LIII. THE ACTIVATION OF CERTAIN OXIDASE PREPARATIONS.

By CAECILIA ELISABETH MARY PUGH.

From the Department of Physiology, The University of Manchester.

(*Received May 1st, 1929.*)

PART I. ACTIVATION BY PEROXIDASES.

1. *Lactarius* FUNGI.

SEVERAL instances have been noted in the past of the activation by one means or another of a preparation containing some oxidising enzyme. The best-known instance is that described by Bach and Chodat [1902; 1903, 1, 2] of the activation of the oxidase of the fungus *Lactarius vellereus* by peroxidases. The oxidase was obtained by precipitation of the juice of the fungus with three volumes of alcohol. By repeated precipitation of this oxidase in 40 % alcohol, it was separated into two fractions, neither of which showed much oxidising power apart from the other. The one fraction, insoluble in 40 % alcohol, was very strongly activated by peroxidases, and especially by the other fraction of the oxidase, which was soluble in 40 % alcohol. These authors concluded from their observations that the complete oxidase consisted of two enzymes, (1) an "oxygenase" able to give rise in air to a peroxide utilisable by peroxidase, and (2) peroxidase. On this basis they developed their well-known theory of the constitution of the "direct" oxidases.

In their quantitative experiments, Bach and Chodat used pyrogallol as substrate and measured the activity of their preparations by noting the oxygen absorbed. The experiments were carried out without control of hydrogen ion concentration, and no allowance was made for the autoxidation of pyrogallol. Because of this and the advances in our knowledge of oxidases which have taken place since Bach and Chodat published these experiments, certain possibilities arise which must be considered before the explanation given by them can be accepted.

(1) A change in hydrogen ion concentration by addition of peroxidase preparations with a p_H different from that of the pyrogallol-oxygenase solution might of itself cause an increased activity of the oxidase in *Lactarius*.

(2) Hydrogen peroxide might be produced by the action of an enzyme on pyrogallol, the hydrogen peroxide being normally broken down by catalase, but utilisable instead by peroxidase for oxidation of pyrogallol with consequent increase in oxygen absorption.

(3) There might be oxidation by the action of peroxidase and peroxide of substances in the *Lactarius* preparation which inhibit the enzyme acting on pyrogallol, the peroxide being either (*a*) hydrogen peroxide produced by the

action of the enzyme, or (*b*) a peroxide utilisable by peroxidase and produced by autoxidation of some substance in *L. vellereus*. The possibility of this explanation is based on observations by Bach [1909], who found by estimation of unchanged tyrosine that the action of tyrosinase from *Russula delica* on tyrosine was retarded by peroxidase, even, to some extent, in presence of hydrogen peroxide, although the same end-point was approached. He found, however, that if the tyrosinase preparation were old, then peroxidase with hydrogen peroxide caused initial activation. Bach concluded that substances inhibitory to the action of tyrosinase were produced gradually in the preparation on keeping, these substances being oxidisable by peroxidase with hydrogen peroxide.

(4) The presence in peroxidase preparations of something other than peroxidase itself might be responsible for the increased oxygen absorption. A co-enzyme might facilitate the action of a *Lactarius* enzyme on pyrogallol, and if such co-enzyme were deficient in the *Lactarius* preparations but present in the peroxidase preparation added, activation would be produced which would not be due to peroxidase itself.

(5) With regard to the use of pyrogallol as substrate, Bach and Sbarsky [1911] described the formation from pyrogallol of two types of oxidation product, insoluble purpurogallin, and soluble brownish substances which they believed to be condensation products of purpurogallin. The possibility was therefore to be considered of the directing by peroxidase preparations of the oxidation of pyrogallol in such a way as to increase the amount of oxygen absorbed.

(6) Finally, the "oxygenase" of Bach and Chodat might be interpreted as an autoxidisable substance rather than as an enzyme, and without being necessarily a constituent of all oxidases. That "oxygenase" was such an autoxidisable substance was the conclusion reached by Gallagher [1923] in his investigation of potato "oxygenase."

The phenomenon described by Bach and Chodat [1903, 2] has therefore been more fully investigated with regard to these various points.

Enzymes concerned.

Lactarius vellereus not being available, other *Lactarius* fungi were used in this investigation, namely, *L. blennius*, *L. quietus*, *L. rufus*, and *L. turpis*[1]. The action of appropriate preparations from these fungi on pyrogallol was found to be activated by peroxidase from horseradish, in the manner described by Bach and Chodat for the oxidase of *L. vellereus*, but effective peroxidase preparations from these juices were not readily obtained.

These fungi all contained, as likewise described by Chodat [1910] for *L. vellereus*, both tyrosinase and laccase, but in varying proportions. Guaiacol affords a convenient test for laccase, as it is not oxidised by tyrosinase. Quinol, also, is relatively slowly attacked by tyrosinase. *L. turpis* juice caused much less rapid coloration of buffered guaiacol and quinol solutions than of tyrosine

[1] I am indebted to Dr J. Ramsbottom for kind assistance in identifying some of the fungi.

and *p*-cresol, whereas *L. blennius*, *L. quietus*, and *L. rufus* juices all acted very rapidly on guaiacol and quinol relatively to their action on tyrosine and *p*-cresol. The tyrosinase in these juices could be freed from laccase by alcohol precipitation. A preparation obtained from *L. blennius* juice by two precipitations in 40 % alcohol was found to behave towards phenols as does crude tyrosinase from mealworms, having no action on guaiacol with or without addition of hydrogen peroxide. A similar preparation was obtained from *L. turpis* by 40 % alcohol, and from *L. rufus* in the fraction from 65–80 % alcohol. Tyrosinase appears to be less definitely separated from the juices by alcohol precipitation than is laccase. Fractions obtained from 40–60 % alcohol acted strongly on guaiacol, but were not free from tyrosinase. The two enzymes appear to possess differing susceptibilities to cyanide. The action of *L. quietus* juice on tyrosine was found to be 95 % inhibited by *M*/500 KCN, that of *L. rufus* juice 97 %; whereas Wieland and Sutter [1928] stated that the action on quinol of the enzyme from *L. vellereus* investigated by them was only 67 % inhibited by *M*/500 KCN.

