THE PROPERTIES AND CLASSIFICATION OF THE OXIDIZING ENZYMES, AND ANALOGIES BETWEEN ENZYMIC ACTIVITY AND THE EFFECTS OF IMMUNE BODIES AND COMPLEMENTS

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(Received March 15th, 1909)

The presence in plant and animal juices of bodies possessing the properties of ferments which act as oxidizing agents for unstable bodies, such as the guaiaconic acid of guaiacum resin, was first demonstrated by Schönbein¹ in 1856.

The action of these bodies was studied in greater detail by Bertrand² in a series of papers beginning in 1894. Bertrand first showed that the formation of Japanese lacquer was due to the oxidation of a substance present in the plant juice of certain species of *Rhus* by a ferment which he termed *laccase*. The browning and blackening of the cut surfaces of fruits and other parts of vegetable tissues is also due to the action of oxidizing ferments.

From the juice of the potato an oxidizing ferment termed *tyrosinase* was obtained, which acted vigorously upon the isolated tyrosin, yielding a dark brown substance, richer in oxygen than the tyrosin from which it was formed.

These browning or blackening ferments are distinct in nature from the oxidizing ferments present in most fresh plant juices, and also in pus, milk, blood, and extracts of some animal organs, which possess the property of turning tincture of guaiacum blue, of oxidizing and thereby rendering coloured certain phenolic bodies, or of setting free iodine from a solution of hydriodic acid.

Bertrand showed that this latter class of oxidizing ferments was present in many different types of plant, and he expressed the view that they were universally present in all plants.

It is, however, certain that they are not present in all plant juices, although they may be present in other parts of the same plant. Our own experiments recorded below show, for example, that the class of oxidizing

1. *Zeitsch. f. Biol.*, Bd. III, S. 325.

2. *Compt. rend.*, p. 1215, 1894.

ferments is absent in the fresh juice of the fruit of the lime, lemon, and orange, but present in the crushed seeds of lemon and orange. Other experiments have demonstrated to us that their distribution in different parts of a root or fruit varies widely, and that they are present in greatest quantity in the region containing the greatest abundance of respiratory vessels.

Thus a section across a carrot stains almost at once in the protoxylem, and the staining radiates out from this along the path of vessels, while internally to the protoxylem there is very little staining (see Expt. XIX).

It was found by many observers that the guaiacum test for these oxidizing ferments often failed, especially in fluids of animal origin and when freshly made tincture of guaiacum was used for testing.

Further, it was found that in such cases the blueing could still be obtained, provided either hydrogen peroxide were added or some organic form of peroxide, containing oxygen linked in the peroxide form.

A similar oxidation is seen in the well-known test for traces of blood in urine or other fluids in which tincture of guaiacum and ozonic ether (a form of peroxide) are added to the suspected fluid. This test also demonstrates that bodies other than oxidizing ferments occurring in living tissues are capable of giving the guaiacum blueing. Many inorganic and organic bodies also give it, such as sulphurous and nitrous acids, ferrous salts, and potassium permanganate.

The substances causing this reaction may be either oxidizing or reducing bodies, so long as they can act as oxygen carriers.

In this connection it may also be pointed out, as has been done by Bach,³ that all the oxidations shown to occur with this class of oxidizing enzymes, viz., oxidation of hydriodic acid, of the aromatic amines and phenols, and of the guaiaconic acid of the guaiacum resin, possess the common factor of a moveable or *dynamic* hydrogen atom in the molecule. Between this hydrogen atom and the atom of the oxygen in the peroxide form there is already a strong tendency to reaction, and the catalyst simply increases this tendency.

On account of the too great readiness with which the guaiacum underwent the oxidation change giving the characteristic blueing, and also on account of the fact that blood, and other oxidizing bodies not ferments, readily yielded the guaiacum test, other more definite tests were sought out. A considerable number of tests have been described in which phenols or amido-phenols are oxidized to bodies which possess various

3. *Berichte d. deut. Chem. Gesellsch.*, Vol. XL, p. 230, 1907.

colours. These colour tests for oxidizing enzymes are all very similar, and when one of them has been obtained, or not obtained, in any given case, a similar result is usually obtained with all the others, probably because the potential of chemical energy required for oxidation lies at about the same level for all of them.⁴

The results of carrying out a number of these colour tests will be given later, but one important difference which we have found may be stated here in general terms, since, in our opinion, it leads to certain important conclusions regarding the classification and mode of action of this class of oxidizing enzymes different from those generally accepted at the present time. This result is that none of the colour tests, with the exception of the guaiacum test, are appreciably increased in velocity, or catalysed, by the solution of the oxidizing ferment alone, except when the fresh juice is taken immediately after its preparation (see Expt. VII), and are only so catalysed when hydrogen peroxide or some other form of peroxide is added at the same time.

The reasons why the guaiacum test is often positive when the others are negative are (1) the presence of organic peroxide in the guaiacum itself, and (2) that the oxidation occurs much more readily than with the other colour tests with the oxy- and amido-phenols, and such like bodies, and so the naturally occurring organic peroxide of the juice or of the reagent is sufficient to give an oxidation. Whereas the more stable amino-acids only break up with appreciable velocity in presence of the more readily decomposed, and hence more powerful, free hydrogen peroxide, which has been artificially added.

No attempt will be made in this paper to give a full account of the very wealthy literature of the oxidizing ferments,⁵ since accounts in English have recently appeared in 'The Nature of Enzyme Action,' by Bayliss,⁶ and the 'Intracellular Ferments' of Vernon;⁷ but some outline of the classification introduced by Bach and Chodat, and of the basis for that classification, must be given in order that our own views and experiments may be more easily followed. It may be added that this classification has been almost unanimously adopted by subsequent writers.

4. Minor differences in reactivity of the different colour reagents are given in the experimental part of the paper.

5. Citations of the literature are also to be found in the comprehensive review by Bach and Chodat, *Biochemischen Centralblatt*, 1903; in 'Über tierische Peroxydasen,' Ernst von Cзыhlarz u. Otto von Fürth, *Beitrage z. Chem. Physiol. u. Path., Zeitsch. f. Biochemie*, Bd. X, S. 358, 1907; Bach, *Berichte*, 1904 to date; Spence, this Journal, Vol. III, p. 165, 1908.

6. *Monographs on Bio-Chemistry*. Edited by Aders-Plimmer and Hopkins. Longmans, Green and Co., 1908.

7. Published by John Murray for Physiological Laboratory, University of London.

The experimental work of Bach and Chodat, especially that of a quantitative nature on the action of these oxidizing ferments, is, in our opinion, sound, and forms an excellent basis for further work; but we cannot agree that their experimental observations give sufficient ground for the classification they have adopted nor for belief in two classes of oxidizing ferments, the *oxygenases* and the *peroxidases*, which they postulate, forming together a mixture of ferments corresponding to the old *oxidases*.

In our opinion there is but one class of such enzymes concerned, which, since they act only in presence of oxygen linked as a peroxide, might still be known as the peroxidases. The oxygenases do not exist as ferments at all; there is no use for such a term, and it might be allowed to drop out. The oxygenases are simply preformed peroxides in juice or reagent, and not in any sense ferments.

The experimental basis, and as far as we can discover the only one, for a belief in the oxygenases is that certain juices, such as that of the potato, give at once a blue colour with fresh guaiacum alone without added hydrogen peroxide, while other fresh juices, such as those of radish and cucumber,⁸ give absolutely no blue coloration with the fresh guaiacum tincture until hydrogen peroxide, or some other peroxide, is added, when almost at once a strong blue is obtained.

Further, if the potato juice be heated for some hours to about 60° C. it in most cases loses the power of blueing spontaneously, and now only gives the blue colour when a peroxide is added.⁹

The explanation of this is easy on Bach and Chodat's classification, which represents that there are two enzymes present in the juices, viz., *oxygenase* which manufactures peroxides from the material at hand in the juice, and a *peroxidase* which then activates this peroxide and causes it to attack the oxidizable body yielding the colour test and oxidize it, so producing the colour.

We submit that there is no proof that there is an enzyme or enzymes forming the class of the oxygenases and producing organic peroxides, and that the whole difference between the two classes of juices is simply that

8. Bach and Chodat, *loc. cit.*

9. This destruction of organic peroxides (or of oxygenases according to Bach and Chodat's views) is not quite so easy as it is usually described, for potato juice may be briskly boiled in a test-tube for half a minute without destroying completely all the store of peroxide. If it is again boiled, however, or if it be thoroughly boiled for some minutes in a beaker so that all parts are boiled, the peroxide is completely destroyed. This refractiveness was discovered by Woods (*Bull. U.S. Dept. of Agriculture*, No. 18, p. 17) in the case of the tobacco oxidase, and by him attributed to part of the oxidase existing as a zymogen. There is little doubt, however, that the result is due to incomplete destruction of the peroxide and peroxidase.

one has a store of peroxides and the other has not, and, further, that these peroxides are thermally unstable and are destroyed by heat.

