ACTION OF OXIDISING FERMENTS.*

By WALTER G. SMITH, M.D., Ex-President, R.C.P.I.

LET us, in the first place, consider for a moment the forms and sources of oxygen which are at our disposal.

They are four in number:—

1. Molecular oxygen: the oxygen of the atmosphere. Its formula is O_2 , or, graphically, either $\overset{+}{O} = \overset{-}{O}$, or $O \equiv O$

For it is held by chemists that oxygen can function in either of two valencies, *i.e.*, as diad or tetrad, and that the atoms are held together by electrical forces.

The doctrine of valency is not finally settled, and still bristles with difficulties. The present view is that chemical valence is reckoned as the chemical expression of the number of mobile electrons or free electrical charges upon the atoms. Transference of electrical charges is the key to all acts of substitution in chemistry, whether organic or inorganic (Jones).

Molecular oxygen is not a highly active form of the element, and it is well known that argon and its companions, iodine, bromine, fluorine, gold and platinum, do not directly combine with oxygen.

Hæmoglobin is an oxygen carrier, but oxygen loosely bound in the form of oxy-hæmoglobin is an inefficient direct oxidising agent.

2. Ozone:—Formula $O \begin{smallmatrix} \diagup O \\ \text{N} \\ \diagdown O \end{smallmatrix}$

It is a condensed triatomic form of oxygen, and is a very powerful oxidising agent.

3. Atomic or ionic oxygen: $\overset{+}{O}$ or $\overset{-}{O}$

It has often been conjectured that the active or nascent state of oxygen represents the gas momentarily in its atomic or ionic state.

For example, if H_2O_2 be added to an acidified solution of potassium permanganate tumultuous effervescence of oxygen

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occurs, and the KMnO_4 is reduced. That is, the two oxides interact and liberate molecular oxygen, formed from the atoms of oxygen in the two oxides being in opposite electrical states. And we have the singular phenomenon of each oxide acting as a *reducing* agent upon the other— $5 \text{H}_2\text{O}_2 + 2 \text{KMnO}_4 + 3 \text{H}_2\text{SO}_4 = 2 \text{MnSO}_4 + \text{K}_2\text{SO}_4 + 8 \text{H}_2\text{O} + 5\text{O}_2$. Active oxygen appears to play an important part in the mechanism of action of oxidising ferments in plants.

We are thus led to consider:—

4. Liberation of oxygen from peroxides, inorganic and organic.

It is generally believed, according to Dr. Dakin, that living cells contain labile substances capable of taking up molecular oxygen from the oxy-hæmoglobin of the blood with the formation of unstable peroxide-like bodies which possess marked oxidising properties.

(*Oxidations and Reductions in the Animal Body*, 1912.)

It is quite likely that substances of this type are concerned with the oxidation of compounds in living tissues which are resistant to oxygen under ordinary conditions.

The specific character of certain animal oxidations is most remarkable, especially when phenomena such as those presented by diabetes and alkaptonuria are concerned. In these affections, oxidation of a single readily oxidisable product of metabolism (*e g.*, glucose or homogentisic acid, respectively) may be completely restrained without in the least impairing the capacity of the body for effecting the oxidation of other substances.

The most familiar super-oxide is hydrogen peroxide H_2O_2 , and its oxygen is probably of tetrad valency.

There are three possible formulæ for H_2O_2 :—

The H_2O_2 of commerce is possibly a mixture of (a) and (b), either of which formulæ has more to recommend it than (c).

Commercial H_2O_2 is often strongly acid, owing to free HCl or H_2SO_4 derived from its mode of preparation. Parke

Davis' preparation is only slightly acid, and as a test liquid it is advisable to dilute it with an equal volume of distilled water.

We conclude, therefore, that biological oxidations are concerned chiefly with the ionic and peroxide forms of oxygen, and, only to a minor degree, with molecular atmospheric oxygen.

Oxidation of Organic Tissues.

It is to this branch of the subject that I especially wish to direct your attention, and it is needless to waste time in showing what a dominant part oxygen plays in biological phenomena.

One phase of the subject has given rise to an extensive literature, and it presents several difficult problems which have not yet been completely solved.

I refer to the action of so-called oxidases, a shibboleth often loosely invoked in explanation of certain well-known phenomena.