After being heated to 90° for 20 minutes none of the *Lactarius* juices used in the present investigation had any appreciable action on either tyrosine or guaiacol; the thermostable catalyst described by Wieland and Fischer [1926] was therefore not present in any appreciable quantity in any of them. The 40 % alcoholic precipitates all contained some tyrosinase and catalase, and some contained laccase. The "peroxidase" fractions of the fungi prepared as described below all contained tyrosinase, and some contained laccase; the guaiacol-hydrogen peroxide test for peroxidase was positive only with *L. blennius* and *L. turpis* "peroxidase."

Experimental procedure.

Juices of *L. blennius*, *L. quietus*, and *L. turpis* were kept in the ice-chest at a slightly alkaline reaction, and acidified before precipitation to imitate as nearly as possible the reaction of the fresh juices. Some *L. rufus* juice was precipitated fresh, and the remainder was kept in the same way as the other juices. "Oxygenase" fractions were obtained by addition of alcohol to the extent of 40 %, the precipitate being centrifuged, dissolved in slightly alkaline water (residue discarded), acidified, and reprecipitated in 40 % alcohol, as many times as possible with the material available. The first 40 % alcoholic liquid after removal of the precipitate was concentrated *in vacuo*, then alcohol was added to bring it to 65 %, the precipitate centrifuged, and alcohol added to the liquid to bring it to 85 %; this last precipitate was dissolved in water and reprecipitated between the limits about 65–85 % alcohol, as many times as possible, the final solution constituting the "*Lactarius* peroxidase" fraction. Peroxidase from horseradish was prepared by the method described by Bach and Chodat [1903, 1], and, unless otherwise stated, such a preparation is what is meant by the term peroxidase.

The action of "oxygenase" fractions on pyrogallol, and the effect thereon of addition of peroxidases, was investigated by the use of microrespirometers

to measure the rate of oxygen absorption. Throughout the experiments the respirometers were shaken in a water-bath at room temperature, in which they were equilibrated for at least five minutes before the shutting of the taps. Corresponding experiments were shaken side by side, and in such experiments identical buffer solution was used. The following mixtures were placed in the bottles.

Respirometer I. Left: 1 cc. 1·2 % pyrogallol in phosphate buffer p_H 6·0; 0·5 cc. "oxygenase" fraction; buffer to 3 cc. *Right:* 0·5 cc. "oxygenase" fraction; buffer to 3 cc. *Respirometer II. Left:* 1 cc. 1·2 % pyrogallol; 0·5 cc. "oxygenase" fraction; 0·5 cc. peroxidase from horseradish or *Lactarius*; buffer to 3 cc. *Right:* 0·5 cc. "oxygenase" fraction; 0·5 cc. peroxidase; buffer to 3 cc. *Respirometer III. Left:* 1 cc. 1·2 % pyrogallol; buffer to 3 cc. *Right:* 3 cc. buffer. *Respirometer IV. Left:* 1 cc. 1·2 % pyrogallol; 0·5 cc. peroxidase; buffer to 3 cc. *Right:* 0·5 cc. peroxidase; buffer to 3 cc. To each mixture a little phenylurethane or thymol was added as antiseptic, and in the small tube of each bottle was placed excess of strong potash to absorb carbon dioxide. The use of pyrogallol solutions stronger than 1·2 % (2·5 and 5 %) did not increase the rate of oxygen absorption.

Any departures from the above arrangements are mentioned in the text. In calculation of the activation of the "oxygenase" fraction by peroxidases, allowance was made for the effect of the peroxidase preparation used on the autoxidation of pyrogallol, as follows:

$$\text{activation ratio} = \frac{O_2 \text{ absorbed in II} - O_2 \text{ absorbed in IV}}{O_2 \text{ absorbed in I} - O_2 \text{ absorbed in III}}.$$

Ratios above unity indicate activation and below unity inhibition.

Control. The presence of substrate was necessary for the phenomenon of activation to occur. Using *L. quietus* "oxygenase" fraction, in absence of pyrogallol, Respirometer I absorbed over the week-end 19·8 mm.^{3} O_2, II 25·7 mm.^{3} O_2, IV 6·4 mm.^{3} O_2.

Experimental results.