After we have put forward the evidence in favour of this view, we shall draw attention to the similarity between this simpler scheme for the activity of the oxidizing ferments, and that for the action of 'immune' body and 'complement' in the case of haemolytic and other cytolytic sera and the cells attacked; and also for the action of ordinary hydrolitic enzymes upon their substrates.

In all the above cases three things are required, viz.:—(1) a body of a ferment nature, (2) a substrate on which it can act, and (3) a body which enables the ferment to act upon the substrate so as to cause hydrolysis, oxidation, or some other type of chemical reaction. Also, the ferment and the substrate are usually much more specific to each other than is the third body, which is simpler in nature, such as an alkali or acid or a peroxide, and we suggest that *complement* is an activating substance of this kind.

Returning to the oxidizing ferments, we may now quote the classification given by Bach and Chodat. In their general review of the subject of oxidizing ferments, published in 1903, these authors give the following list:—

'I. *Oxygenases*. Protein-like bodies which take up molecular oxygen with peroxide formation.

'II. *Peroxidases*, which enormously raise the oxidation power of the peroxides which by themselves oxidize very sluggishly, at the dilutions in question.

'III. *Katalases*, which destroy peroxides with evolution of oxygen.'

In our opinion, all these three names are ill-chosen, because they break the general law that the name should, as, for example, in lactase, tyrosinase and lipase, indicate by the root of the word the substrate acted upon, and by the termination 'ase' the fact that the body designated is an enzyme. Now, in the first two classes named above, oxygen and peroxide respectively are not the substrates, but the bodies which the particular enzymes bring into action upon the substrates, and much more resemble the alkali in trypsin action or the acid in pepsin action. Further, in the third class, the '*katalases*,' there is no reliable evidence of this destructive action upon hydrogen peroxide being due to an enzyme at all. Although it is clearly a distinct activity from that of the oxidizing ferments, yet it is not an oxidation, and it is not specific, occurring, as it does, with every ferment solution of whatever type, with nearly all animal

or vegetable fluids, and with numberless inorganic catalysts. In any case, it is absurd to give it a name which belongs to or includes the whole vast range of catalytic actions. Every true enzyme is a '*katalase*' in the sense that it acts catalytically, and why a catalytic agent, which happens to act upon hydrogen peroxide, and of which it has never been clearly shown that it is a specific enzyme, should be dignified with the name of '*katalase*' it is difficult to conceive. Whatever the body is (or the large number of bodies), it is quite certain no oxidizing enzyme is in question, since nothing is oxidized and the oxygen is simply discharged as molecular oxygen.

The statement which is made by Bach and Chodat¹⁰ that the so-called oxydases are mixtures of *peroxide bodies* and peroxydases, appears to have led some of their English reviewers to entertain the view that they regard the oxygenases as unstable peroxides rather than as true enzymes producing peroxides. But that this is an error is shown by the above classification, as also by the repeated statement in their works that the oxygenases are ferments which *produce* the organic peroxides, and that it is only in the presence of the peroxides that the peroxidases have any action whatever.

Thus the rationale of the complete reaction of oxidation by these ferments, according to Bach and Chodat, is as follows:—

First an enzyme, called *oxygenase*, acts upon certain substances present in the plant and forms organic peroxides; and, secondly, another and distinct enzyme, *peroxidase*, which is entirely unable to act in absence of formed peroxide now comes into activity and causes a reaction which transfers the oxygen, previously attached to the peroxide by the oxygenase, to the oxidizable substrate, which may be one of several substances.

We have also looked carefully through the later papers by these authors, and have not been able to find any abandonment of the position that the oxygenases are peroxide-producing enzymes.

Thus, Bach¹¹ states the oxydases are nothing else than mixtures of peroxydases and oxygenases, that is, of peroxide-activating and of peroxide-building enzymes. In this paper, because he obtained by alcohol precipitation, and re-dissolving in water, a solution which alone only slowly attacked tyrosin, but was strongly activated towards tyrosin by addition of small amounts of hydrogen peroxide, Bach claims to have

10. *Bio-chemisches Centralblatt*, 1903, and elsewhere.

11. *Berichte*, Vol. XXXIX, No. 10, p. 2126, 1906.

shown that the usual tyrosinase which attacks tyrosin at once is also a mixture of an oxygenase and of a peroxidase which is specific for tyrosin. No further proof is here given of the enzymic nature of the supposed oxygenase of the tyrosinase.

Again, in 1908, in a polemical paper against Chodat, who had been unable to repeat his observations as to the activation of tyrosinase by hydrogen peroxide, Bach¹² states that the failure of Chodat to repeat his results was due to the use of too high concentration of hydrogen peroxide, and in this paper he once more enunciates that 'tyrosinase, like the 'ordinary oxydases, is composed of an oxygenase, that is, a body which 'produces ("bildet") peroxide accompanied by uptake of oxygen, and is 'replaceable by hydrogen peroxide, and of a peroxidase, which activates 'the so-formed peroxide, or the added hydrogen peroxide.'

There is proof given by Bach and Chodat that peroxide is present in fresh plant juice which has been treated with air,¹³ but none that this peroxide is produced by an enzyme. The proof as to the presence of peroxide, which is not entirely free from experimental suspicion, was obtained as follows. Air was passed through a sample of fresh juice from *Lathraea squamaria* containing oxydase, and at the same time a 1 per cent. solution of barium hydrate was slowly dropped into the juice. A barium precipitate was obtained which did *not* give the hydrogen peroxide reaction with the titanium reagent, after washing and decomposing with dilute sulphuric acid. The solution did, however, intensely blue potassium iodide and starch, and as the absence of nitrate was shown otherwise, it was assumed that an acylated hydroperoxide was present.

It does not appear to us that much that is new is proven by this experiment, since the oxidase known to be present in the original juice would have similarly acted on iodide and starch solution, and there is no reason why it should not be precipitated unchanged by the baryta.

On other grounds it is highly probable that traces of organic peroxides are present in most fresh plant juices. (See Expt. XX.)

This does not, however, prove that they are formed either in the plant or after separating the juice by an enzyme such as the postulated 'oxygenase' of Bach and Chodat, and all experimental proof of such enzymic origin is hitherto lacking.

12. *Berichte*, Vol. XLI, p. 216, 1908.

13. We shall show in our own experiments later that *excess of air or oxygen*, as by standing in shallow layers in open vessels, again destroys the peroxide first formed, so that once more no result is obtained without added peroxide.

Again, the presence of the elusive oxygenase is not proven by the thermal instability of the spontaneous blueing by guaiacum. For the peroxides are also very unstable bodies thermally, and the failure to obtain a blue with juice and guaiacum alone after heating to 50° — 60° C., and then obtaining it on adding hydrogen peroxide, may easily be due to the destruction of the thermally unstable organic peroxides, and not to that of an enzyme which produces such peroxides.

In fact, the failure of the blueing after heating is all in favour of the simple presence of unstable peroxides. Because, if it were an enzyme (oxygenase) only that were destroyed by the heat, then this enzyme previously to the process of heating would have had ample opportunity to preform a good supply of organic peroxide, and although the enzyme were destroyed on heating to 50° — 60° C., there would be enough preformed peroxide to still give the blueing afterwards.

The simplest hypothesis, accordingly, is that the heating merely destroys the organic peroxides which are essential to the action of the only ferment required, viz., the peroxidase, and on now replacing this loss of organic peroxide by the simple hydrogen peroxide, the blueing is obtained because the essential chemical linkages are present for the peroxidase to act.

In regard to the experimental fact discovered by Bach and Chodat that the juices of certain plants, such as cucumber and radish, do not give a blue with guaiacum alone, but at once give a fine blue on addition of traces of hydrogen peroxide, while other juices, such as those of potato and carrot, give a blue at once, with guaiacum alone, we can completely confirm the experimental observation, but believe it is susceptible of a much simpler explanation than that the potato and carrot contain two ferments, oxygenase and peroxidase, which act as above described, while in cucumber and radish the oxygenase is absent and peroxidase only present.