It is not easy to get clear ideas on this matter from the text-books, and my object in this communication is three-fold, viz.: to clarify my own mind; to attract the interest of the members of the Academy to a difficult and perplexing topic; and to indicate the intimate relations which subsist between animal and vegetable physiology.

All attempts at complete explanation of this phenomena may be regarded, at present, as tentative and provisional.

Over and above direct oxidation by means of molecular oxygen, or by ozone, as exemplified in numerous cases in inorganic and organic chemistry, we have to deal with phenomena of a different order in relation to organic life.

Here we meet with a mechanism whereby inert oxygen is "activated" and acquires enhanced powers.

This mechanism is carried out through the agency of catalysis. A catalyst is a substance which, in minimal quantity, accelerates, *i.e.*, alters the rate of, chemical change without itself necessarily appearing in the final product.

Now most of the processes going on in living structures are performed through the medium of enzymes, which may be briefly defined as the catalysts produced by living

organisms. Physiological chemistry is largely catalytic chemistry, and most enzyme reactions are hydrolytic in nature. There are very many kinds of enzymes which differ from each other in their chemical behaviour, and in the specificity of their action, *e.g.*, 10 enzymes are recognised in yeast.

Enzyme processes and reactions fall into two chief categories:—

- (a) Many of them are hydrolytic, *i.e.*, water is either taken up or eliminated, *e.g.*, those concerned in the digestive functions. Many of these are specific in their action.
- (b) A large number represent an oxidising mechanism, less specific in action, and to this group I will now invite your attention.

In order to simplify matters as much as possible, my remarks will be distributed under three headings, *viz.*:—

- (1) The browning of vegetable tissues.
- (2) Colour reactions with guaiacum or other aromatic reagents.
- (3) Theory of the processes involved.

Browning of Plant Tissues.

Darkening of sap pressed from plants is a well-known and common phenomenon. This can be brought about by various agencies:—

- (a) Action of the vapour of chloroform, and some other volatile liquids.
- (b) Action of tyrosinase on tyrosin, *e.g.*, potato, bean.
- (c) Auto-oxidative processes.

Browning is a frequent occurrence, and is met with in some of the largest natural orders, *e.g.*, umbelliferae, labiatae, and compositae, while it is rare, or absent, in other orders, *e.g.*, cruciferae.

A few simple experiments will demonstrate these points.

I show you a Portugal laurel leaf which has been exposed to the vapour of chloroform.

The mottled green and yellow leaf is turned a dark chocolate brown.

The chloroform injures the vitality of the cells and so

allows of physico-chemical changes which do not occur in normal fresh tissues.

Potato is similarly affected.

But potato also contains another ferment, tyrosinase, as is easily shown.

Here is a slice of potato which has been treated with a small quantity of tyrosin. Note the brown colour produced by the action of the enzyme tyrosinase, which exists in the potato and bean.

The action of tyrosinase is not to be confounded with the oxidising ferments present in most plants, as evidenced by the blueing of guaiacum. Tyrosinase has no action on guaiacum. P-cresol also gives brown with tyrosinase.

Now let us turn to the more difficult problem of colour changes induced by oxidising enzymes, and revealed to us by various indicators.

Numerous reagents have been suggested and employed successfully from time to time for the detection of organic oxidation.

Guaiacum resin was the first reagent employed, and is still in frequent use. I am quite aware of its limitations and fallacies, and that it must be employed with caution.

Although I have tested several other reagents, nevertheless, for the sake of simplicity, and through want of time, I will chiefly resort to guaiacum this evening.

Guaiacum resin contains guaiaconic acid, a body which is very susceptible to oxidation with the production of a fine blue colour.

As a colour reagent, the guaiacum resin should be thoroughly boiled with rectified spirit and animal charcoal, and filtered. This tincture keeps well for several days. The treatment with charcoal removes traces of peroxide which are liable to occur in guaiacum resin, owing to impurities or otherwise (B. Moore).

Examples of the blueing of guaiacum by oxidation, direct or indirect:—

Tinct. Iodi.

Liq. Ferri Perchlor.

Vapour of HNO_3 .

The clinical use of guaiacum + H_2O_2 as a test for blood is

extremely valuable as a negative test, and its failure is certain proof of absence of blood.

The positive reaction given by blood is difficult to account for, and there are several fallacies to be guarded against.