Using "oxygenase" fractions from the fungi named, and peroxidase (*a*) from horseradish, (*b*) from the fungus itself, the following results were obtained:

Fungus	No. of precipitations in 40 % alcohol	Duration of experiment in hours	mm.^{3} O_2 absorbed in respirometers I	II	III	IV	Activation ratio
			(*a*) Horseradish peroxidase				
L. blennius	2	6	32·6	42·5	19	15	2·02
L. quietus (1)	4	6	101	288	18·5	11·7	3·33
„ (2)	4	1	313	356	10·5	8·7	1·15
		3	533	667	21	15	1·27
		6	783	1110	29	22·5	1·44
L. rufus	3	6	371	401	30	23	1·11
L. turpis	2	6	55	58	38	33	1·47
			(*b*) Fungus peroxidase				
L. blennius	2	6	32·6	49·7	19	27·3	1·65
L. quietus	4	6	101	111·5	18·5	29	1
L. rufus	1	6	384	454	30	113	0·96
L. turpis	1	4	44	292	28	268	1·50

In no instances were activations observed as great as those described by Bach and Chodat. This may be due to the phenomenon being better shown by *L. vellereus* than by the varieties of *Lactarius* used in the above experiments.

Most of the horseradish peroxidase preparations were found to retard the autoxidation of pyrogallol, and either to retard the action of the "oxygenase" fraction of *Lactarius* juices on pyrogallol in its early stages, or to produce less activation at the beginning of the experiment than later: figures quoted for *L. quietus* (2) in the first table show this increase in activation ratio with time. The following experiments, in which diluted, unprecipitated juice was used in place of "oxygenase" fraction, and the p_H buffered at 6·5, also show increase in activation with time.

Fungus	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers I	II	III	IV	Activation ratio
L. rufus	½	13	10	5	3	0·87
	1	20	21	9	7	1·27
	2	35	40	15	12	1·40
	4	64	83	25	20	1·62
	10	154	224	45	40	1·69
L. quietus	1	120	99	9	11	0·79
	2	206	199	15	17	0·95
	3	287	297	20	23	1·02
	6	475	551	33	37	1·16
	10	629	794	45	53	1·27

The various points raised in connection with the observed activation phenomenon will now be considered in turn, and experiments bearing on them described.

(1) *Possibility of change in hydrogen ion concentration.* Since the solutions were buffered, this effect is minimised. Some of the horseradish peroxidase preparations, which consisted of alcoholic precipitates dried *in vacuo* and dissolved in distilled water, were found to be acid, and the buffering was not able to prevent slight decrease in p_H on addition of such peroxidases. Since Wieland and Sutter [1928] found the optimum p_H for the action of the enzyme from *L. vellereus* investigated by them to be 4·6, using quinol as substrate, a decrease in p_H might of itself activate the *Lactarius* oxidase. The effect of change in p_H on the oxygen uptake accompanying the action of *L. rufus* juice on pyrogallol was therefore examined.

p_H	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers I	III	I–III
6·0	8	74	27	47
6·5	8	142	34	108
7·0	8	279	242	37

It is shown in the middle column that decrease in p_H did not increase the oxygen taken up by the autoxidation of pyrogallol, and in the last column that, when allowance was made for the autoxidation of pyrogallol, decrease in p_H from 6·5 did not increase the oxygen taken up by the action of enzymes in the juice on pyrogallol. Hence, change in hydrogen ion concentration from a higher to a lower p_H cannot explain the phenomenon of activation.

(2) *Possibility of hydrogen peroxide production* by the action of an enzyme or enzymes in *Lactarius* preparations on pyrogallol. Some catalase was present in all *Lactarius* preparations, so that if hydrogen peroxide were produced and normally broken down by catalase, but on addition of peroxidase utilised for oxidation instead, then there would be an increase in oxygen absorption. This increase, however, would depend on the rate of production of hydrogen peroxide, and in no way could exceed a doubling of the rate. Higher rates than this are here recorded, and the activations observed by Bach and Chodat were even greater. Hydrogen peroxide production in this way, therefore, cannot provide a complete explanation of the phenomenon. The possibility is not precluded that it does take place, and in conjunction with (3), the oxidation of inhibitory substances in *Lactarius* might explain the phenomena.

The work of Wieland and Sutter [1928] furnishes no evidence of the production of hydrogen peroxide by enzymes in *L. vellereus* other than the thermostable catalyst described by Wieland and Fischer [1926]. The former investigators failed to detect any hydrogen peroxide production by their enzyme preparation from *L. vellereus* (apparently a laccase) under conditions in which its production by the Wieland and Fischer catalyst was readily demonstrated.

(3) *Possibility of oxidation of inhibitory substances.* Since the activation ratio increased with time, it seems probable that inhibitory substances either in the *Lactarius* or in the peroxidase preparations added were being oxidised on addition of peroxidase. In this connection the work of Szent-Györgyi [1928] on an inhibitory hexuronic acid oxidisable by hydrogen peroxide in presence of peroxidase and a phenol is of interest.

A consideration against the presence of inhibitory substances in the *Lactarius* preparations is the control experiment described earlier in this paper, which shows that there cannot be present both inhibitory substances oxidisable by peroxidase and peroxide, and substances autoxidisable with production of peroxide. If present along with such autoxidisable substances, they would in any event be oxidised even without addition of peroxidase, by means of peroxidase in the juices. If, however, hydrogen peroxide were derived from the action of an enzyme on pyrogallol, the presence of inhibitory substances in the *Lactarius* preparations, oxidisable by peroxidase + peroxide, might explain the phenomena, since then much greater activation than a doubling in the rate would be possible. They cannot be of the type described by Bach [1909], since, on keeping the *Lactarius* juices under slightly alkaline conditions, their activation by peroxidase did not increase, but eventually disappeared before either tyrosinase or laccase had lost all their activity, tyrosinase being most persistent.