The oxidation in presence of added hydrogen peroxide is easily demonstrated by means of any of the colour tests, in the many vegetable juices or animal extracts or secretions which contain an oxidizing ferment. But it is an exceedingly difficult matter to trace to their true causes those cases in which a positive result is obtained without the addition of hydrogen peroxide.

Such a positive result is more often obtained when the guaiacum test is employed than with the other reagents we have used for testing. This very frequent positive result is not always due to development of peroxide

in the reagent on standing, as is usually said to be the case, but is present or absent in a most capricious way. In our opinion, the variation in result is due to the presence or absence of preformed peroxide of organic nature in the particular pieces of resin from which the tincture was made, although no doubt there may be a tendency for the amount of this peroxide to increase as the reagent grows older.

The only similar variation with the other test substances we have observed was in the behaviour of potato juice to *p*. phenylene-di-amine in giving the characteristic green without added peroxide of hydrogen when the potato juice was tested immediately after grating the potatoes. Within half an hour the same juice gave not a trace of green until hydrogen peroxide had first been added. Further, even on adding hydrogen peroxide to its juice, if the mixture was left for some few minutes before the *p*. phenylene-di-amine was added no green was obtained. Thus, the peroxide had been destroyed in the meantime, instead of any having been formed as would have occurred if an oxygenase or peroxide-forming ferment had been present.

In this same potato juice, even when quite fresh and giving the *p*. phenylene-di-amine reaction, the positive result of an amethyst colour with α -naphthol could *not* be obtained until hydrogen peroxide had also been added.

With this one exception of the potato juice immediately on preparation, all the colour reagents except guaiacum invariably gave us a negative result until hydrogen peroxide was added. The guaiacum gave us a positive result in the capricious way described above in many instances, such as potato, carrot, wheat, oats, apple, banana, and several species of nuts.

The causes of the variations with the guaiacum will presently be traced out as we followed them up experimentally, but before taking this up we would like to point out that in order to justify the views of Bach and Chodat, that there are two enzymes present, one of which produces peroxides, while the other activates reaction between such peroxides and the oxidizable chromogenic body, the evidence obtainable from the use of different colour-reagents must be consistent and concordant. That is to say, in the same juice one reagent must not indicate the presence of peroxidase only and absence of oxygenase, and another reagent indicate both oxygenase and peroxidase.

Now this is precisely what occurs experimentally, for with the exception of the freshly made potato juice reacting positively to

p. phenylene-di-amine at the same time that it reacted negatively to α -naphthol, the evidence was conclusively negative in regard to 'oxygenase' throughout, save for the capricious results with guaiacum only. If now it be admitted that these variable guaiacum results were due to varying amounts of peroxide in the guaiacum, and hence afforded no evidence of 'oxygenase' in the juice being tested, then all evidence for the existence of oxygenase disappears, and we are left with one enzyme, or type of enzyme only, which produces its effect by activating towards each other peroxide and the substrate to be oxidized.

In support of the view that the different behaviour of guaiacum tincture was due to varying amounts of peroxide in the tincture itself, we submit the results of Expt. XX detailed later (see p. 158), which show that when means are taken to exclude peroxide from both guaiacum and juice being tested, a negative result is always obtained until peroxide has been added from outside.

But if this be accepted we are left only with the positive result with perfectly fresh potato juice and *p.* phenylene-di-amine, and since this rapidly disappeared as the juice stood, and, further, as traces of added hydrogen peroxide similarly disappeared on standing, we are disposed to attribute this effect to traces of peroxide in the fresh juice which disappeared on standing.

If such a ferment as the postulated 'oxygenase' had really existed in the potato juice, then the amount of peroxide would not have decreased on standing, but, on the contrary, there would have been an increase all the time, and the test would have grown stronger instead of disappearing. The disappearance was probably due, in part, to the substances ('katalases') present in all such juices which decompose peroxides after the juice is shed from the cells, and in part due to the using up of peroxide in the oxidations brought about by the tyrosinase and other oxidizing ferments which we have been calling peroxidases.

That the rate of disappearance of peroxide is in some way connected with exposure is shown by the fact that the positive reaction with *p.* phenylene-di-amine is lost in a very short time (inside half an hour) when exposed to the air in a thin layer in a flat-bottomed glass dish, while it is still present after several hours in a sample of the same juice preserved in a tightly corked flask which has been completely filled. A positive reaction to a sample of guaiacum tincture which gives no colour with carrot juice is still given by this potato juice, for long after it has ceased to react with *p.* phenylene-di-amine. This is probably due to two reasons:

first, that the guaiaconic acid is more readily oxidized (or at a lower chemical potential) than the *p*. phenylene-di-amine, and secondly, that there is a conjoined effect of the minimal traces of peroxide in both reagent and juice in one case, and of that in juice only in the other. .

It would thus appear that all those cases in which a colour test of oxidation is obtained without adding hydrogen peroxide are due to peroxide already present in traces either in the juice or in the reagent. Also, that the amount of peroxide in the juice decreases instead of increasing on standing, and that the peroxide which is specially present in varying amount in the guaiacum reagent exists in the resin and its products quite at the beginning, and so may be present in absolutely freshly made tinctures, from which it may be removed by treatment with charcoal.

It was further found that a mere trace of a reducing agent, such as a drop of dilute ammonium sulphide, or sulphuretted hydrogen water added to half a test tube full of a fresh juice showing the guaiacum test without addition of hydrogen peroxide, was quite sufficient to permanently destroy all the trace of organic peroxide present, so that now the reaction became negative.

RECORD OF EXPERIMENTS

The chief substances used by us for demonstrating oxidation were (1) Guaiacum in fresh 10 per cent. tincture made from the resin. (2) A 1 per cent. solution of *p*. phenylene-di-amine in distilled water. This gives on oxidation a fine green colour, which usually strikes out quite suddenly after a pause of several seconds; it occurred positively only after addition of hydrogen peroxide, saving the exception above mentioned of potato juice, where it is obtainable without added peroxide for a period of about half an hour in the fresh juice; after that it is only obtainable when hydrogen peroxide is also added. (3) A 1 per cent. solution of α naphthol in 50 per cent. alcohol. This reagent never gave a positive result with any of the juices, however fresh, until hydrogen peroxide was also added; it strikes a fine amethyst colour in presence of the peroxidase and peroxide; the reagent turns slowly amethyst coloured on standing, and should be made fresh. (4) Indo-phenol or Spitzer's reagent, which we prepared fresh in all cases by mixing equal parts of the two previous solutions of *p*. phenylene-di-amine and α naphthol, and of a 2 per cent. solution of sodium carbonate. This on oxidation yields a fine

purple; the test never gave a positive result unless hydrogen peroxide was added, the development of colour being no greater than occurs spontaneously in the diluted reagent or in *boiled* juice of equal concentration, to which the reagent has been added in equal quantity. (5) Hydrochinon in 1 per cent. solution in water. (6) Synthesized 'guajacol' (Merck). This behaves quite differently from the natural guaiacum resin, giving a strong brown colour, and on standing a brown precipitate, obtainable only in presence of peroxide. (7) Guaiaconic acid (Merck) used in 2 per cent. solution in absolute alcohol.

In making comparative tests care was taken to use as nearly as possible corresponding amounts of juice equally diluted, of reagent, and of hydrogen peroxide where that reagent was added. In the earlier experiments a solution of hydrogen peroxide, the usual laboratory strength (10 vols. of H_2O_2 per cent.) ten times diluted was employed, and in the later experiments the pure perhydrol of Merck was used, first diluted ten-fold, as a stock solution made up in small volumes at a time, and this was then again ten times diluted immediately before use.

As a general rule, about 5 c.c. of juice was taken or a given dilution of the juice with distilled water, and to this about 1 c.c. of the diluted peroxide was added and 1 c.c. of the reagent being used.

Where necessary three tubes were used, of which one contained the juice after boiling, another the juice without boiling, with reagent only added, and a third, unboiled also, to which both reagent and hydrogen peroxide had been added.

In other cases where the juice was of known character and the presence of peroxide was not being proven, but rather the effect of reagents upon its activity, this procedure was not necessary, and the experimentation was modified accordingly.

Experiment I.—Testing of *wheat* for oxidizing ferments. One part of wheat by weight, extracted with three parts by volume of distilled water at 30° C., filtered to an almost clear filtrate—water in contact with the powdered grain for about one hour.

Reagent	1. Boiled extract alone	2. Unboiled extract plus hydrogen peroxide	3. Unboiled extract alone
1. Guaiacum	Blue*	Blue	Blue
2. <i>p</i> -phenylene-diamine	Nil	Green	Nil
3. α naphthol	Nil	Amethyst	Nil
4. Hydrochinon	Nil	Brown	Nil

* This result was afterwards shown to be due to insufficient boiling of the extract.

