XLVI. THE ALDEHYDE OXIDASE OF THE POTATO.

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THE aldehyde oxidase of the potato was first studied by Bach [1913]. He showed that the potato reduces sodium nitrate to nitrite in the presence of acetaldehyde and that an enzyme is responsible. He stated, however, that the system potato plus aldehyde would not reduce methylene blue. In this respect it differed from the aldehyde oxidase of animal origin. Michlin [1927] was able to precipitate the enzyme from a watery extract of potato by acetone, and stated that the dried powder reduced nitrates but not methylene blue. It was therefore thought interesting to compare further the properties of the potato with the animal aldehydase, and in the course of the investigation it was found that preparations of the potato enzyme would under suitable conditions reduce both nitrate and methylene blue. It is thus possible to correlate the specificities of the plant and animal aldehydases.

Preparation of the enzyme.

Old and new potatoes have been used with similar results. When potatoes are minced, the juice squeezed out contains approximately half the enzyme. The other half seems to be firmly fixed to the potato pulp, and is not extracted by washing with large quantities of water, by grinding with ether and then extracting with water, or by dilute alkalis. Therefore, for the preparation of the enzyme the juice was used. Two pounds of potatoes were finely minced and 300 cc. of juice pressed out through linen. The cloudy liquid was saturated with ammonium sulphate and the precipitate filtered off with suction. The precipitate contains all the enzyme. It was then washed several times with saturated ammonium sulphate solution and finally dissolved in 150 cc. of distilled water. The solution contains a cloudy suspension which is filtered off. For the further purification the method used by Dixon and Kodama [1926] for the purification of the Schardinger enzyme was used. 100 cc. of the clear brown filtrate are treated four times with 3 g. of charcoal (Merck's medicinal). The resulting solution has lost none of its activity, but about 90 % of its protein, and is very light brown in colour. It is then made slightly acid and 5 g. of kaolin are added. All the enzyme is adsorbed and is eluted by 5 % sodium carbonate solution. It is neutralised with dilute acetic acid and

dialysed for 4 hours. The solution has about 5 % of its original protein and about one-third of its activity as the following experiment shows.

Exp. 1. The activity of the enzyme during the various stages of preparation was determined as follows: 1 cc. of the solution to be tested was put in a vacuum tube with 2 cc. buffer p_H 7·3, 1 cc. 5 % sodium nitrate and 0·5 cc. 1 % acetaldehyde. The tubes were thoroughly evacuated and kept at 37° for 2 hours. The nitrite formed was determined by adding 2 cc. of the Griess-Ilosvay reagent to each tube and comparing the colour resulting, after 15 minutes, in a colorimeter. The protein was estimated by taking 1 cc. of the solution to be tested and adding 5 cc. of a 2 % solution of sulphosalicylic acid, and after a given interval comparing in a nephelometer. Tube *A* contains the original juice, tube *B* the ammonium sulphate precipitate dissolved in water and filtered, tubes *C*, *D*, *E*, *F*, the solution of the precipitate after the first, second, third and fourth treatment with charcoal respectively, and tube *G* after treatment with kaolin.

Tube	Activity	Protein %
A	+ + +	100
B	+ + +	50
C	+ + +	32
D	+ + +	26·5
E	+ + +	18·3
F	+ + +	12·5
G	+	4·7

It is obvious that there is no advantage in the treatment with kaolin and unless otherwise specified the enzyme was used after the fourth treatment with charcoal in the following experiments.

The solution was then saturated with ammonium sulphate and the precipitate centrifuged off and dried in a vacuum desiccator. A grey powder was obtained which is readily soluble in water.

Specificity of the enzyme.

(*a*) *Hydrogen donators.* Using nitrate as the acceptor various substances beside aldehyde were tried as hydrogen donators, all of which were negative. They included the following: ethyl alcohol, acetone, diethyl ketone, glucose, sucrose, the sodium salts of lactic, citric, succinic, malic, tartaric, acetic, formic, and pyruvic acids, quinine, morphine, tyrosine, cysteine, catechol, hypoxanthine, and reduced glutathione. None of these reduce nitrate alone. All the aldehydes tried were distinctly positive except *p*-dimethylamino-benzaldehyde which, probably because of its insolubility, reduced but slightly. The figures in the following table represent the amount of nitrate reduced in arbitrary units, the reduction by acetaldehyde being taken as ten units.

Exp. 2. 1 cc. potato juice, 5 cc. buffer p_H 7·3 and 1 cc. 5 % sodium nitrate were placed in each of nine tubes with 0·05 cc. of the aldehyde to be tested. The tubes were then evacuated and incubated at 37° for 2 hours. 2 cc. of the Griess-Ilosvay reagent were then added and the resulting colour compared in a colorimeter.