(4) *Possibility of effect not being due to peroxidase itself.* The activation by peroxidase of the action on pyrogallol here discussed appears to be of an entirely different type from the activation of the action of tyrosinase on a monohydric phenol by boiled tyrosinase preparations, or by *o*-dihydric phenols, discussed later in this paper.

In some qualitative experiments on the liberation of iodine from potassium iodide and the blueing of guaiacum by "oxygenase" with peroxidase, Bach and Chodat [1902] record a control with boiled peroxidase preparation, which did not cause activation. In the present work it was shown that the phenomenon under investigation was likewise not brought about by boiled peroxidase preparation, since, when peroxidase which had been boiled for some time to destroy its activity was used, no activation of the action of *L. rufus* juice was observed.

Duration of experiment in hours	p_H	mm.³ O_2 absorbed in respirometers I	II	III	IV	Activation ratio
9	6·5	132	139	37	46	0·98

Use of an amino-acid, glycine, in place of peroxidase preparation did not cause activation of the action of the juice. (Amino-acids are not oxidised by the oxidation products of pyrogallol produced by mealworm tyrosinase. *Respirometer. Left:* 1 cc. 0·2 % pyrogallol in phosphate buffer, p_H 6·0; 1 cc. 0·45 % glycine in buffer; 0·5 cc. mealworm preparation; buffer to 3 cc. *Right:* 1 cc. 0·2 % pyrogallol; 0·5 cc. mealworm preparation; buffer to 3 cc. No change in levels occurred. Tyrosine in place of pyrogallol yielded a similar result, as also did quinol.)

L. rufus juice which had been dialysed to remove any hypothetical dialysable co-enzyme was not more activated by peroxidase than juice which had not been dialysed.

Description of preparation	p_H	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers I	II	III	IV	Activation ratio
Undialysed	6·5	8	134	183	20	43	1·23
Dialysed	6·5	8	47	65	29	44	1·17

There was, therefore, no dialysable co-enzyme in the juice, which was supplied by the peroxidase preparations.

(5) *Possibility of change in nature of oxidation products.* Unless excess substrate be present, the rate of action cannot be assumed to be a measure of the activity of the enzyme. When dilute (0·2 %) pyrogallol solutions were used no difference in rates was detectable with or without addition of peroxidase. The oxygen absorption, however, with or without addition of peroxidase, exceeded in a few hours the total oxygen absorption produced by the action of another enzyme preparation, tyrosinase from mealworms, on the quantity of pyrogallol used.

Required for 1 cc. 0·2 % pyrogallol:

calculated value for the production of purpurogallin	266	mm.³ O_2
by action of tyrosinase from mealworms; final figure	311	,,
,, *L. quietus* "oxygenase" fraction; 1 day	448	,,
,, ,, ,, ,, + peroxidase; 1 day	398	,,

Since the excessive oxygen absorption took place with or without addition of peroxidase, however, it cannot account for the phenomenon of activation[1].

When 2 cc. saturated tyrosine in phosphate buffer p_H 6·0 was used as substrate in place of pyrogallol, activation by peroxidase was still observed, showing that the phenomenon of activation did not depend on choice of substrate.

Fungus	No. of precipitations in 40 % alcohol	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers I	II	Activation ratio
L. quietus	4	1	279	267	0·96
		4	403	498	1·24

Wieland and Sutter [1928], using quinol as substrate, tested the action of peroxidase from horseradish on the oxidase preparation obtained by them from *L. vellereus*. The duration of their experiment was 30 min. They found that 4·96 cc. O_2 were absorbed without, and 5·60 cc. O_2 with, addition of peroxidase. These figures they considered scarcely to exceed their experimental error.

(6) *Possibility of the presence of autoxidisable substance in* Lactarius. This appears to be the most likely explanation of the phenomena observed by Bach and Chodat.

Juices which had been exposed to air were, when afterwards tested on pyrogallol, more readily activated by peroxidase than juices which had been kept *in vacuo*, the initial retardation on addition of peroxidase being counterbalanced sooner by the activating action. The following experiments were carried out with *L. quietus* juice. One sample of juice was exposed to air for about one day while another sample stood in an evacuated desiccator. The activation by peroxidase of the action of each sample on pyrogallol was then determined. 1 cc. was used of a peroxidase preparation which had initially a slightly retarding action, and it was only after a number of hours—given in column A of the following table—that the oxygen absorption with peroxidase became equal to that without peroxidase, the oxygen absorption at which this occurred being given in mm.³ in column B. Beyond this point activation set in. It is seen that activation set in more readily when the fungus juice had previously been exposed to air than when it had been *in vacuo*. A 1 and B 1 denote the results obtained with a sample previously exposed to air, and A 2 and B 2 the corresponding results with a sample previously kept *in vacuo*. The whole experiment was repeated three times.

Experiment	A 1 hrs.	B 1 mm.³	A 2 hrs.	B 2 mm.³
1	2½	288	4½	507
2	2	200	4	300
3	5	209	11½	331

A similar effect was observed with the *L. rufus* "oxygenase" fraction. The initial retardation on addition of peroxidase was less when the "oxygenase" preparation had stood for one day than when it was tested immediately after

[1] It appeared to be due to the oxidation of pyrogallol by *Lactarius* giving rise to products different from those produced by the oxidation of pyrogallol by tyrosinase from mealworms.

preparation. It was usually observed also that the initial retardation was slightly greater when 1 cc. peroxidase was used (II b) in place of 0·5 cc. (II a).

