176. ON THE SO-CALLED "IODIDE OXIDASE". MECHANISM OF IODIDE OXIDATION BY *ASPERGILLUS*

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It is well known that different organisms have the property of oxidizing iodides to free iodine. This reaction was first described by Schönbein [1848]. It was used by Bach & Chodat [1904] and among other workers by Wolff [1914; 1917] who suggested that the oxidation was due to a secondary reaction between the iodide and the oxidation products of polyphenols. Raciborski [1905], however, found that *Aspergillus niger* excreted an iodide-oxidizing system into its medium, but that both phenol oxidases and peroxidase were absent. Hence he postulated the existence of a specific iodide oxidase.

Korczewski [1922] confirmed Raciborski's observation and showed that the iodide-oxidizing system was destroyed by heat and acid and inhibited by KCN, NH_2OH and peroxide. Further, if the iodine is not removed an equilibrium is reached and the reaction soon comes to an end. He always found peroxide present in the medium, but, since its concentration was very low, he concluded that it could not be responsible for the oxidation of iodide. He found, however, that the addition of peroxidase caused a marked acceleration of iodide oxidation. Korczewski concluded that the culture medium contains an oxygenase similar to that postulated by Bach & Chodat [1902; 1903; 1904], and that this enzyme forms with molecular oxygen a superoxide which is responsible for the oxidation of iodide.

Methods

The methods used for preparing an active iodide-oxidizing medium were based on Korczewski's work. *A. niger* was grown on a medium composed of: 4% sucrose, 0.2% K_2HPO_4 , 1% $(\text{NH}_4)_2\text{SO}_4$, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. It was found most convenient to buffer the medium to pH 7.8 with *M/15* phosphate, inoculate it heavily with spores and incubate for 36–44 hr. at 30°; this usually gave a light continuous mycelium and the pH seldom fell below 4.5.

Before inoculation all media were sterilized by heating under pressure to 120° and allowing to cool rapidly. Cultivation was carried out in 500 ml. Erlenmeyer flasks containing 100 ml. of medium. The stock cultures were grown on wort agar slopes, and were subcultured once a month.

Manometric experiments were carried out at 39° in Barcroft manometers, all results being checked by control experiments. When any volatile or possibly autoxidizable substances (e.g. acetaldehyde) were used, the same quantity of these substances was placed in the compensating vessel as in the reaction vessel. The total volume of solution in each vessel was always 3.3 ml.

Factors involved in the oxidation of iodide

Six flasks of medium were prepared by Korczewski's method. The pH of the contents of each flask was determined and the iodide-oxidizing activity estimated. 10 ml. from each flask were pipetted into 100 ml. conical flasks which received

also 2 ml. of 0.4% KI solution and two drops of starch. The free iodine formed was titrated with $1.199 \times 10^{-2} N$ $\text{Na}_2\text{S}_2\text{O}_3$ at intervals during $2\frac{1}{2}$ days. Iodide oxidation appeared to proceed continuously, the rate of oxidation being greater with the lower pH. This was in agreement with Korczewski's results (see Table 1). The contents of all flasks gave also a positive reaction for H_2O_2 with benzidine and peroxidase but a negative reaction for peroxidase.

Table 1. *Effect of pH on the rate of oxidation of iodide by the culture medium of Aspergillus niger*

Oxidation expressed in ml. of $1.199 \times 10^{-2} N$ $\text{Na}_2\text{S}_2\text{O}_3$						
Time	pH 3.6	pH 3.8	pH 4.4	pH 4.6	pH 5.2	pH 6.8
70 min.	0.05	0.05	+	+	—	—
95 min.	0.20	0.20	0.07	0.10	0.03	+
20 hr.	0.80	0.85	0.42	0.45	0.18	0.10
24 hr.	0.90	0.95	0.47	0.50	0.18	0.10
43 hr.	1.25	1.10	0.62	0.62	0.25	0.14
45½ hr.	1.50	1.25	0.77	0.88	0.60	0.64
48 hr.	1.50	1.25	0.77	0.93	0.68	0.82
63 hr.	1.58	1.25	0.81	0.93	0.76	0.86

Since Korczewski had asserted that peroxide was always present in the active medium, it was thought advisable to check his two findings, namely: (1) that the oxidation of iodide is not due to peroxide, and (2) that it is inhibited by potassium cyanide.

For this purpose the culture medium was prepared as usual and the following quantitative estimations of the oxidation of iodide were carried out: (1) iodide oxidized by the media alone, (2) by media in the presence of $M/1000$ KCN, (3) media in the presence of $M/100$ KCN, (4) boiled media, (5) dialysed media and (6) the O_2 present as H_2O_2 was also determined.