Aldehyde added	Reduction of nitrate
Formaldehyde	2
Acetaldehyde	10
Propaldehyde	4
Benzaldehyde	9
Valeraldehyde	8
Anisaldehyde	9
Cinnamaldehyde	7
Citral	8
p-Dimethylaminobenzaldehyde	0·5

The differences in the amount of reduction are probably due to differences in toxicity and solubility.

(*b*) *Hydrogen acceptors.* It has been found that a variety of hydrogen acceptors can be substitued for nitrate in the system. Methylene blue, the Clark indicators (dyes of the indophenol and indigo groups with known oxidation-reduction potentials), quinone, and *m*-dinitrobenzene are reduced by the enzyme plus aldehyde but not by the enzyme alone. These results have been obtained a great number of times and with a variety of potatoes.

The reduction of methylene blue has been studied most extensively. The rate of reduction varies according to the preparation of the enzyme. In the untreated juice 1 cc. of 1 : 5000 methylene blue is reduced in about an hour; and the accelerating effect of aldehyde is comparatively small. The probable reason for this is that there are in the untreated juice substances which reduce methylene blue spontaneously, or others, being aldehydic in nature, which act as hydrogen donators. Consequently addition of aldehyde has little effect. The well-washed pulp, which contains about half the enzyme (as estimated from its nitrate reduction), on the other hand, reduces methylene blue slowly in the presence of aldehydes and much more slowly (negligibly for the purposes of the experiment) alone. This slow reduction in contrast to the free enzyme might be explained on the grounds of adsorption. The pulp adsorbs the dye in considerable quantities and may render it less available for the enzyme, thus increasing the reduction time. The ammonium sulphate preparation of the enzyme reduces rapidly in the presence of aldehyde and not at all alone. The following experiment shows the time relations of the various reductions using roughly equivalent amounts of enzyme.

Exp. 3. 2 cc. buffer p_H 7·3 and 1 cc. methylene blue 1 : 5000 were placed in each of six tubes. In tube *A* 2 cc. untreated potato juice were added and in tube *B* the same, plus 0·5 cc. 1 % acetaldehyde. In tube *C* was placed 0·4 g. washed potato pulp, and in tube *D* the same, plus 0·5 cc. acetaldehyde. In tube *E* 2 cc. purified juice were added, and in tube *F* the same, plus 0·5 cc. acetaldehyde. The reductions were carried out anaerobically at 37°.

Tube	Reduction time (mins.)
A	70
B	60
C	No reduction
D	480
E	No reduction
F	120

The reduction of the Clark dyes was carried out with the enzyme purified with charcoal and kaolin. The dyes were not reduced by the enzyme alone but were when aldehyde was added to the system. The time of reduction, however, was not obviously dependent on the r_H of the dye or on its chemical constitution. No. 5 which was reduced first was in the red form, but so was No. 7 which reduced several hours more slowly than No. 4, the last, like all the others, being in the blue form. Inasmuch as similar irregularities appear with a preparation of the aldehydase from liver, it must be concluded that the free energy of the reaction is not the factor determining the rate in these aldehyde systems. The determining factors are unknown. Boiled enzyme as control plus aldehyde does not reduce or at most very slowly in times far exceeding those of the enzyme aldehyde reduction. Finally *m*-dinitrobenzene which gives no oxidation potential is easily reduced. The following experiment gives the reduction times of the dyes.

Exp. 4. The p_H of the solutions was 7·3, and the experiments were carried out at 37°. The dyes, through the kindness of Dr Needham, were obtained from Dr Clark, and were made up in solutions equivalent to a 1 : 5000 methylene blue solution. 1 cc. enzyme purified with charcoal and kaolin, 2 cc. buffer, 1 cc. of the dye to be tested, and 0·5 cc. 1 % acetaldehyde were placed in each tube and the reduction time was determined. All the dyes when subsequently shaken in air were re-oxidised completely. The dyes are given by their numbers (for a list of names and numbers see Dixon [1926]). Number 3 *a* is methylene blue.

Dye	Reduction time with aldehyde	Reduction time without aldehyde
1	Slightly in 18 hrs.	No reduction
2	,, 18	,,
3	,, 18	,,
3 *a*	182 mins.	,,
4	140	,,
5	88	,,
6	660	,,
7	510	,,
8	540	,,
9	340	,,

Acetaldehyde was the only aldehyde studied systematically. Benzaldehyde was tried with methylene blue. The reduction time was a little longer than when acetaldehyde was used with the same amount of enzyme.