The blood test appears to be conditioned by the presence of iron, for the guaiacum test for blood is given by the iron-containing hæmatin, but not by the iron-free hæmatoporphyrin.

In mollusca and crustacea, hæmocyanin, a copper-containing substance, acts like hæmoglobin as an oxygen carrier (Beatty).

Guaiacum is also blued by pus, milk, and extracts of some animal organs.

Before entering upon the discussion of the mechanism of oxidation processes, it will, I think, be convenient to demonstrate some simple experiments which will bring out clearly the chief points at issue.

If a number of plant tissues, or their expressed juices, be tested in regard to two reagents, viz.: hydrogen peroxide and guaiacum, we can readily distinguish three groups:—

- (a) Tissues which do not blue guaiacum, but immediately decompose H_2O_2 and liberate molecular and relatively inactive oxygen. A very widespread phenomenon and one independent of (b) or (c). It is due to enzyme A. (catalase), *e.g.*, alyssum, iris, tropæolum, wallflower, yeast, soybean.
- (b) Tissues which blue directly with guaiacum, *e.g.*, potato, apple, carrot. These contain enzyme B, which may be associated with the catechol group.
- (c) Tissues which do not blue guaiacum until H_2O_2 is also added. This is termed indirect reaction, *e.g.*, alyssum, wallflower, tropæolum, soybean. These contain enzyme B, but are devoid of the catechol grouping.

How are we to account for these singular facts? The argument runs on these lines:—

If some bodies react directly with guaiacum, and others only indirectly, *i.e.*, after the addition of H_2O_2 , plainly

there must be, in the former case, some substance present which is absent in the latter case, and which can be replaced by addition of H_2O_2 .

(a) In the first class, *i.e.*, decomposition of H_2O_2 alone, the reaction is referred to the presence of an enzyme, because the change is nullified by first boiling the tissue.

This enzyme has been unfortunately named catalase, and is the commonest enzyme met with in plants.

In this connection I wish to observe that the inherent difficulties of the subject are exaggerated by a perplexing nomenclature on which authorities are not agreed. The terms employed are more definite than our present knowledge justifies, and, in my opinion, in the existing fluid state of enquiry it is wiser to abstain from crystallising into words concepts which are not susceptible of proof.

The term "catalase" is especially unfortunate because it lays undue stress on the idea of catalysis, an agency common to *all* enzymes, and it has never been proved to be a distinct enzyme. Whatever the body, or bodies, may be, it is not an oxidising enzyme, and the name is absurd and misleading. If it were not that the term "peroxidase" is commonly used in another sense, the word would be quite appropriate and consistent to denote an enzyme that operates only on H_2O_2 . I will simply call it enzyme A.

Other terms in current nomenclature are also vague and confusing, *e.g.*, oxygenase, oxidase, and peroxidase, and we will do well to avoid for the present all such indeterminate words, which assume more knowledge than we possess, and confine ourselves to the simple description of experimental facts.

Catalase (enzyme A) is widely diffused not only in plants, but also in the animal fluids and tissues. Thus, pus and blood, *e.g.*, briskly effervesce on the addition of H_2O_2 ; also fat and meat. Its function in plant life is not well understood.

Organic peroxides are unaffected by enzyme A (catalase).

(b) Tissues that blue directly with guaiacum alone.

This is an oxidation phenomenon, and is often ascribed to a so-called "oxidase" reaction, *e.g.*, potato, horse-radish.

The term "oxidase" was originally supposed to connote

a specific entity, something or other, which had the capacity, in its own right, to activate oxygen when brought into contact with certain substances.

This conception, however, was neither satisfactory nor illuminative, and failed to account for the difference noted above, between direct and indirect oxidation.

The activity of oxidising ferments is checked by many things, *e.g.*, acids, NH_4SH , and coal gas.

Oxidising ferments are absent from the fresh juice of limes, lemons, and oranges, but are easily demonstrated in the crushed seeds of lemon or orange.

This is a significant fact in relation to the dietetic value of lemons and oranges in respect of diseases such as rickets and scurvy (B. Moore).

Fresh milk frequently responds to the guaiacum test, but Swiss condensed milk and glaxo gave negative results with all tests. B. Moore reminds us that this absence of oxidising ferments distinguishes preserved foods from fresh foods.

From this it appears that among "accessory food substances" we should take into account not only the so-called vitamins, but also the group of oxidising ferments.