Experiment II.—Similar experiment with powdered and extracted oats, giving exactly similar results.

Experiments III and IV.—*Swiss Condensed Milk* and a proprietary food called *Glaxo* gave negative results on testing with all the usual reagents. This is to be expected, since all such foods and also canned fruits and patent foods for children and invalids are sterilized by boiling. It is a point worth bearing in mind that this absence of oxidizing ferments distinguishes all preserved foods from fresh foods.

Experiment V.—*Fresh milk*, tested as above to guaiacum, *p.* phenylene-di-amine, *a* naphthol, and hydrochinon, gave a reaction only in presence of added hydrogen peroxide, except in the case of guaiacum, where a slow blueing occurred without the peroxide.

Experiment VI.—*Fresh carrot juice* was positive to guaiacum with and without addition of peroxide, but positive to other reagents only after peroxides. In latter experiments with a guaiacum free from peroxide carrot juice was often found negative, especially after standing in air for some time.

Experiment VII.—*Fresh potato juice* was found the most strongly positive of all the juices tested towards guaiacum; no preparation of guaiacum was used throughout which did not give a blue with it, but when both potato juice and guaiacum were deoxidized as much as possible, the blueing effect practically disappeared. Potato juice when just drawn off is slightly positive to *p.* phenylene-di-amine but negative to the other tests, and turns negative to the *p.* phenylene-di-amine also after about half an hour.

Experiment VIII.—Potato juice was dried in an air oven at about 50 to 55° C. and then extracted with water at 48° C. for forty-eight hours. This very materially reduced the oxidizing power, a faint blueing was still obtainable both with and without added peroxide, but stronger with; hydrochinon both negative; *a* naphthol negative without, slightly positive with; *p.* phenylene-di-amine, both negative.

Experiment IX.—*Wheaten flour* gave strong plus to guaiacum with and without peroxide; faint reactions with other reagents and only in presence of peroxide in each case.

Experiment X.—Serum of pig's blood, three days old, gave negative to guaiacum both in presence and absence of hydrogen peroxide; to *p.* phenylene-di-amine slight reaction without and strong reaction with peroxide; to *a* naphthol, peroxide tube strongly positive, doubtful without peroxide.

Experiment XI.—Effect of minute amounts of acid and alkali and of acid and alkaline phosphates on the oxidizing reactions. This was tested as follows, using guaiacum as reagent:—

The acid stops in minute amounts; much more alkali is required to stop the oxidation, but the colour changes from blue to a yellowish green. The tests were carried out with potato and carrot juices:—

Fresh potato juice diluted ten-fold with distilled water, and 5 c.c. of juice taken to quantity named below of reagent and then 0.5 c.c. of tincture of guaiacum added.

1. Normal control. Blue at once.
2. Added 0.1 c.c. of $\frac{M}{10}$ HCl ($=\frac{M}{500}$ approximately). Faint blue, very slowly deepening.
3. Added 0.1 c.c. of $\frac{M}{10}$ NaOH. As blue as control.
4. Added 0.5 c.c. of 5 per cent. NaH_2PO_4 ($=\frac{M}{25}$ approximately) As blue as control.¹
5. Added 0.5 c.c. of 5 per cent. Na_2HPO_4 ($=\frac{M}{25}$ approximately) Blue much weakened.

1. This reversed behaviour of the phosphatic solutions as compared to the acid and alkali is very peculiar, and difficult to understand, but it was several times observed.

Carrot juice treated exactly the same way gave:—

1. Control. Strong blueing.
2. Added 0.1 c.c. of $\frac{M}{10}$ HCl. No blueing.
3. Added 0.1 c.c. of $\frac{M}{10}$ NaOH. Strong blueing.
4. Added 0.5 c.c. of 5 per cent. NaH_2PO_4 . Strong blueing.
5. Added 0.5 c.c. of 5 per cent. Na_2HPO_4 . Weak blueing.

Experiment XII.—Fresh juice from grated *radishes* was taken and tested to (1) guaiacum, (2) *p.* phenylene-di-amine, (3) *a* naphthol, (4) pyrogallol, (5) indo-phenol (Spitzer's reagent).

The same result was obtained throughout, viz., negative in absence of hydrogen peroxide; positive in presence of the peroxide. There is absolutely no blueing with guaiacum alone, even with guaiacum which alone gives a good blue with potato juice. On standing over night in a stoppered vessel the same radish juice now gives a fair blue with the same guaiacum. The remaining portion of the radish juice was centrifuged, first alone and then after fractional precipitation, with alcohol added up to 25 per cent. in the mixture. It was found that both sediments were very strongly active, much more so than the supernatant liquor.

Experiment XIII.—This experiment was made with *carrot* juice and with *apple* juice. In each case the fresh juice got, as usual, by grating

and filtering was tested alongside an extract made by drying the grated mass at 45° C. for some days. The carrot gratings had been in the oven for three days and the apple gratings for eight days.

The contrast in the filtrates from the fresh and the dried preparations is most striking. The dried is throughout completely negative, and the fresh positive. With guaiacum alone there is little blueing, even in the case of the fresh juices, but immediate effect with addition of peroxide; in neither case is there an effect with the filtrates from the dried materials. With *p.* phenylene-di-amine, *a* naphthol, and indo-phenol there is a very strong reaction, but only in presence of hydrogen peroxide with the fresh preparations, and nothing with preparations from the dried material.

Experiment XIV.—Effects of partial or fractional precipitation of potato juice with alcohol. The properties of the different alcoholic precipitates, and results of combustion of the dried alcoholic precipitates.

A quantity of 320 c.c. of fresh potato juice was taken and thoroughly centrifuged. The deposit consisted of starch underneath with a thinner greenish brown layer on top like very fine mud, or ooze. The upper layer could be easily washed off from the strongly impacted starch granules underneath.

It was so removed, shaken up with distilled water, re-centrifuged, and separated again from the small amount of starch mechanically removed with it at the first separation. It was once again shaken up with distilled water, and the brownish colloidal solution or suspension which frothed strongly was found to be strongly active, giving a good blue with guaiacum alone. Examined under the microscope it shows a field crowded with exceedingly minute particles much less than 1μ in active Brownian movement.

This deposit is like an excessively fine mud, which readily passes into suspension; it closely resembles the different fractional precipitates with alcohol, about to be described.

The supernatant fluid after centrifuging was still opalescent and gave a strong blue with guaiacum, to this one-quarter of its volume of absolute alcohol was added, making a 20 per cent. alcoholic solution, in which a copious greyish brown precipitate appeared.

Throughout it was found difficult and tedious to separate this exceedingly fine precipitate by filter and pump, and that they settle excellently and quickly into a compact mass with the centrifuge so that they can readily be separated by decantation.

The precipitate so separated from 20 per cent. alcohol on shaking up

with a considerable quantity of water gives a fine and durable suspension, which gives a fine blue with guaiacum alone and strongly positive tests with the other reagents after addition of hydrogen peroxide. The supernatant fluid is still opalescent and still gives a good blue with guaiacum. A quantity amounting to 350 c.c. of this supernatant fluid was taken, and to it 88 c.c. of absolute alcohol was added, bringing up the alcohol concentration to 36 per cent., giving rise to a considerable amount of a second alcoholic precipitate, which, on separation and shaking up with water, was likewise found to be strongly positive. Separated supernatant fluid again taken and treated with one-fourth its volume of absolute alcohol, so bringing strength up to approximately 49 per cent. by volume; the third alcoholic precipitate was obtained, also giving fine blue with guaiacum alone and positive result with other reagents *plus* hydrogen peroxide. Again added one-fourth volume of absolute alcohol to supernatant fluid, bringing percentage of alcohol up to 60 per cent., and giving fourth alcohol precipitate, which also gives strong positive tests.

Finally, this supernatant fluid, which was now quite clear of suspended particles, had an equal volume of absolute alcohol added to it, so bringing the alcoholic content up to 80 per cent. by volume. A heavy flocculent precipitate was so obtained and separated by centrifuging and decanting. The supernatant liquid now gave no positive tests, but the fifth and last precipitate did give such positive tests after suspending by shaking in water.