Oxygen may or may not act as a hydrogen acceptor. It was impossible to measure the oxygen uptake of the enzyme aldehyde system because it was found that aldehyde and oxygen rapidly destroy the enzyme. Two tubes, one containing 1 cc. of enzyme, 3 cc. buffer p_H 7·3, and 1 cc. water, the other the same except that 1 cc. 1 % acetaldehyde was substituted for the water, were allowed to stand open to the air for an hour at 37°. At the end of that time aldehyde was added to the tube containing none, methylene blue or nitrate added, and the tubes were evacuated. It was found that the incubation with aldehyde inhibited both reactions completely. On the other hand, incubation

with aldehyde in the absence of oxygen inhibits the activity of the enzyme comparatively slightly. Therefore, when the enzyme is shaken in a Barcroft apparatus with aldehyde, there is a very slight initial uptake and then a complete cessation. Nor does the addition of catalase to break up hydrogen peroxide that may be formed, and may be responsible for the inactivation of the enzyme, increase the uptake considerably.

In order to determine whether quinone is reduced, the reduction potential of the system was measured before and after adding aldehyde. The reduction potential of the purified juice alone shows a regular drift, the rate of which is unaffected by the addition of aldehyde. The enzyme plus quinone, however, gives a perfectly steady potential which when aldehyde is added shows a marked reduction. Enzyme boiled for 15 seconds plus aldehyde shows a reduction, but a much slower one, and enzyme thoroughly boiled for 5 minutes gives no reduction as the following curves show. 1·5 cc. of enzyme and 5 cc. of buffer p_H 7·3 were used. The experiments were carried out in nitrogen at 20°, and the potential measured with gold electrodes.

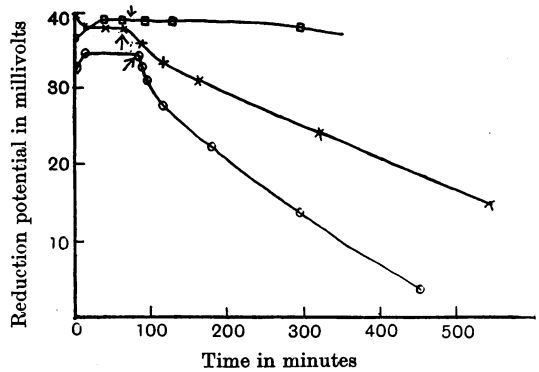

Fig. 1. Reduction of quinone.

□——□ Enzyme boiled 5 minutes. ×——× Enzyme boiled fifteen seconds.
○——○ Enzyme not boiled. → 1 cc. acetaldehyde added.

The effect of nitrate on the reduction of methylene blue was then tried. If the reductions of the dye and nitrate are due to the same system it would be expected that the two hydrogen acceptors would compete, that is, the methylene blue reduction would be retarded in the presence of nitrate. This was found to be the case, as the following experiment shows, and suggests that the same system is responsible for the nitrate and methylene blue reductions. If different systems are responsible it would be necessary to assume that the presence of nitrate is in some way toxic to the methylene blue-reducing system.

Exp. 4. Two tubes were set up; tube *A* containing 1 cc. of enzyme, 3 cc. buffer p_H 7·0, 1 cc. methylene blue, 0·5 cc. 1 % acetaldehyde, and 1 cc. water; tube *B* containing the same except that 1 cc. 5 % sodium nitrate was substituted for the water. The reductions were carried out at 37°.

ERRATUM

Vol. **22**, p. 348, Fig. 1. The description of the ordinates should read "Observed E.M.F. against a saturated calomel electrode."

Tube	Reduction time (mins.)
A	90
B	115

It was shown by adding Griess-Ilosvay reagent to the tube containing nitrate after the reduction of the methylene blue had been completed, that nitrite had been formed.

On the other hand, there is a possibility that the enzyme activates nitrate so that the active nitrate will reoxidise methylene white, thus retarding the methylene blue reduction [Quastel, Stephenson and Whetham, 1925]. The following experiment was therefore tried. One side of a branched vacuum tube of the type used by Dixon and Tunnicliffe [1927] was filled with 1 cc. enzyme, 0·5 cc. aldehyde and 1 cc. methylene blue. In the other 1 cc. 5 % sodium nitrate was placed. The tube was thoroughly evacuated and the reduction of the methylene blue was allowed to go to completion. The nitrate was then tipped over. If the enzyme activates nitrate, an immediate blueing of the solution ought to occur owing to the reoxidation of methylene white. This did not, however, happen and the retardation of the methylene blue reduction by nitrate is due therefore to its competing as a hydrogen acceptor.

Properties of the enzyme.