Description of "oxygenase" fraction	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers		
		I	II a	II b
Fresh	1	92	75	—
	2	162	152	—
One day old	1	97	91	89
	2	163	168	162

These results are consistent with the presence in the juices of a substance which on autoxidation produces a peroxide, and consequently increases the action on pyrogallol when peroxidase is added. There appear also to be inhibitory substances in the peroxidase preparations, which cause initial retardation, and are oxidised away by peroxide with peroxidase.

It follows from the consideration of the experiments so far described that the most likely alternative explanation to the presence of an autoxidisable substance in *Lactarius* juices is the production of hydrogen peroxide by the action on pyrogallol of an enzyme in *Lactarius* precipitated by 40 % alcohol, and the oxidation by peroxidase and this hydrogen peroxide of a further amount of pyrogallol, and also of inhibitory substances in *Lactarius* juices. If this had been the complete explanation, then previous exposure to air of the juices would have been expected to have either no influence on the effect of addition of peroxidase, or, if the inhibitory substances became oxidised in air, to diminish rather than accentuate the observed effect on addition of peroxidase. Since accentuation was observed, the effect was probably due to the alternative explanation, namely, the presence of autoxidisable substance. Although this is evidence of the presence of autoxidisable substance, the production of hydrogen peroxide by the action of an enzyme is not excluded as a partial explanation of the phenomenon.

Further support of the above view was obtained. If by any means the oxidising activity of the juices could be diminished without a corresponding diminution in the degree of activation by peroxidase, then the activation would appear to be a phenomenon not entirely dependent on the action of the enzyme which oxidises pyrogallol. Cyanide was found to affect the action of the enzyme in the juice on pyrogallol much more than it did the process responsible for activation, thus causing a relatively large increase in activation.

Exp. 1. A sample of *L. quietus* juice was tested without cyanide, using 1 cc. peroxidase, and working at p_H 6·5.

	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers				Activation ratio
		I	II	III	IV	
Exp. 1	7	675	766	36	29	1·15
Exp. 2	7	156	335	—	—	2·15

Exp. 2. 0·5 cc. $3M/250$ neutralised KCN in buffer p_H 6·5 was added to each bottle of each respirometer, bringing the cyanide concentration to $M/500$, and using otherwise the same materials as in Exp. 1. No alteration in p_H of the

liquids during the experiment was detectable. *M*/500 KCN practically stopped the autoxidation of pyrogallol.

Retardation by cyanide of the action of the juice on pyrogallol was as follows:

Duration of experiment in hours	mm.³ O_2 absorbed in respirometers		Percentage retardation
	Without KCN	*M*/500 KCN	
6	634	132	·79

The phenomenon of activation disappeared when the juice had been heated. *L. quietus* juice, which had been heated at 90° for half an hour, gave the following results:

Treatment of juice	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers				Activation ratio
		I	II	III	IV	
			Activation			
Heated	7	64	50	29	29	0·60
			Activity of juice alone			
Unheated	6	634	—	25	—	—
Heated	6	55	—	25	—	—

The loss of activity of the juice itself on heating was 95 %.

Discussion.

The investigation which has been carried out shows that the phenomenon discovered by Bach and Chodat [1903, 2] of the activation by peroxidase of the action on pyrogallol of *Lactarius vellereus* oxidase takes place when certain other *Lactarius* fungi are used. In recent years it has been considered likely that the explanation of the observations which led Bach and Chodat to the formulation of their theory of the constitution of "direct" oxidases was the presence of substances autoxidisable to peroxides. This explanation has been found to be applicable in the present instance. There are in the fungi, in all probability, substances autoxidisable to peroxides utilisable by peroxidase; the addition of peroxidase therefore causes oxidation of further pyrogallol, an effect which is enhanced by removing peroxidase already present in the juices by fractional precipitation with alcohol. It is possible that, besides this, one of the oxidases in the juice may produce hydrogen peroxide when acting on pyrogallol, and may therefore be able to form with peroxidase a system analogous to that discovered in milk by Thurlow [1925], thus providing a partial explanation of the phenomenon of activation by peroxidase. As already pointed out, no thermolabile oxidase in *Lactarius* has been shown to have this function, but it is possible that one may be present able to act in this way, and it is intended to investigate the matter when further material is available. The presence of autoxidisable substance precludes the co-existence in *Lactarius* juices of inhibitory substances oxidisable by peroxidase and peroxide, without which the production of hydrogen peroxide by the action of an enzyme on pyrogallol cannot furnish a complete explanation. The increase in activation ratio with time is probably due to the presence of such inhibitory substances in peroxidase prepared by the method used. The presence of these inhibitory

substances may account for the difference in activating power of peroxidases from different sources observed by Bach and Chodat.

Evidence was not obtained that the oxidases in *Lactarius* juices are separable solely into enzyme-like peroxide and peroxidase, the original view of Bach and Chodat [1903, 2]. A 60–80 % alcohol precipitate from *L. turpis* juice, which was allowed to stand in the ice-chest in contact with 80 % alcohol for 10 days, lost its activity on tyrosine and on guaiacol, although acting slightly on guaiacol after addition of hydrogen peroxide. Since neither the "oxygenase" nor "peroxidase" fraction of *L. vellereus* oxidase obtained by Bach and Chodat had much oxidising power apart from each other, it seems possible that by repeated alcoholic precipitation Bach and Chodat largely destroyed tyrosinase and laccase in both fractions, and separated from one another autoxidisable substance and peroxidase. The view that tyrosinase and laccase are separable into peroxidase and autoxidisable substance does not appear tenable. It seems necessary at present to regard tyrosinase, laccase, and peroxidase with autoxidisable substance as separate distinct systems.

