and with the aldehydrase, to determine quantitatively the hydrogen peroxide which was to be expelled as an intermediate product. A prerequisite for this was the use of a reagent to trap the hydrogen peroxide by chemical union, and so preserve it from further transformation, and at the same time protect the enzyme from its action. For oxygen destroys also the enzymes of milk, and does so by means of its first stage of hydrogenation, *i.e.*, by hydrogen peroxide.

An excellent reagent for the above purpose was found in the hydroxide of tervalent cerium, which combines very rapidly with hydrogen peroxide to form the yellowish-brown cerium peroxide, $Ce(OH)_3 \cdot O \cdot OH$. In the presence of this trapping agent both xanthine and aldehyde used up exactly one molecular proportion of oxygen, which was estimated to the extent of almost 100% as hydrogen peroxide. In this way the first stage of aerobic dehydrogenation has been demonstrated quite definitely and quantitatively.

The enzyme reactions of milk have also contributed substantially to an understanding of catalase action. Wherever oxygen is used up by a cell, catalase is also present, and from this the more or less legitimate conclusion has been drawn that the specific function of the enzyme—of destroying hydrogen peroxide catalytically—is biologically related to this fact.

Allusion has already been made to the susceptibility of milk dehydrase to oxygen; it shows itself by the fact that enzyme solutions, acting on xanthine or aldehyde in the presence of air, lose their activity in course of time. If, however, a little catalase is added to the solution at the outset, the reaction proceeds at a linear rate, without any disturbance, as was first shown by Dixon with xanthine as substrate. The reaction with aldehydes behaves in exactly the same way. Thus the rôle of catalase as a protective ferment has been established experimentally.

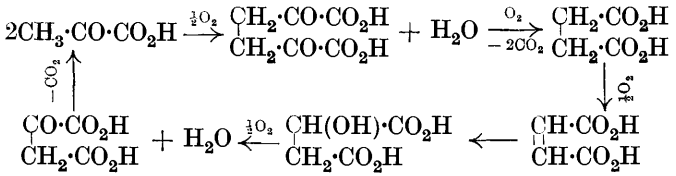
The two dehydrogenating ferments of milk have been found also in the liver and in other organs. This disposes of the suggestion that they have no general biological importance. They are, however, evidently of no importance in the nutrition of the calf. The milk serves as an overflow for a function which exceeds the needs of metabolism, much in the same way as the sexual hormones are excreted in the urine.

A third enzyme system, sharply defined with respect to its chemical transformations, was found in 1915 by Thunberg in muscle. Since then it has been studied in great detail by various investigators. Its action consists chiefly in the dehydrogenation of succinic to fumaric acid, and both oxygen and methylene-blue act as acceptors of the hydrogen. The reaction is of special interest because it can be followed further biologically to the pyruvic acid stage and probably, as we shall see, even beyond this. The action of washed muscle is restricted to the above-mentioned dehydrogenation, which may be followed by the hydration of fumaric to malic acid, a transformation which is catalysed by a separate ferment. With unwashed whole muscle pulp the dehydrogenation of the malic acid is, according to A. Hahn, carried further and pyruvic acid is formed *via* oxalacetic acid. In the reaction

$$CO_2H{\cdot}CO{\cdot}CH_2{\cdot}CO_2H \longrightarrow CO_2H{\cdot}CO{\cdot}CH_3 + CO_2$$

carbon dioxide, the final stage in the " combustion " of carbon, is formed as the last link in a chain consisting exclusively of dehydrogenations. A second molecule of carbon dioxide could be formed by the enzymic decarboxylation of pyruvic acid to acetaldehyde. But beginning with the acetic acid stage, which would succeed this acetaldehyde, experimental verification is lacking.

This being so, it is of great interest that Toenniessen has recently demonstrated the transformation of pyruvic into succinic acid, in the surviving liver of the dog. Should his observations be confirmed, and should it also be found that $\alpha\delta$-diketoadipic acid, the dimeric dehydrogenation product of pyruvic acid, is converted in the tissues into succinic acid, then the cycle of the dehydrogenative degradation to carbon dioxide and water would be closed.



It is only in the first of these reactions that the replacement of

oxygen by another hydrogen acceptor, such as methylene-blue, has not yet been realised. The whole chain of these reactions leads to the following summarised equation :

$$CH_3 \cdot CO \cdot CO_2H + \frac{5}{2}O_2 \longrightarrow 3CO_2 + 2H_2O$$

Pyruvic acid, as a product of the dehydrogenation of lactic acid, is on the main line of biological degradation.

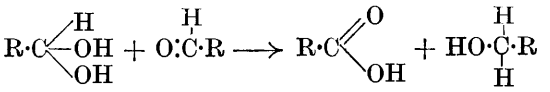
The difficulty of demonstrating the formation of hydrogen peroxide in the aerobic dehydrogenation of succinic acid is specially great for the reason that muscle tissue is endowed with an extraordinary catalytic power. After the action of the dehydrogenase has been completely stopped by means of hydrocyanic acid, the catalase is still able to decompose added hydrogen peroxide.