For these estimations 10 ml. of each medium were used and 5 ml. of 0.4% KI solution, except in the case of experiment (6), which was carried out in Barcroft manometers using 3 ml. of medium, 0.1 ml. of water and 0.2 ml. of catalase. The catalase was placed in hanging tubes which were dislodged after equilibration of temperature and closing of the taps.

The results of these experiments (Table 2) show that (1) the presence of $M/1000$ KCN accelerates iodide oxidation while $M/100$ KCN causes inhibition, and what is more important (2) the peroxide present in the medium is not

Table 2. *Iodide oxidation in 10 ml. of culture medium in 70 hr.*

Estimated in ml. of $1.199 \times 10^{-2} N$ $\text{Na}_2\text{S}_2\text{O}_3$ and expressed as $\mu\text{l.}$ of oxygen equivalent

Flasks	Untreated	+ $10^{-3} M$ KCN	+ $10^{-2} M$ KCN	H_2O_2 as $\mu\text{l. O}_2$
1	6.05	60.5	3.36	17.7
2	10.08	80.6	3.36	17.6
3	15.45	137.8	15.45	13.2
4	8.74	39.0	0.00	16.1

sufficient to account for all the iodide oxidation. The results with cyanide do not agree with those of previous workers, who, unfortunately, do not state what strength of cyanide was used, or how long the reaction mixture was allowed to stand. If they used cyanide at concentrations of about $M/100$, they could not expect any oxidation of the iodide to be apparent for some time, since any free iodine would immediately react according to the equation $\text{KCN} + \text{I}_2 = \text{KI} + \text{ICN}$, and not until all cyanide had been thus oxidized could any free iodine appear.

That this is the explanation of the apparent inhibition obtained by previous workers is clearly demonstrated by further experiments in which the effects of cyanide and thiosulphate are compared.

In another series of experiments the estimation of peroxide and of oxidation of iodide were carried out in media: (1) untreated, (2) dialysed, (3) boiled and (4) mixture of boiled and dialysed after standing for 24 hr. The iodide oxidations were only allowed to proceed for 24 hr. The results of these experiments (Table 3)

Table 3. *Estimation of H_2O_2 and iodide oxidation*

H_2O_2 in μ l. of O_2 and iodide oxidation in μ l. of O_2 equivalent in 10 ml. of culture medium (untreated, dialysed, boiled and mixture of boiled and dialysed media). The peroxide in the mixture of boiled and dialysed media was determined after standing for 24 hr.

Flask	Untreated		Dialysed		Boiled		Boiled and dialysed	
	H_2O_2	Iodide	H_2O_2	Iodide	H_2O_2	Iodide	H_2O_2	Iodide
1	60.8	16.8	0	1.34	60.8	0	60.8	8.06
2	41.2	10.75	0	0	41.2	0	56.6	6.05
3	52.0	12.1	0	0	52.0	0	62.4	5.38
4	20.33	16.8	0	0	30.5	5.37	60.9	16.8
5	36.13	16.8	0	0	51.6	0	56.7	27.55
6	52.13	30.2	0	0	62.6	2.69	62.6	34.6

clearly show that at least two factors are involved in iodide oxidation: one is heat-labile and the other is dialysable. Further, the high peroxide concentrations in the mixtures of boiled and dialysed media indicate that there must be a source of peroxide in the medium. Thus, the fact that the iodide oxidation could exceed in its equivalent the amount of peroxide present in the medium before addition of iodide, could no longer exclude the participation of peroxide. This was ultimately confirmed by the experiments carried out in the presence and absence of catalase. For this purpose, four flasks of medium were prepared as before except that after sterilization and before inoculation 1 ml. of sterilized catalase was added to two of the flasks. The catalase was sterilized by precipitation with absolute alcohol, and redissolved in sterile distilled water. The following estimations were carried out on these media: (1) peroxide in 10 ml. of media, (2) iodide formed in 24 hr. in 10 ml. of media in the presence and absence of catalase and (3) in the presence of $10^{-3} M$ cyanide added to media devoid of catalase.

The results of these experiments (Table 4) show that catalase added either before or after incubation entirely destroys the iodide-oxidizing activity of the

Table 4

KI oxidation per 24 hr., and H_2O_2 concentration expressed as μ l. O_2

Flask 10 ml. of media	No catalase		Catalase	
	1	2	3	4
H_2O_2	30.25	110.3	0.0	0.0
KI oxidation	11.4	13.4	0.0	0.0
KI oxidation in $10^{-3} M$ KCN	33.6	40.3	—	—
KI oxidation after catalase addition	0.0	0.0	—	—

medium. This clearly demonstrated that the oxidation is due entirely to the reaction: $H_2O_2 + 2HI = 2H_2O + I_2$.