All the different precipitates caused by successive additions of alcohol are alike in appearance, all contain oxidizing enzyme, and all give similar composition on combustion; they are, moreover, exactly like the suspension naturally present in the potato juice and partially thrown out by the centrifuge. All the precipitates have the appearance of a fine alluvial mud, and pass up into a fine suspension with fine particles in Brownian motion when worked up into water. This suspension remains permanent over night, and settles very slowly. Notwithstanding this, quite clear solutions yet exceedingly active as oxidizers are obtainable when fresh potato juice is allowed to stand untouched for twenty-four hours, so that there must also be a portion of oxidizing ferment in true solution. It is probable that a part of the fine deposit can pass into solution and show oxidizing properties, for even after thorough washing the suspensions show strong oxidizing properties.

An explanation of the peroxidase results obtainable both with the water clear juice, and with the thoroughly washed colloidal suspension

of the same juice may possibly be that the oxidizing ferment exists in two phases, a portion being in true solution and a portion in excess as colloidal particles in fine suspension.

Iodine gives no distinctive colour, although the suspensions show marked reduction with Fehling's solution.

When the neutral suspensions are boiled there is very marked frothing, the test-tube filling with froth. Part of the emulsion breaks down on boiling, but a good deal still remains as a milky suspension. After just boiling, there is a very slow blueing with guaiacum alone, much increased on adding hydrogen peroxide, and on further boiling for about one minute, all oxidizing power is lost in either presence or absence of peroxide.

Addition of a trace of hydrochloric acid causes no immediate effect, but on standing for a few minutes the emulsion settles completely and the supernatant fluid has lost all trace of oxidizing properties with or without peroxide.

A trace of alkali does not cause loss of oxidizing properties, but a greenish yellow shade appears in the blue colour produced, more alkali inhibits the activity and destroys the ferment.

In a second experiment with potato juice a similar fractional precipitation with alcohol was carried out after all starch had been thoroughly thrown out by prolonged centrifugalization, and then, instead of taking up in water, the several precipitates were dried in a desiccator over sulphuric acid to a constant weight, and the dried scale preparations so obtained were first examined qualitatively by the protein tests, tested for phosphorous and manganese, and a portion was combusted of each in order to ascertain if the composition varied as the percentage of alcohol was increased. Similar testing was also carried out with similar precipitates from turnip and carrot juices.

The following table shows the ultimate analyses of the precipitates caused by the alcohol in the potato juice after drying to constant weight; the first precipitate, with 20 per cent. of alcohol, was too small for analysis:—

COMPOSITION OF DIFFERENT OXIDIZING PRECIPITATES THROWN OUT BY THE GIVEN PERCENTAGES OF ALCOHOL FROM FRESH POTATO JUICE

No. of pp. and percentage of alcohol by volume in fluid from which thrown out		Ash Percentage	Percentages reckoned in ash-free substance of—			
No.	pp. from 36 per cent. of alcohol		C	H	N	O, etc.
2.	pp. from 20 " "	7.07	48.9	7.3	10.2	33.6
3.	pp. " 49 " "	4.71	46.9	7.0	11.1	35.0
4.	pp. " 60 " "	10.96	46.3	7.2	12.2	34.3
5.	pp. " 80 " "	29.89	45.1	6.9	9.9	38.1
Average for organic material		—	46.8	7.1	10.8	35.3

It is to be observed that while the ash varies considerably, the composition of the ash free organic matter remains fairly constant. The composition approaches more nearly that of the mucins or gluco-proteins than that of any other class of bio-chemical bodies. In this connection it is noteworthy that the suspensions in water of all the precipitates very strongly reduce Fehling, and they are also thrown down as above stated by very dilute acids, and even the less strongly ionized organic acids, which destroy their oxidizing activities. On the other hand, as we shall point out, the precipitates either fail altogether with certain of the protein tests, or in other cases indicate traces only of proteins.

The ash always contained manganese, as shown by Bertrand, and iron in small amount, and phosphates in fair quantity were invariably present after incineration and applying the molybdate test.

As a general rule, a biuret test could only be obtained after boiling, and then only a trifling show of colour, nothing whatever of the colour given by free protein being seen in the cold. The xanthoproteic test was negative in the cold, and after boiling only the palest yellow was obtainable, but a certain amount of deepening towards an orange colour was obtained on adding ammonia in excess. The Millon's reagent gave quite negative results throughout.

Our general impression from the qualitative protein tests is that protein is absent in these highly active precipitates, although there is a high nitrogen percentage, as shown by the above figures, which indicate a carbohydrate moiety joined on to a radicle rich in nitrogen but not containing the groups which are present in ordinary protein and give the colour tests.

In order to find out whether prolonged drying to a constant weight over sulphuric acid in the desiccator had any effect upon the oxidizing effects, the remaining portions of the four dried precipitates which yielded the analytical results given above, and which had been kept dry from November 11th till November 26th (fifteen days), were taken and ground up in distilled water. All yielded again very fine emulsions like those originally obtained, showing exceedingly minute particles, under the one-sixth objective, in active Brownian movement. The only change from the original condition is that now scarcely any blueing is obtained with the guaiacum test until hydrogen peroxide also is added, when a fine blue is obtained; this shows that all the preformed peroxide of the fresh juice has disappeared, but that the peroxidase enzyme is still present and active and ready to induce interaction between substrate and peroxide. The

other colour tests are, as usual, completely negative in absence of hydrogen peroxide, and strongly positive in its presence.

Carrot juice behaves much like potato juice in regard to fractional precipitation by alcohol, all the precipitates being less in amount from the same amount of juice, and all being active; the 80 per cent. alcohol finally left after precipitation is inactive. With turnip juice nearly all the oxidizing ferment comes down in the third fraction (57 per cent. by volume), and the 80 per cent. supernatant fluid is clear and free from oxidizing action. The precipitates by showing a reducing action on Fehling, and by failure to give protein colour tests, behave exactly identically with the potato juice precipitates.

Experiment XV.—Effects of exposure to oxygen, and of shutting off from air, or of exposing to air in shallow layers respectively.

Here we came across one of the most puzzling and difficult to understand of all our results. So far as we are able to follow them, there appear to be two opposed effects, one of a more rapid formation of peroxide *in very small amount* from some precursor in the very fresh juice, and the other of a slow breaking up of the preformed peroxide which is slowed or avoided in tightly sealed up full vessels.

For example, a freshly cut surface of cucumber, radish, carrot, or certain other vegetable tissues shows no blueing with a guaiacum preparation which is itself free from peroxide either naturally or by special treatment. (See Expt. XX.) But merely grating the cucumber to get the juice and filtering from debris of tissue through a clean cloth is enough to give origin to a certain amount of blueing power in absence of added hydrogen peroxide. Further, vigorous shaking up of this juice with air, or bubbling oxygen through it for some minutes, materially increases the amount of natural peroxide, and hence the blueing effect with guaiacum alone. Now, if the juice be separated into two parts, one of which is used to fill a small bottle, which is then tightly corked, and the other is spread out in a thin layer on the bottom of a Petri dish, then, entirely contrary to what might have been anticipated, the tightly corked up sample juice will be found after a few hours, or even after a couple of days, to give a good blue with guaiacum alone, while the juice exposed to the air will probably have lost all blueing power except on addition of hydrogen peroxide. This change occurs probably in a few hours, certainly within twenty-four hours. Hence, in the early stages shaking with air develops peroxide, but the peroxide formed is unstable in presence of atmospheric oxygen, which ultimately robs the juice of all its small store of peroxide.

Not only can the exposure destroy the natural peroxide, it (or some peroxide-destroying enzyme such as catalase present in common solution) can also destroy added hydrogen peroxide, for if to a juice which is not giving an oxidizing reaction with guaiacum alone one adds a minute amount of hydrogen peroxide, sufficient to give a good immediate blue on further addition of the guaiacum reagent, and if now the juice so treated be divided into two equal parts, to one of which guaiacum is added, at once giving a good blue, and the other allowed to stand for five minutes, then it is found that no colour is given by the latter owing to destruction of the added peroxide in the interval.

That this is the true explanation is shown by the immediate blueing obtained on adding more peroxide after the guaiacum has failed to give the colour in the second portion.

By taking a large quantity of juice, adding small quantities of hydrogen peroxide at a time, and testing small portions at intervals after each addition of peroxide, this peroxide destruction may be followed out as often as desired.

This experiment further demonstrates that there is no firm linkage between ferment and peroxide such as can prevent the latter from destruction, for then enough peroxide would be permanently preserved linked on to the ferment to give a blue colour at once when the substrate (guaiacum) was added even after a time interval.

Experiment XVI.—Effect of reducing agents upon ferment and oxidizing reactions.