If we accept enzymic reactions involving hydrogen peroxide, together with hydrolysis and condensation, as conditioning factors in cellular metabolism, we facilitate an understanding of the energy changes which constitute the life of the cell. This has of late been pointed out by Knoop, in particular. Without discussing further these relationships, which have not yet been examined experimentally, we merely cite, as an example, the conversion of carbohydrates into fats.

Further discussion in this connexion will be limited to the correlative oxidation and reduction of aldehydes, associated with the name of Cannizzaro. It is more than twenty years ago that liver tissue was found to contain an enzyme, capable of converting two molecules of aldehyde into one of acid and one of alcohol (Battelli). This case has been found to be quite analogous to that of Schardinger's enzyme in milk. The above-mentioned liver enzyme not only accelerates the Cannizzaro reaction, but at the same time accelerates also the " oxidation " of the aldehyde by molecular oxygen, and its dehydrogenation by methylene-blue. It is merely an accident that the former activity was observed first.

We now understand why the aldehydrase, which was first described as a reducing ferment of milk and later recognised as an oxidase also, is likewise able to dismute aldehydes. In this particular case the proof has been forthcoming that three seemingly quite different reactions depend on one and the same ferment. When the velocity of any one of these three reactions is measured under reproducible conditions, whereby the ferment is damaged irreversibly, for instance, by using it in the aerobic dehydrogenation of an aldehyde, all three of its functions are impaired in the same proportion. The enzymic dismutation of aldehydes must accordingly be regarded as a de-

hydrogenation of aldehyde hydrate by means of aldehyde as hydrogen acceptor :



If oxygen, methylene-blue and aldehyde are simultaneously present, the extent to which each of these three acceptors participates in the reaction depends on its affinity for the active surface, which affinity is greatest in the case of methylene-blue. But since there will be an absorption equilibrium, the three reactions which have here been specially discussed will proceed together and it is not a matter for surprise that under the conditions of aerobic dehydrogenation the Cannizzaro reaction may also come into play to a subordinate extent.

The conception of the nature of biological oxidation developed from the hypothesis that a heavy metal takes part, more particularly iron. Through a number of reactions and phenomena in this field, and especially through the investigations of Warburg, this theory has acquired a certain measure of probability. It is in particular the character of the inhibition by chemical agents which shows a parallelism between the action of oxidative ferments and the catalytic rôle of iron in non-biological oxidations. The recognition of iron as a constituent of oxidising enzymes in no way, however, implies that it functions as an oxygen activator. Indeed, observations on the catalytic action of iron rather indicate that it is a pronounced dehydrogenating catalyst. To begin with, it is only bivalent iron which has the power of activating oxygen. In this ferrous condition iron is capable of accelerating the oxidation of suitable substances, not only with oxygen, but also with methylene-blue or quinone. Its participation therefore entirely resembles that of a dehydrogenating enzyme. The most suitable view, to which these considerations lead us, is that the bivalent metal associates itself with the substrate of the oxidation, forming a labile complex in which those hydrogen atoms become labile which are to be removed in the dehydrogenation process. Instead of the activation of oxygen, we have here that of hydrogen. The demonstration that hydrogen peroxide is an intermediate in the hydrogenation of oxygen, when iron is the catalyst, has been attempted, but without success. The cause of this failure became evident when the reaction velocities with oxygen and with hydrogen peroxide were measured comparatively in the same system. At the most unfavourable p_H the velocity was about one thousand times greater for hydrogen peroxide than for oxygen. This shows the fundamental impossi-

bility of demonstrating the formation of hydrogen peroxide in catalysis by iron. In the analogous reactions with copper and with cobalt, in which the ratio of the reaction velocities is very much more favourable, hydrogen peroxide can be detected as an intermediate product in catalytic dehydrogenation.

There is no doubt that the results which various investigators of dehydrogenation processes have obtained during the last decade in no way permit of a clear understanding of the complex course of biological oxidation. We believe, however, that it is essential to study in the first place such chemically intelligible partial reactions as can be followed accurately in every detail; from these we can advance step by step. It may indeed be desirable to extend our knowledge of the nature of the whole complex of the respiratory ferment, whether it be Warburg's iron compound, related to hæmin, or Keilin's cytochrome. But the investigations of these authors do not at present give us information about the way in which material is broken down in cellular metabolism; for this their material is far too complicated. It should once more be emphasised that, in accordance with the conception we have outlined, we regard the complete degradation of food material in the organism as the result of many reaction stages. From this standpoint, we cannot recognise the validity of the objection that individual steps in the oxidation, such as we have described in small number, are dissociated from the living cell, are foreign to it, are, as it were, denatured. When the liver of a dead animal furnishes a ferment system which accelerates the dehydrogenation of aldehydes or of purine bases, it seems unreasonable, or at least illogical, to regard this merely as a post mortem manifestation, not characteristic of the living organ. Thus there is to my mind not the slightest reason for denying the power of living muscle to dehydrogenate succinic acid aerobically, the less so since this acid has been isolated from meat. It is just in this series of reactions that the individual enzymes taking part in the process happen to show a particularly clear gradation in stability.

The whole of enzyme research is based on the supposition that the phenomena studied *in vitro* furnish a picture of the processes taking place in the cell. Why should this supposition not apply also to the enzymes of biological oxidation?