The O₂ uptake by culture medium

Examination of samples of medium in Barcroft manometers reveals the presence of an oxidizing system with an O₂ uptake of about 45 μ l. per hr. per ml. If all the O₂ absorbed were reduced to peroxide, the latter would be sufficient to account for the iodide oxidations already observed. The effect of KI on the O₂ uptake was examined and is shown in Fig. 1. The free iodine formed was titrated with thiosulphate and was found to vary directly as the square of the initial iodide concentration (Fig. 2) which forms additional evidence for the reaction $\text{H}_2\text{O}_2 + 2\text{HI} = 2\text{H}_2\text{O} + \text{I}_2$, since H_2O_2 may well be kept at a fairly constant value by the enzyme system.

That the inhibition of O₂ uptake by KI in these experiments is due not to the iodide itself but to the free iodine formed was easily shown by the fact that the addition of $\text{Na}_2\text{S}_2\text{O}_3$ removes this inhibition (Fig. 3).

A more striking result was obtained in the presence of $M/170$ KCN instead of thiosulphate. The cyanide not only prevented the accumulation of free iodine, but by inhibiting all traces of catalase made the total peroxide formed in the medium available for the oxidation of iodide, with the result that the O₂ uptake was almost double that of the medium alone (Fig. 4).

The primary oxidation system in the medium and origin of H₂O₂

It can be definitely stated now that the oxidation of iodide in the culture medium is a secondary oxidation reaction due to H_2O_2 formed as a result of the activity of the primary oxidation system present in the medium. This is strongly supported by the results of experiments related in previous sections which can be summarized as follows:

(1) The oxidation of iodide in the culture medium depends on two kinds of factors, (a) thermolabile and non-dialysable—the enzyme, and (b) thermostable and dialysable—the substrate and H_2O_2 formed in the primary reaction.

(2) The medium alone has a distinct O₂ uptake, which shows that it contains a complete oxidizing system, namely the enzyme and its substrate.

(3) The H_2O_2 is gradually formed in the medium exposed to air, which shows that the O₂ in the primary oxidation reaction is reduced to peroxide.

(4) The iodide oxidation in the medium is inhibited by the addition of catalase which destroys the H_2O_2 .

(5) The O₂ uptake by the medium is doubled on the addition of iodide and cyanide, the latter acting only as inhibitor of catalase present in the medium.

The nature of the primary oxidation reaction and the mechanism of iodide oxidation

The simplest way of determining the nature of the primary oxidizing system consists in separating the enzyme from its substrate and in identifying the latter. For this purpose the culture medium obtained in the usual way was dialysed for at least 16 hr. against running tap water and after dialysis precipitated with five volumes of acetone. The precipitate was centrifuged off and redissolved in 10 ml. $M/5$ acetate buffer pH 5.6. The activity of this preparation tested with boiled medium used as a substrate shows a yield of at least 80%.

In order to identify the specific substrate which is oxidized by this enzyme preparation, its catalytic activity was tested with about 20 different substances such as succinic, lactic, malic and glycollic acids, alanine, glutamic acid, uric acid, alcohol, acetaldehyde, glucose, fructose, *p*-phenylenediamine and different

Fig. 1.

Fig. 1. The effect of iodide on the O_2 uptake of the medium.

Fig. 2.

Fig. 2. The relation between the concentration of iodide and the free iodine formed.

Fig. 3.

Fig. 3. The effect of iodide on the O_2 uptake of the medium in the presence of thiosulphate. 2.7 ml. medium; 0.4 ml. $M/4$ KI; 0.4 ml. $M/40$ $\text{Na}_2\text{S}_2\text{O}_3$.

Fig. 4.

Fig. 4. The effect of iodide on the O_2 uptake of the medium in the presence of cyanide.

phenols such as phenol, catechol, quinol, resorcinol, phloroglucinol. Of all these substances only glucose acted as a substrate for this enzyme preparation.

The enzyme responsible for the primary oxidation reaction in the medium can therefore be identified with the glucose oxidase of *A. niger* discovered and described by Müller [1928; 1929, 1, 2; 1931], reinvestigated by Franke & Lorenz [1937] and purified by Franke & Deffner [1939]. This view is strongly supported by further experimental results which can be summarized as follows:

(1) In absence of glucose the acetone preparation of the enzyme prepared from culture media has no O_2 uptake and does not oxidize iodide.

(2) In presence of $M/6$ glucose the preparation shows a marked O_2 uptake and iodide undergoes a secondary oxidation doubling the O_2 uptake of the enzyme-glucose system alone.

(3) This secondary oxidation of iodide is abolished on the addition of catalase.