The oxidizing ferment is extremely susceptible to the merest traces of reducing agents, such as sulphuretted hydrogen or ammonium sulphide, for the addition of a drop of diluted ammonium sulphide or sulphuretted hydrogen water to a half test tube full of potato juice will not only stop blueing with guaiacum alone, but will prevent it even in presence of a large excess of hydrogen peroxide. The ferment appears to be completely and irrecoverably destroyed, either by molecular change or by firm anchorage of the sulphide. At any rate, we have never been able to restore activity once the reducer has been added.

Experiment XVII.—Thermo-labile nature of the peroxides.

Except that we prefer to call it peroxide instead of oxygenase, we can experimentally confirm the statements of Bach and Chodat, that the substance giving oxidizing reactions without added hydrogen peroxide is less stable than the ferment or peroxidase which gives rise to the oxidation in presence of peroxide. We have pointed out that our dried precipitates

from alcohol had lost practically all their peroxide, although they had plenty when freshly thrown down and at once taken up in water. Heating to about 55° C. destroys the unstable peroxide but leaves the peroxidase; this occurs more quickly in some juices than in others. Thus, potato juice takes some hours, and even then although the amount is very greatly diminished a slight trace remains which is most difficult to get rid of. For example, a portion of freshly prepared potato juice was divided into eight similar portions, which were placed in an air oven kept at 55° C. on February 7th, 1909, at 12-20 p.m., and the tubes were tested one at a time at the following intervals afterwards:—Tube 1, tested at 12-45, still gives intense blue immediately; tube 2, at 1-20 p.m., blue not so intense and comes more slowly; tube 3, at 2-20, more slowly still; tube 4, at 3-50 p.m., still a good deal of blue, partial precipitation by the heat; tube 5, at 4-30 p.m., still blue; tube 6, at 11-40 a.m. (February 8th), very faint blue only coming after prolonged shaking; tube 7, at 3-40 p.m., no longer gives blue until hydrogen peroxide has been added. Thus a period of about twenty-seven hours was required to destroy all the natural peroxide at 55° C.

The same result occurs more slowly, as pointed out, on standing in air at ordinary temperatures, and here again potato requires longer than any other juice we have experimented with, taking several days, while carrot juice will be almost free within twenty-four hours. We have not investigated whether this is due to a larger original supply, or to slower reduction or greater resistance of the natural potato peroxide. Great variations are experienced of a perplexing nature both as to the rate at which the maximum amount of peroxide is developed, and the rate at which it disappears, for which we are quite unable to account.

Experiment XVIII.—Effects of germination on distribution of oxidizing ferment and peroxide.

A small quantity of oats, from the same sample as the grain used in Experiment II, were sown on moist cotton wool, and left in the incubator to germinate from November 4th till November 16th, being kept moist during the period and at a temperature of 32° C. At the end of the period the sprouts were about 10 centimetres long. They were pulled out from the oats, and the sprouts and residues of seeds were tested for oxidizing ferment with the following results:—

Both gave a positive result with ordinary guaiacum tincture alone; both were quite negative to all the other coloured indicators for oxidation until hydrogen peroxide had also been added, and both contained

'katalase,' as shown by vigorous discharge of oxygen when added to hydrogen peroxide (5 vols. per cent.).

Experiment XIX.—Absence of oxidizing enzymes in the fresh juice of lime, lemon, and orange; but presence of such in seeds of lemon and orange.

One of our incentives in commencing this research on oxidases was the necessary absence of oxidizing enzymes by reason of the sterilization, or other form of preparation, from all preserved foods, vegetables, and fruits, as also from sterilized milk and milk substitutes, such as children's and infants' food. While such enzymes are present, and in considerable quantity, in fresh vegetables and juices, in fresh fruits eaten uncooked, and in fresh unboiled milk.

This is a difference of a tangible nature in the chemistry of food, of these two classes, and it appeared possible that it might give some basis for a better understanding of the etiology of such diseases as rickets or scurvy, the former of which is said to be associated with exclusive use of boiled and proprietary foods, while the latter, which is, however, a distinct condition, appears to arise from prolonged abstention from fresh vegetable food.

It was these considerations which led us to try those antiscorbutic vegetable juices which are accredited with the most powerful properties, such as lime juice, lemon juice, and orange juice.

We were greatly surprised to find that this group amongst the many which we tested was the only one which did not yield good oxidase reactions either in presence or absence of peroxide. Entirely negative results were given both by juice and rind, and the crushed seeds only in the case of lemon and orange (we were unable from lack of material to test the lime seeds) gave a somewhat feeble positive effect.

The absence of oxidase in the juice of the fruit of this group is very interesting, although we are at a loss at present to account for it. The peroxidase is not simply concealed by the somewhat high acidity of the juices, for no more success is obtained in the testing on neutralizing the juice. It may be that the organic acids present inhibit the production of the ferment, as they certainly inhibit its action when added from without, and tend to destroy it. Thus, if a quantity of potato juice be mixed with the acid (or even almost neutralized but still faintly acid) lemon juice, on now adding guaiacum a negative result is obtained; but if the acid be completely neutralized the usual blueing follows, especially on adding hydrogen peroxide.

In testing the lime juice care must be taken to use a sample which

has not any sulphurous acid as preservative, as this gives a transient oxidation and blueing with guaiacum even in minimal amounts.

The crude juice gives not a trace of effect with any of the tests for oxidizing enzymes.

Experiment XX.—Direct application of guaiacum test for peroxidase and peroxide, to fresh cut surface of vegetables.

This was carried out by slicing, after thorough washing of the outer surface, and then applying an 'old' solution of guaiacum to one surface and a 'new' solution of guaiacum to the other surface. The test is a convenient one for peroxide and peroxidase distribution in different parts of the tissue, and also demonstrates that even in those plants which yield most easily the reaction with guaiacum alone, such as the potato and carrot, the natural organic peroxide is not present as such in the plant tissue, but is formed in the first few minutes after cutting from some precursor in the plant juices.

It may be pointed out that although the terms 'old' and 'new' are used in describing the results of this experiment, this is done because at the time we thought the difference was due to age of the two tinctures. Later we found (see Expt. XXI) that the amount of peroxide present in any given sample of guaiacum was more an accident of amount of impurities of vegetable origin in the piece of resin from which it was originally made up. Accordingly 'old' means containing more peroxide and sufficient to give direct test in presence of peroxidase, and 'new' means comparatively peroxide-free, and hence enables to give blueing with peroxidase in absence of added hydrogen peroxide.

A potato was taken, washed thoroughly, dried, and sliced through with a clean knife. *At once* tincture of guaiacum was placed on the two cut surfaces; 'old' guaiacum on one, and 'new' guaiacum on the other. The surface exposed to the 'old' guaiacum blues instantly all over; that with the 'new' guaiacum only at one or two spots and much more faintly. The blueing with the 'new' tincture occurs more especially at a bruised spot or just close to the epidermis. On cutting away about a centimetre all around the peel to remove any injured portions, only a very slight blueing is obtained, for a few minutes, but comes on standing.

In order to test whether the residual amount of blueing in the case of the potato were due to age, as the potato had been removed from the earth for some days or weeks, and since quite negative results had previously been got with fresh radish and fresh cucumber treated with 'new' guaiacum tincture, the following similar experiment was next tried.

A fresh clean carrot was taken, a sectional cut made across it, and the two clean cut surfaces were treated one with 'old' and the other with 'new' guaiacum.

The 'old' tincture caused instant blueing, the colour being most marked for about a zone of half a centimetre thickness round the outside, and again most marked in the protoxylem, the central part is less intensely stained, but the whole surface is still distinctly blue.

The 'new' tincture, on the contrary, gives for over ten minutes' time after first application no blueing except at two minute spots where there happen to be old bruises just under the skin; ¹⁴ at about the expiry of ten minutes a faint blue appears as a ring sending out radiating branches, or veins distinctly marking out the protoxylem. It is noteworthy that this is the part of the section where most oxygen would probably be present in the plant tissues.

On adding to the section of fresh carrot treated with 'new' guaiacum tincture, a minute trace of hydrogen peroxide, by touching with a fine glass rod moistened with the diluted peroxide, there is produced at once an intense blueing, showing the failure to obtain blueing initially is due to absence of peroxide and not to absence of ferment.

Experiment XXI.—Effects of destruction of peroxide in both reagent (guaiacum tincture) and vegetable juice by treatment with animal charcoal before use.