2. Other oxidase preparations.

A. Potatoes. Potatoes were minced and pressed, and the juice was centrifuged; the liquid was precipitated once in 40 % alcohol and the precipitate dissolved as described for *Lactarius* juices. The potato preparations contained tyrosinase, laccase, and catalase. The action of such potato preparations on pyrogallol at p_H 6·5 was not activated by peroxidase, neither was new potato juice unprecipitated by alcohol activated by peroxidase.

Source of preparation	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers				Activation ratio
		I	II	III	IV	
Old potatoes	11	138	161	31	46	1·07
Old potatoes	8	132	131	22	54	0·70
New potatoes	11	112	102	31	76	0·32

These results are unexpected in view of the work of Gallagher [1923] on an autoxidisable substance in potatoes. The peroxidase preparations were all neutral; the presence in them of true inhibitory substances is therefore probable. Dialysis of the potato preparations did not result in activation by peroxidase.

Source of preparation	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers				Activation ratio
		I	II	III	IV	
Old potatoes	11	101	68	31	46	0·31
New potatoes	11	137	134	31	76	0·55

B. Mealworms. Owing to the absence of laccase, a preparation from mealworms affords convenient material for investigation of the action of peroxidase on the activity of tyrosinase. The preparation contains much catalase, but appears to contain no peroxidase, since it does not act on guaiacol even after addition of hydrogen peroxide, under which conditions a trace of added peroxidase causes immediate coloration of guaiacol. Using the mealworm preparation, prepared according to Raper [1926], filtered, the results (1) were

obtained; dialysed and centrifuged, the results (2). A similar preparation several months old, filtered, was activated (3). The results (4) were obtained by using 2 cc. saturated tyrosine in buffer as substrate in place of pyrogallol, with the mealworm preparation used in (1). The p_H was 6·5, and all peroxidase preparations were neutral.

Description of experiment	Duration of experiment in hours	mm.³ O_2 absorbed in respirometers I	II	III	IV	Activation ratio
(1)	10	314	285	23	26	0·89
(2)	7	373	319	31	28	0·85
(3)	10	55	99	23	26	2·28
(4)	8	210	143	—	—	0·68

Thus, only the old preparation was activated by peroxidase. This supports the observations made on *Lactarius* preparations, which indicate that the presence of tyrosinase in the "oxygenase" fractions was not responsible for the activation phenomenon. Since the peroxidase preparations even when neutral had an inhibitory action, the presence in them of inhibitory substances is probable.

The observation recorded by Bach [1909], of the action of peroxidase on tyrosinase, was confirmed qualitatively, using a preparation from mealworms obtained by preliminary extraction with 60–80 % saturated ammonium sulphate, followed by extraction with alkaline water. The darkening of tyrosine by tyrosinase was initially impeded by peroxidase, in presence or absence of hydrogen peroxide, but more in its absence. After one day, all four mixtures were equally dark.

PART II. ACTIVATION OF TYROSINASE.

1. Activation by *o*-dihydric phenols.

It was shown by Raper [1926] that the oxidation of tyrosine by tyrosinase from mealworms was initially activated by 3 : 4-dihydroxyphenylalanine. During the course of the present research, the oxygen absorption accompanying the action of tyrosinase on tyrosine was measured[1]. It was found that the absorption did not start at once, but gradually increased to a constant rate, indicating that the reaction is autocatalytic. This is in agreement with the "catechol" theory of Onslow [1923], as pointed out by McCance [1925]. The addition of dihydroxyphenylalanine or of catechol caused activation by eliminating the initial lag. That this is the interpretation of the phenomenon appears from a recalculation of Raper's figures as rates.

Times (minutes)	0	65	125	205
	Tyrosine oxidised per 20 cc. (mg.)			
Without dihydroxyphenylalanine	0	1·15	2·44	3·54
With dihydroxyphenylalanine	0	1·49	2·54	3·47
Whence, rates per 5 minutes—				
Without dihydroxyphenylalanine	0·088	0·107	0·07	—
With dihydroxyphenylalanine	0·114	0·09	0·06	—

The enzyme used by Raper had not been dialysed.

[1] It amounts to a little over five atoms per molecule of tyrosine; the action on dihydroxyphenylalanine is accompanied by an absorption of a little over four atoms per molecule.

In the present experiments, the following mixtures were placed in respirometers. I. *Left:* 0·4 mg. tyrosine; 0·5 cc. tyrosinase from mealworms; buffer to 3 cc. *Right:* 0·5 cc. tyrosinase; buffer to 3 cc. II. *Left:* 0·4 mg. tyrosine; 0·5 cc. tyrosinase; 0·06 mg. catechol; buffer to 3 cc. *Right:* 0·5 cc. tyrosinase; 0·06 mg. catechol; buffer to 3 cc. The p_H was 6·0. The oxygen absorbed in mm.^{3} was as follows:

Rates per 20 min.

I 2; 12; 14; 11; 9; 7; 5.
II 6; 6; 6; 7; 7; 7; 6.