(c) Tissues which do not blue directly with guaiacum, but give a blue after addition of H_2O_2 .

It is clear that in these cases a third substance must intervene in the reaction.

What is the nature of this third component?

In the current literature it is styled "peroxidase," but I will merely name it enzyme B.

There is some justification for regarding it as essentially consisting of an active colloidal metallic hydroxide of either Fe or Mn, associated with some stable organic colloid (gum, protein, etc.) which protects the hydroxide from aggregation.

This enzyme operates upon both inorganic peroxides and on organic peroxide-like substances—and liberates from them active oxygen which gives colour reactions with guaiacum and other indicators.

The action of an organic peroxide in the living organism is the most feasible hypothesis which will account for the fact that many of the oxidation processes which take place

in vivo can be imitated *in vitro* only by H_2O_2 , *e.g.*, in diabetes, the oxidation of B-oxybutyric acid into acetoacetic acid. Indole is oxidised into indoxyl in the body, but outside the body by H_2O_2 only (Beatty. *Enzyme Action*, 1917).

Glucose may be oxidised within the body to glycuronic acid, while H_2O_2 is the only reagent capable of effecting this change outside the body.

But it will naturally be asked—Where does this peroxide-complex come in, when a tissue, *e.g.*, potato, directly blues guaiacum without the help of H_2O_2 ?

The answer is that, in such cases, an organic peroxide-like substance is developed, which, under the influence of enzyme B, liberates active oxygen and, hence, blues guaiacum.

In the case of potato and pear, Mrs. Wheldale Onslow has carried out some instructive experiments which show that these tissues contain a colourless aromatic grouping which affords the reactions characteristic of catechol (pyrocatechin), *i.e.*, a dioxy-phenol body. When the injured tissue is exposed to the air it undergoes auto-oxidation by the free oxygen of the air and forms an unstable peroxide-like system.

Then steps in enzyme B, which acts upon this peroxide system, liberates from it active oxygen, and so gives rise to colour reactions.

Enzyme B (peroxidase) is extraordinarily resistant to decay, and the ferment has been recognised in samples of wheat over 200 years old. Peroxidase outlasts the power to germinate by 100 years (Beatty).

Not to weary you with further speculations upon an obscure topic, I will endeavour to summarise what appears to me to be our present stock of knowledge, based mainly upon the work of Benjamin Moore, Bayliss, and Mrs. Onslow.

Take first the action between potato and guaiacum as a type.

We can picture to ourselves the chain of events as embracing 5 links:—

(1) Free molecular oxygen of the air.

(2) An unstable auto-oxidisable substance, the precursor of (3).

(3) An organic peroxide-complex formed by action of (1) on (2), *i.e.*, it does not exist preformed in the plant. In a recent paper Mrs. Onslow postulates a distinct enzyme for effecting this change (*Bio-Chem. Journal*, October, 1920).

(4) Enzyme B (so-called peroxidase of authors), which splits off active oxygen from (3) and accelerates the transfer

(5) Of oxygen to guaiacum, or other suitable acceptor.

No. 4, *i.e.*, enzyme B, is the true active catalytic ferment of oxidation.

It will be remembered that most plants contain enzyme A (catalase), which does not blue with guaiacum; effervesces with H_2O_2 , and is not an oxidising ferment, and has nothing to do with the colour reactions.

Now look at the question from another point of view. We have already seen that the cut or injured tissues of various plants are browned on exposure, and, further, that in response to the guaiacum test they fall into 2 sub-classes, *viz.*: those that give a direct blue with guaiacum, and those which do so only after the addition of H_2O_2 .

The results may be thus tabulated:—

I.	II
Turn brown on injury, or exposure to CHCl_3 . An auto-oxidation process.	Do not turn brown.
Give direct blue with guaiacum.	Do not respond directly to guaiacum.
Contain an unstable aromatic substance of the catechol group which produces a peroxide-like substance under influence of a special enzyme. Contain enzyme B (peroxidase).	Are devoid of catechol substances, <i>i.e.</i> , of peroxide-like bodies. Give blue with guaiacum + H_2O_2 .

The inference, then, is that in Class II. the addition of

hydrogen peroxide supplies the place of the missing link of the catechol grouping, which functions as an organic peroxide. In potato, after extraction with cold alcohol (which dissolves the aromatic body), I found that an aqueous solution of the residue gave, when concentrated, green with FeCl_3 , *i.e.*, evidence of the presence of the catechol grouping. It is better, however, to extract the catechol substance with boiling alcohol.