This experiment was conducted in order to make a closer study of certain results of the previous experiments which show rather perplexing variations in behaviour towards the guaiacum test (*a*) with different types of vegetable juice, or the juice of the same origin with the age of the juice, and (*b*) taking the same juice and at the same time, variations according to whether one or another tincture of guaiacum made from the same stock of the resin was employed for the test.

With the single exception of potato juice, *for an hour or so after it is made*, giving a positive result with *p.* phenylene-di-amine without addition also of hydrogen peroxide, all the oxy- and amido-phenol coloured indicators of oxidation had concordantly given that a ferment peroxidase alone was present, which required to have peroxide added in order that it might act.

The guaiacum test, on the other hand, as above indicated, gave most

14. A similar application to a section of an apple showing a bruise did not, however, demonstrate any more marked blueing in the neighbourhood of the injured and browned cells. Still, the browning naturally occurring must be an oxidizing effect upon tyrosin or similar chromogenic substances giving colour on oxidizing.

perplexing variations. Thus, even with fresh guaiacum and potato juice as fresh as it can be separated from the grated tuber, a considerable blueing was obtained; fresh carrot under like conditions sometimes gave a blue fainter than that with the potato, sometimes nothing; and fresh cucumber or radish juice, even with moderately stale guaiacum tincture, gave but a poor effect and nothing with fresh tincture.

The most feasible explanation of these peculiar variations which occurred to us, and that which suggested the following experiments, was that there are two natural sources of peroxide, viz. :—(a) the plant juice, and (b) the tincture of guaiacum. The summation of these two effects causes the guaiacum test to be positive, especially with a strongly peroxide containing tincture, in cases where the small amount of peroxide in the juice is insufficient yet to start the oxidation of the oxy- and amino-phenols. In further support of this, the potato juice, which of all the juices we examined is most strongly positive to guaiacum alone, reacts positively, when it is quite new and highest in its content of natural peroxide, to *p*. phenylene-di-amine, but just fails to appreciably quicken the oxidation of *a* naphthol, unless hydrogen peroxide be added.

We hence had to examine separately the tincture and the vegetable juices, using mildly destructive agents for peroxides which would not destroy the ferment also. Direct reducing agents, such as sulphuretted hydrogen or ammonium sulphide could not be employed, since, as above stated, they appear permanently to destroy the ferment also, either by altering its constitution or by firmly linking on to it.

An attempt to clear the guaiacum of colour by means of animal charcoal although it failed in its immediate objective, giving a much deeper green coloured solution, happily had the advantage of destroying the peroxide of the tincture, and yielding a clear filtrate which had no power of blueing until hydrogen peroxide was also added.

The usual explanation of the variations in peroxide content of guaiacum tincture is that the peroxide is formed as the tincture stands, and hence, for purposes of the test, that freshly made tinctures must always be employed.

While we are not prepared to deny that peroxide may be so formed, as the tincture stands and darkens in colour in the course of several weeks, we have clear evidence that this is not the main source of the variations, and that a quite freshly made tincture just filtered off may give marked blueing, while another sample of tincture a week old may give quite a negative result, although it was made from some pieces of the same stock of resin.

For example, we had in hand two samples of guaiacum tincture, both made with the same absolute alcohol in 10 per cent. solution from the same stock of guaiacum resin, but one was made about ten weeks previously and the other four days previously. The 'old' tincture gave fine blue with all our vegetables on the cut surfaces, as detailed in Experiment XIX, and with the separated juices, while our 'new' tincture gave scarcely a trace of effect. In order to make sure that it was a question of ageing and accompanying peroxide formation, we determined upon making an absolutely fresh preparation, and for this purpose we took some pieces of the guaiacum resin, and washed the outer surface carefully with alcohol in order to remove a green powder which forms on the other surface of the broken guaiacum, and which we thought might be oxidized. These washed pieces were dissolved as far as they would dissolve to make a 10 per cent. tincture in previously boiled absolute alcohol, and a clear filtrate was obtained and used immediately for testing.

To our great surprise, this gave at once a fine blue immediately with the sections of vegetables and juices without adding any peroxide whatever.

We therefore had now a tincture just made which reacted positively without added peroxide, a four days old tincture made without any special precaution which acted negatively under like conditions, and a ten weeks old tincture which behaved exactly like the just made one.

Further, a tincture made from the green powder on the outside of the pieces of guaiacum gave, like the four days old tincture, a negative result.

It was also noticed that while the ten weeks old tincture and the 'new' tincture possessed the deep yellow brown colour of guaiacum tincture, the four days old tincture was much paler in colour, and the tincture made from the surface powder was green in colour. This led us to boil up some of the 'old' tincture with animal charcoal, in order to try to decolourize it, when we found that instead of decolourizing it went darker, and the filtrate had a green colour.

This filtrate tested now upon carrot juice gave no blueing until hydrogen peroxide was added, showing that its peroxide had been removed by the charcoal; it still, however, gave a blue with potato juice alone. Boiled up once more with charcoal, and again added to potato juice, it still caused blueing, but only slowly and less intensely. This final amount of blueing was probably due to the peroxide in the potato juice itself (*vide infra*).

On examining now the residue left behind on the filter paper in filtering the alcohol extract of the guaiacum resin before the addition of animal charcoal, the chief source of the peroxide of the guaiacum tincture, and the cause of the variations was discovered.

The greater part of this residue consisted of broken seeds and tests of seeds, and other vegetable material. These impurities evidently had been collected with the resin, and since, like all such fresh vegetable material they would contain peroxides, it can easily be understood that they would yield peroxide to the tincture. Also, in making up tinctures from the same stock, the amount of such impurities varying in different pieces, would explain the variations in degree of spontaneous oxidizing power shown by the different tinctures in absence of added peroxide. This all the more so because the pieces of seed are quite large, and whole seeds even were seen, much larger than barley seed. It was on this account, in all probability, that the green outside powder was free from peroxide, and by accident, probably, some pieces more free than usual had been picked up for the preparation of the four days old ('new') tincture.

We would accordingly recommend that in preparing a guaiacum tincture for peroxidase experiments this débris be avoided, and that the filtrate be thoroughly boiled with animal charcoal and filtered. Under such conditions the filtrate can be safely used for several days at least, and any spontaneous blueing observed may be certainly set down to the action of peroxide in the juice or vegetable tissue being tested, and not in the guaiacum itself.

The view that the animal charcoal acts by destroying peroxide in the guaiacum is supported by the fact that animal charcoal added in small amount to diluted (1 in 10) hydrogen peroxide solution causes an immediate effervescence.

Having ascertained that the peroxide could be discharged from guaiacum tincture by animal charcoal, we turned the method upon those vegetable juices giving blueing alone, such as potato and carrot, and found that here the peroxide could also be removed by treating in the cold with animal charcoal and filtering.

Carrot juice, potato juice, and a sample of tincture of guaiacum which gave a good blue with each of them without hydrogen peroxide, were severally treated at laboratory temperature with animal charcoal and left to stand for one and a half to two hours, then they were filtered, the guaiacum having turned green in colour.

The carrot juice so treated when now tested with the ordinary untreated guaiacum gives a dirty greyish green instead of the previous deep blue, whereas with the charcoal treated guaiacum it no longer gives a trace of blue or green. Here peroxide has been completely destroyed both from juice and reagent.

The potato juice which before gave an intense blue when now, after charcoal treatment, tested with the ordinary untreated guaiacum gives a dirty greyish blue, and when both charcoal treated potato juice and charcoal treated guaiacum are used only a grey with the faintest suggestion of blue is obtainable.

This experiment, therefore, supports the view that a positive result with the guaiacum test in the absence of added hydrogen peroxide, indicates traces of peroxide either in the guaiacum or in the plant or other juice being tested. By the charcoal treatment the guaiacum can be freed of peroxide, and then the test may be utilised as a fairly delicate one for peroxides naturally occurring in plants, or formed soon after the juice is set free.

Experiment XXII.—Dialysis in parchment paper.

A quantity of 500 c.c. of fresh carrot juice was placed in a sausage tube of parchment paper, and dialysed against 1,500 c.c. of distilled water in a tall cylinder. Next morning the outer fluid gave with guaiacum (ordinary) alone a slow but distinct blueing. This subject, however, requires further investigation.

Experiment XXIII.—Supplementary experiment on presence of oxydases in bananas, in certain species of nuts, viz., Spanish chestnut, almond, filbert, walnut and Brazil nut and in hyacinths.

None of these gave positive results with the phenol or amido-phenol derivatives, or with guaiacum free from peroxide, except when hydrogen peroxide was added, when they reacted in varying degree.