1 cc. saturated tyrosine in buffer was then used as substrate and 0·1 mg. dihydroxyphenylalanine as activator, p_H 7·0:

Rates per 15 min.

I 0; 5; 20; 27; 22; 23; 17; 13.
II 25; 26; 20; 21; 15; 13; 10; 6.

There was no initial lag when, in place of tyrosine, dihydroxyphenylalanine was used as substrate. Using 1 mg. dihydroxyphenylalanine at p_H 6·0:

Rates per 7 min.

I 12; 10; 9; 8; 8.

The enzyme used in the above experiments had been dialysed.

Undiluted tyrosinase preparation acted too rapidly on *p*-cresol for the initial lag to be obvious, but on dilution of the enzyme it became apparent. Using 2 mg. *p*-cresol as substrate, and 0·05 mg. catechol as activator, at p_H 7·0:

Rates per 10 min.

I 3; 9; 10; 13; 13; 12; 11.
II 10; 13; 12; 12; 12; 12; 11.

There was no initial lag when catechol was used as substrate.

Using 2 mg. catechol, at p_H 7·0:

Rates per 5 min.

I 10; 9; 8; 10; 7; 6; 6.

Using enzyme still further diluted:

Rates per 5 min.

I 3·6; 2·9; 3·9; 3·3; 4·2; 2·6; 3·6; 2·9.

The enzyme used in the above experiments had not been dialysed.

In the following experiments the enzyme had been dialysed.

Using 2 mg. *p*-cresol at p_H 7·0:

Rates per hour

I 0; 3; 15.

Using 2 mg. catechol at p_H 7·0:

Rates per 15 min.

I 5; 5; 4.

A similar effect was observed when a *L. turpis* "oxygenase" fraction was used instead of mealworm tyrosinase. Using 2 mg. *p*-cresol as substrate, and 0·06 mg. catechol as activator, at p_H 7·0:

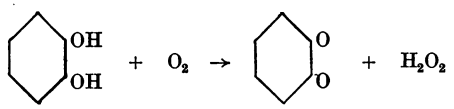
Rates per hour

I 0; 0; 0; 19; 26.
II 5; 11; 22; 22; 26.

Discussion.

The phenomena here described have a significant bearing on the mode of action of tyrosinase. Since the action on the monohydric phenols tyrosine and *p*-cresol is autocatalytic, and the lag is eliminated by addition of an *o*-dihydric phenol, it appears as if some product of action on an *o*-dihydric phenol enabled the enzyme to act on the monohydric phenol. Since dialysis of the enzyme preparation did not destroy its action on the monohydric phenol, the necessary product appears not to be dialysable. It is quite likely, however, that in some instances the requisite *o*-dihydric phenol may be present as an impurity in the monohydric phenol, a possibility included by Onslow [1923]. Some old samples behaved towards peroxidase and hydrogen peroxide as if they contained some corresponding *o*-dihydric phenol, and such samples showed no initial lag when tyrosinase acted on them. This possibility is under further investigation, although it cannot furnish a complete explanation, as follows from the work of Onslow and Robinson [1928] described below.

The production of hydrogen peroxide by the action of tyrosinase has not so far been demonstrated, and if the preparation from *L. vellereus* used by Wieland and Sutter [1928] contained tyrosinase, then the production of hydrogen peroxide by tyrosinase is unlikely. The production of *ortho*-quinones has been demonstrated [Happold and Raper, 1925; Pugh and Raper, 1927]. If, nevertheless, the reaction



expresses what occurs when tyrosinase acts on an *o*-dihydric phenol, in accordance with the suggestion of Onslow and Robinson [1926], then both *ortho*-quinone and hydrogen peroxide are possible activators of the action on a monohydric phenol. Onslow and Robinson [1928] believe the effective substance to be an *ortho*-quinone. Using an enzyme obtained from the potato by preliminary treatment with alcohol, followed by aqueous extraction of the enzyme, they obtained, by means of treatment with charcoal, preparations which acted strongly on catechol, but scarcely at all on *p*-cresol or tyrosine. Addition of a trace of catechol caused action on *p*-cresol, and slight action on tyrosine. They concluded from their experiments that the oxidation of the monohydric phenols is a secondary phenomenon depending on the presence of an *ortho*-quinone and not directly on the action of the enzyme. They assumed that alcohol did not extract the *ortho*-quinone from plant tissue, and that it was an *ortho*-quinone which was removed by charcoal.

An *ortho*-quinone has not been shown to convert a monohydric to an *o*-dihydric phenol. Also it seems unlikely that an *ortho*-quinone is the effective substance, because dihydroxyphenylalanine will serve as activator, although the *ortho*-quinone formed from it is not "free" (as shown by its inability to oxidise an external amino-acid), but condenses at once with another part of its own molecule [Raper, 1927]. The following considerations indicate that

neither *ortho*-quinone nor hydrogen peroxide is able to bring about the conversion of a monohydric to an *o*-dihydric phenol at the requisite rate (the rate of a series of reactions being determined by the rate of the slowest), unless possibly it is catalysed in some way. Peroxidase with hydrogen peroxide, as well as tyrosinase, produces *ortho*-quinones from *o*-dihydric phenols such as catechol [Pugh and Raper, 1927], yet peroxidase with hydrogen peroxide does not act on tyrosine, even in presence of a trace of added catechol, although peroxidase with hydrogen peroxide is known to act rapidly on dihydroxyphenylalanine, the *o*-dihydric phenol produced from tyrosine by tyrosinase [Raper, 1926], to give products indistinguishable from those produced by tyrosinase. Neither does peroxidase with hydrogen peroxide act on phenol. It follows, since peroxidase with hydrogen peroxide is able to carry out all further stages in the reactions, that neither hydrogen peroxide nor *ortho*-quinone is able to convert monohydric to *o*-dihydric phenol, unless the change is catalysed by something specifically present in tyrosinase preparations. Until enzymes catalysing action on monohydric and *o*-dihydric phenols have been shown to be distinct from one another, the name "tyrosinase" must be applied to both, since action on tyrosine by means of a co-enzyme is a distinctive property of the enzyme.