It is quite probable that traces of organic peroxide substances are present in most plant juices.

A direct positive reaction (Class I.) in the absence of added H_2O_2 indicates the presence of traces of organic peroxide.

B. Moore holds that guaiacum resin itself may sometimes contain small amounts of organic peroxide (due to impurities) and can be freed from this by boiling with alcohol and animal charcoal. Hence, guaiacum alone is not always trustworthy, and requires to be checked by other indicators. Potato and carrot can also be freed from natural peroxide by treating in the cold with animal charcoal and filtering (Moore).

To sum up:—

Among the host of enzymes known to occur in plants, the experiments shown this evening have demonstrated the existence of three ferments:—

1. *Enzyme A* (catalase).—The most common, and almost of universal occurrence. Reacts only with H_2O_2 , and is not an oxidising ferment, and liberates molecular oxygen, O_2 , relatively inactive.
2. *Enzyme B* (peroxidase).—Very widely diffused. Reacts with organic peroxides. Varieties: (a) acts on catechol group; (b) acts independently.
3. *Tyrosinase*.—Not so widely diffused. Reacts only with tyrosin or related *para*-compounds, *e.g.*, *p*-cresol, and gives rise to brown, dark red, or black colours.

Thus we have two tests for tyrosinase:—(1) Forms a dark brown colour with tyrosin; (2) gives dark red with *p*-cresol.

Here I may digress for a moment to point out that tyrosin, a derivative of phenyl-alanin, $\begin{array}{c} \text{<} \\ \text{---} \\ \text{>} \end{array} \text{CH}_2\text{CHNH}_2\text{COOH}$, together with its enzyme, tyrosinase, have several points of contact with human physiology and pathology.

Pure gelatin, as is well known, does not respond to Millon's test, owing to the absence of tyrosin, and this is one reason why, in dietetics, gelatin cannot replace protein food.

In pathology, there is good reason to believe that the mutual action of tyrosinase and tyrosin is responsible for various pigments met with under abnormal conditions. As examples, three instances may be adduced:—

1. Melanuria and Melanotic Sarcoma.—(Specimen shown.)
2. Alkaptonuria, of which I have seen more than one case.—This condition is connected with the presence of homogentisic acid, a descendant of tyrosin. (Specimen shown.)
3. Ochronosis, a rare condition, first described by Virchow in 1866, and characterised by dark pigmentation in cartilage and elsewhere. In 1904, Osler reported 2 cases, in brothers, who also exhibited alkaptonuria. (Plate shown.)

Finally, I may remark that Benjamin Moore draws an ingenious and suggestive parallelism in mode of action between hydrolytic enzymes, oxidising enzymes, and the active bodies developed in immune sera.

This conception brings the class of oxidising ferments into line both with the great division of hydrolytic enzymes engaged in the processes of digestion and metabolism, and, with the active bodies in the natural and immunising sera, which combat and offer defence against disease, so that the oxidising enzymes form a connecting link between the other two classes.

In all three classes of enzymic action it is to be observed that three interacting bodies are required. These three are:—

- (a) The substrate upon which the ferment is to act.

- (b) The body which is to be combined, directly or indirectly with the substrate, and which alters its chemical and physiological properties.
- (c) The enzyme, or ferment, which activates the reaction.

Thus, for example, in the case of the immune sera, cytolysins, etc., we have:—

- (c) The enzyme, or ferment, which activates the reaction of the toxic or foreign substance in the serum to be attacked and rendered inert.
- (b) *Combining body* (complement), or thermo-labile substance, in the absence of which the reaction cannot proceed.
- (c) *Catalyst*, the specific immune body, or anti-body, which attacks and disintegrates the foreign cell, and “ neutralises ” the toxic substances.

The poisons formed by living cells may be termed cytotoxins, and their characteristics remind us of the properties of enzymes. Further, we know that cytotoxins, quite in the same way as enzymes, induce the formation of specific anti-substances when introduced into the blood. So the formation of anti-toxins is strictly analogous to the formation of anti-enzymes; and anti-toxins have the specific effect of rendering the cytotoxins to which they correspond inefficacious (Czapek).