(4) If in sucrose media the oxidation of iodide was due to the peroxide formed by the glucose oxidase system, it might be expected that in media having no source of glucose iodide would not undergo oxidation. Accordingly, the mould was cultivated on two fresh media, one containing galactose, the other fructose. After 68 and 44 hr. growth respectively, the media were filtered and to 5 ml. of each of them were added 50 mg. of KI. On allowing to stand for 48 hr. no oxidation of iodide was apparent. Further, when the media were examined manometrically the galactose medium showed no O_2 uptake and the fructose medium only a slight uptake of $0.7 \mu\text{l. } O_2$ per ml. per hr.

(5) I_2 was found also to act as a hydrogen acceptor which was demonstrated by anaerobic experiments carried out in the usual way in Thunberg tubes. These experiments, summarized in Table 5, suggest that the decrease in the O_2 uptake

Table 5. *Reduction of I_2 by glucose oxidase $\pm$ glucose in absence of oxygen*

Thunberg tubes	Enzyme ml.	Acetate pH 5.6 ml.	$M/100 I_2$ ml.	$M/10$ glucose ml.	Water ml.	Time of decoloration min.
1	1.0	0.5	0.1	1.0	—	9-9.5
2	—	0.5	0.1	1.0	1.0	55-60
3	1.0	0.5	0.1	—	1.0	≥ 60

by the system in presence of iodine is partly due to its competition with O_2 in acting as hydrogen acceptor. The reduction of iodine by this system explains also Korczewski's observation that during the oxidation of iodide to free iodine an equilibrium is reached which prevents the further accumulation of iodine in the medium.

(6) The enzyme system of the medium shares with the glucose oxidase and other oxidizing systems which reduce molecular oxygen to H_2O_2 the property of promoting secondary oxidation reactions. The addition of alcohol and catalase to such systems doubles their O_2 uptake. In this reaction, as was shown by Keilin & Hartree [1936], the oxidation of alcohol to aldehyde by H_2O_2 formed in the primary oxidation reaction is catalysed by small concentration of catalase. An increase in O_2 uptake by the media was also observed on addition of acetaldehyde which is oxidized to acetic acid. This secondary oxidation reaction, like that of iodide, is on the contrary inhibited by catalase. Using phosphate instead of acetate buffer, the formation of acetic acid can be easily demonstrated by the

lanthanum test. The secondary oxidation of the aldehyde was also followed manometrically and as shown in Fig. 5 the addition of acetaldehyde to the primary oxidation system doubles its O_2 uptake.

Fig. 5. The secondary oxidation of acetaldehyde by the glucose oxidase system in phosphate buffer. 5 mg. glucose; 30 mg. freshly prepared acetaldehyde.

(7) Finally the enzyme system of the medium, like Müller's glucose oxidase, is not inhibited by KCN, NaN_3 , H_2S , NH_2OH or $Na_4P_2O_7$. On the contrary these reagents, as shown in Table 6, if anything accelerate the O_2 uptake of the system. On the other hand NaF was found to inhibit slightly and $NaHSO_3$ considerably the activity of this enzyme.

Table 6. Effect of inhibitors on the O_2 uptake by culture media

Expressed as % acceleration (+) or inhibition (-)

	%		%		%
$M/250 NaN_3$	+55	$M/50 Na_2S$	+86	$M/50 NaF$	-18.7
$M/500 NaN_3$	+47	$M/50 Na_4P_2O_7$	+11.1	$M/100 NH_2OH$	+23
$M/1000 NaN_3$	+29	$M/50 CH_3I.COONa$	+11.7	$M/100 KCN$	+28
		$M/200 NaHSO_3$ (pH 4.4)	-60		

All these experiments clearly demonstrate that there is no indication of the existence in the culture medium of *A. niger* of a special enzyme oxidizing iodide, as was postulated by Raciborski [1905] and Korczewski [1922]. It shows on the contrary that the oxidation of iodide in this medium is due solely to H_2O_2 formed in the primary oxidation of glucose catalysed by the glucose oxidase of Müller which diffuses out from the mycelium of the mould into the culture medium. Moreover, the secondary oxidation of iodide can be obtained in the presence of every primary oxidation system which like glucose oxidase or xanthine oxidase systems reduces molecular O_2 to H_2O_2 , provided that the peroxide does not undergo destruction by catalase or by acting as hydrogen acceptor.

SUMMARY

1. The culture medium of *Aspergillus niger*, as was shown by previous workers, oxidizes iodide to free iodine.

2. This oxidation is not however catalysed by a specific iodide oxidase or by an oxygenase as was suggested by previous workers, but by hydrogen peroxide formed in the medium.

3. This peroxide is formed as a result of a primary oxidation reaction of glucose catalysed by glucose oxidase.

4. The oxidation of iodide is therefore a secondary oxidation reaction which can be promoted by every primary oxidizing system which reduces molecular oxygen to H_2O_2 .

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