The facts may possibly indicate that the conversion of monohydric to *o*-dihydric phenol is brought about by hydrogen peroxide (Dakin's reaction), catalysed by tyrosinase, possibly by way of an oxidised form of the enzyme; but further work is required before the mechanism responsible for action on a monohydric phenol can be known.

2. Activation by boiled preparations.

The observation was made by Haehn [1919], and confirmed by Raper and Wormall [1923], that the action of tyrosinase from potatoes on tyrosine was activated by boiled potato juice. This has been confirmed for tyrosinase prepared from mealworms.

Recalculation of Raper and Wormall's figures in terms of rates shows that the phenomenon consists in a tendency to elimination of initial lag.

	Tyrosine present in g. per 330 cc.				
Ordinary potato juice	0·1427	0·1269	0·1062	0·0905	0·0321
Potato juice + boiled potato juice	0·1442	0·1185	0·0980	0·0804	0·0287
Time (min.)	0	130	220	360	1380

Whence rates per 10 min. (mg.)—

Without boiled juice 1·21; 2·30; 1·12; 0·57.
With boiled juice 1·98; 2·28; 1·26; 0·51.

Using the arrangement of respirometers described for activation by *o*-dihydric phenols, 1 cc. saturated tyrosine as substrate, and 1 cc. boiled mealworm preparation as activator, at p_H 7·0:

Rates per 15 min.

I 0; 5; 20; 27; 22; 23; 17; 13.
II 25; 30; 23; 21; 14; 12; 7; 7.

The boiled preparation had been dialysed before boiling.

That activation by boiled preparations is related to the activation by *o*-dihydric phenols does not appear unlikely.

Summary.

1. The phenomenon discovered by Bach and Chodat of the apparent activation by peroxidases of a constituent of the oxidase of the fungus *Lactarius vellereus* separable from it by 40 % alcohol is considered.

2. Using peroxidase from horseradish, the phenomenon has been confirmed for some other *Lactarius* fungi, and certain alternative explanations to that put forward by Bach and Chodat are discussed.

3. The most probable interpretation of the phenomenon is the presence in *Lactarius* fungi of an autoxidisable substance. The possible presence also of some enzyme able to produce hydrogen peroxide by dehydrogenation of the substrate may contribute to the phenomenon. No evidence was obtained of the dual constitution ascribed by Bach and Chodat to oxidases in general.

4. The effect of peroxidase from horseradish on the action of potato juice and of tyrosinase from mealworms is described.

5. The action of tyrosinase on tyrosine and on *p*-cresol is shown to be autocatalytic, the lag being eliminated by addition of a small amount of catechol or of dihydroxyphenylalanine.

6. The suggestion put forward by Onslow and Robinson [1928], that an *ortho*-quinone is responsible for initiation of action on a monohydric phenol, is discussed.

7. The elimination of lag by boiled tyrosinase preparations is demonstrated.

I wish to express my thanks to Professor H. S. Raper, under whose direction this work was carried out, for his kind criticism and help throughout the investigation.

REFERENCES.

Bach (1909). *Ber. deutsch. chem. Ges.* **42**, 594.
—— and Chodat (1902). *Ber. deutsch. chem. Ges.* **35**, 3943.
—— —— (1903, 1). *Ber. deutsch. chem. Ges.* **36**, 600.
—— —— (1903, 2). *Ber. deutsch. chem. Ges.* **36**, 606.
—— and Sbarsky (1911). *Biochem. Z.* **34**, 473.
Chodat (1910). Abderhalden's Biochem. Arbeitsmethoden, **3**, 42.
Gallagher (1923). *Biochem. J.* **17**, 515.
Haehn (1919). *Ber. deutsch. chem. Ges.* **52**, 2029.
Happold and Raper (1925). *Biochem. J.* **19**, 23.
McCance (1925). *Biochem. J.* **19**, 1022.
Onslow (1923). *Biochem. J.* **17**, 216.
—— and Robinson (1926). *Biochem. J.* **20**, 1138.
—— —— (1928). *Biochem. J.* **22**, 1327.
Pugh and Raper (1927). *Biochem. J.* **21**, 1370.
Raper (1926). *Biochem. J.* **20**, 735.
—— (1927). *Biochem. J.* **21**, 89.
—— and Wormall (1923). *Biochem. J.* **17**, 454.
Szent-Györgyi (1928). *Biochem. J.* **22**, 1387.
Thurlow (1925). *Biochem. J.* **19**, 175.
Wieland and Fischer (1926). *Ber. deutsch. chem. Ges.* **59**, 1180.
—— and Sutter (1928). *Ber. deutsch. chem. Ges.* **61**, 1060.