In the case of the banana, the inner surface of the peel rapidly blues with peroxide containing guaiacum, but the pulp only blues very slowly, and the reagent applied to the cut surface shows marked veining accompanying the course of vessels.

Cross sections of a Spanish chestnut, tested at once with both peroxide containing and peroxide free guaiacum, show blueing with the former at once, but no blueing with the latter, even after ten minutes. The blue first produced by the peroxide containing guaiacum bleaches in a few minutes, but can be brought out again even more strongly by re-applying more of the reagent; this is repeated several times. On touching a spot

on one of the sections treated with peroxide free guaiacum with a glass rod previously dipped in dilute hydrogen peroxide solution, there is an instant blueing at this spot.

Brazil nut gives exactly similar results. Almond, filbert and walnut give no colour with peroxide free guaiacum. Walnut and almond give a slight colour, filbert rather more colour, with peroxide containing guaiacum. Haricot beans, old and very dry, gave no colour either with peroxide containing or peroxide free guaiacum, but an extract gave a feeble positive effect with ordinary guaiacum alone after vigorous shaking, heightened by adding hydrogen peroxide.

The green leaves, bulb, and rootlets of a hyacinth, taken fresh from the garden were examined with peroxide free guaiacum, and only the rootlets gave a rather slow blueing with this alone, but all three parts gave a fine blue on also adding hydrogen peroxide.

Experiment XXIV.—Effect of synthetic guaiacol (Merck).

This reagent behaves quite differently from the natural resin, giving a deep brown colour in oxidation which is very distinctive. Tested with fresh potato and carrot juice it gives no effect until hydrogen peroxide is also added, when the mixture at once turns brown, which deepens in colour to reddish brown, and a reddish brown precipitate is thrown down.

Experiment XXV.—Effect of guaiaconic acid (Merck).

This substance is contained in guaiacum resin and turns blue on oxidation. A supply, obtained from Merck was found to dissolve completely to a brown solution in absolute alcohol. A 2 per cent. solution was used for testing the fresh juice of potato, carrot, apple, and cucumber. The colour obtained was identical with that given by the tincture of the resin, and, as the following results show, it contained a trace of peroxide, about equal to that in the best of the tinctures made from the resin, and distinctly greater than the tincture when freed from peroxide by thorough treatment with animal charcoal.

Potato juice gave with guaiaconic acid alone a greyish blue, deepening on standing; on the addition of a few drops of diluted hydrogen peroxide an immediate blue was obtained, rapidly deepening on standing.

Carrot juice gave practically a negative result with the guaiaconic acid alone, there being only a slight dulling of the natural carrot colour and no trace of blue in fifteen minutes; addition of a few drops of dilute hydrogen peroxide to half of the test gave an instantaneous deep blue.

Apple juice gave with hydrogen peroxide a dirty greyish green, slowly blueing on standing; on adding peroxide also the usual deep blue was obtained.

Cucumber juice gave with guaiaconic acid alone a faint greenish yellow, increasing and showing just a shade of blue on standing; addition of hydrogen peroxide also caused a deep blueing at once.

THE PARALLELISM IN MODE OF ACTION BETWEEN HYDROLYTIC ENZYMES, OXIDIZING ENZYMES, AND THE ACTIVE BODIES DEVELOPED IN IMMUNE SERA

The experiments recorded in the previous section appear to us to demonstrate clearly that the whole difference between the various juices and other fluids showing an oxidizing action consists in the presence of a variable small amount of peroxide which is chemically unstable and destructable by the agencies recorded.

All the juices showing oxidizing properties possess one type of ferment which, since it acts only in presence of either naturally present or artificially added peroxide, may provisionally be styled a peroxidase, and there is no proof of the existence of any other type of enzyme engaged in oxidation processes.

This not only materially simplifies our conceptions regarding the oxidizing ferments, but, in our opinion, brings the class into line both with the great division of hydrolytic ferments engaged in the processes of digestion and metabolism and with the active bodies in the natural and immune sera which combat and immunize against disease. So that the oxidizing enzymes form a connecting link between the two classes.

In all three classes of enzymic action it is to be observed that three interacting bodies are required. These three are (1) the substrate on which the ferment is to act, (2) the body which is to be combined directly or indirectly with the substrate and alter its chemical and physiological properties, and (3) the enzyme or ferment which is to activate the reaction.

In the case of the ordinary hydrolytic enzymes these are as follows:—*Substrate*, the foodstuffs, protein, carbohydrate, or fat; the *Combining Body*,¹⁵ the elements of water finally, intermediately the acid or alkali, in presence of which alone the ferment is active; the *Catalyst*, one of the digestive or other hydrolytic ferments, such as pepsin, trypsin, diastase, zymase, lipase, &c.

15. No generic name, so far as we are aware, has yet been given to this substance usually of a simpler nature than the substrate, which is added to or taken away from the substrate in a catalytic action. We suggest that it might conveniently be called the *Combineate*.

In the case of the oxidizing ferments: *Substrate*, the oxidizable substance, such as tyrosin, naturally occurring phenols in the plant, or the chromogenic indicators used in the preceding section; the *Combining Body*, oxygen yielded by peroxide bodies present in some form, either as simple hydrogen peroxide or as organic peroxides; the *Catalyst*, the enzymes, such as tyrosinase, and the peroxidase experimented with in the preceding section.

In the case of the immune sera, cytolytins, etc.: *Substrate*, the cell or bacterium to be dissolved or the toxic or foreign substance in the serum to be attacked and rendered inert; the *Combining Body*, the complement, or thermo-labile substance, in the absence of which the reaction cannot proceed; the *Catalyst*, the specific immune body or anti-body which attacks and disintegrates the foreign cell or 'neutralizes' the toxic substance.

Between two of these three reacting substances, viz., the substrate and catalyst, there exists usually a considerable amount of specific relationship. This specific relationship is in most cases not narrowed to a single chemical substance, but is closely confined to the members of a class of bodies possessing in their molecular constitution a certain definite grouping. Even very nearly allied groupings are quite inert to the particular catalyst, as, for example, the fermentation by organisms of certain sugars or other substances, which are stereo-isomers of others which are not attacked in the least. But given the *identical* molecular conformation at a certain portion of the molecule, there may be, and will be, attack and chemical action, although the molecular structure at other parts may be such as to render the two bodies in other respects very different physically and chemically.

For example, pepsin and trypsin attack all classes of proteins, down to certain well-marked stages of hydrolytic cleavage so long as certain connections in the molecular aggregate exist, although in physical properties, in reactions to precipitates and indicators, and even in ultimate chemical composition, these proteins are very distinct from one another.

Similarly, the peroxidase ferment in presence of peroxides attacks a large number of oxidizable substances, such as those experimented with in the preceding section, but leaves other classes of oxidizable substances, both those which are more readily and those which are more difficultly oxidizable, unattacked.

Turning to the third member of the group of three, represented by

the alkali or acid in the case of the hydrolytic ferment, by the peroxide in the oxidizing ferments, and by the complement in the immune sera, we find that this is much less specific in character. Thus, an immune body or cytolsin of very specific character may be activated by practically any serum, including the natural serum of the same species as the animal which had been immunized. Similarly, any body containing a peroxide linkage, organic or inorganic, will activate a peroxidase, and any type of acid or alkali which increases hydrogen or hydroxyl ion concentration, respectively, will activate a hydrolytic enzyme.

The ferment character of the reaction in the case of immune sera is also shown by the disproportionately large amount of complement from any normal serum which can be bound to lipoid, and so rendered inert for haemolysis in the second stage of the Wasserman reaction, by very small amounts of syphilitic 'immune' body. In this reaction it is clear that an active principle in the syphilitic serum acts as a catalyst or ferment, the lipoid from liver or elsewhere as a substrate, and the complement from any serum as a combining body with the substrate under the influence of the catalyst.

Finally, attention may be drawn to the similarity between the oxidizing ferments and immune sera in regard to thermo-stability. When an immune serum is heated for some time at 55° C. the complement is destroyed, but the 'immune' body remains untouched; the heated serum, however, is inactive until complement is added by mixing with some unheated serum which may be drawn from any animal.

Quite similarly, when a vegetable juice is heated to 55° C. the peroxide is destroyed, but the ferment or peroxidase is untouched, and although the heated juice is inactive as an oxidizing agent it is at once re-activated on adding hydrogen peroxide or other form of peroxide.

Again, if serum be preserved unheated it slowly loses its complement, and is inactive until fresh complement from an inert serum is added. Similarly, if a vegetable juice be kept it loses gradually its spontaneous power of oxidizing, but regains it as soon as a peroxide is added.