The p_H-activity curves for the nitrate and methylene blue reductions are not the same. The nitrate reduction has a wide range from about 3 to 8·6 with an optimum at 5·5. The range with methylene blue is more restricted. In it two factors come into play; namely, the reduction of the dye by the enzyme-aldehyde system in neutral and slightly acid solution and the reduction by the aldehyde in the presence of protein at p_H 8 and over. In the alkaline range acetaldehyde in the presence of protein will reduce methylene blue spontaneously. This is shown by using boiled enzyme and aldehyde at varying hydrogen ion concentrations. No reduction of methylene blue occurs when boiled enzyme and acetaldehyde are incubated below p_H 8. Above p_H 8, however, a reduction occurs. And finally at still higher p_H acetaldehyde alone will reduce methylene blue.

As the following curves show, the methylene blue reduction range for the enzyme aldehyde system is from p_H 5·6 to 7·8. As the protein plus aldehyde does not begin to reduce until p_H 8, there is a discontinuity in the curve. At p_H 7·8, the enzyme becomes least active with methylene blue. When enzyme purified with kaolin is used there is no reduction of methylene blue at all at this p_H.

The hydrogen ion concentrations were determined by the quinhydrone electrode. All experiments given in previous sections of this paper were carried out between p_H 7 and 7·5 where there is a good nitrate reduction and the methylene blue reduction is near an optimum.

The inability of Michlin to obtain the reduction of methylene blue seems to be explained by these p_H-activity curves. It will be seen that at p_H 7·8 the

reaction velocity has a minimum value. In other words, at this hydrogen ion concentration the reduction of methylene blue occurs, at best, slowly, or if the activity of the enzyme is less, not at all. Now Michlin carried out his experiments at p_H 7·8, and it may well be that his inability to obtain any reduction is merely due to this fact. Bach, on the other hand, uses a watery extract of potato, but does not state the p_H of his solutions. Extracts made according to his directions have, however, p_H 5 to 5·3. As seen in the following curves this is too acid for the methylene blue reduction to take place appreciably and explains why Bach failed to get it.

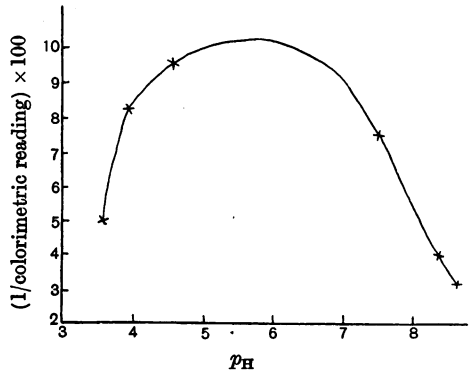

Fig. 2. p_H-activity curve of nitrate reduction.

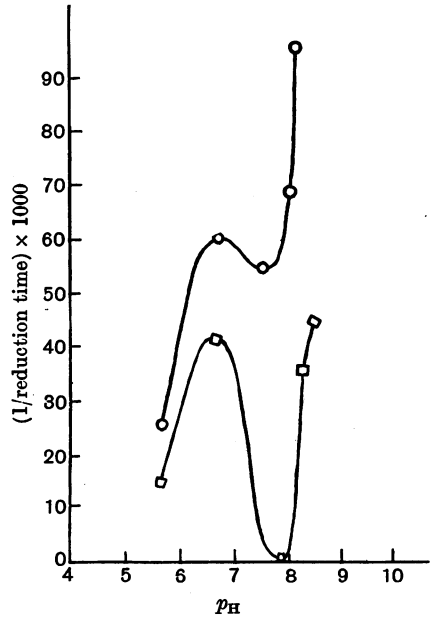

Fig. 3. p_H-activity curve of methylene blue reduction.

□——□ Enzyme treated with charcoal+kaolin.

○——○ Enzyme treated with charcoal.

A preparation of the enzyme was made exactly according to Michlin's directions, and it was found that at p_H 7·3 a good reduction of methylene blue occurred which fell off towards p_H 7·8 showing that this explanation is probably correct.

It should be mentioned here that great care was taken when testing the reduction of methylene blue by any of these preparations to ensure the absence of any substances that might reduce methylene blue spontaneously, or by condensing with the added acetaldehyde, like certain amino-acids. This was done by careful washing of the preparations before the reduction was tested. At p_H 7·5 acetaldehyde and protein will not reduce methylene blue at all in 3 days.

The effect of cyanide was then tried. When *M*/600 KCN is added the reductions of methylene blue and nitrate are slightly inhibited. The fact that methylene blue reduction is inhibited, and that *M*/300 KCN produces a marked inhibition of both reductions seems to indicate that KCN is toxic to the enzyme structure itself, and that the reduction of nitrate is not dependent on the presence of traces of iron. If it were, *M*/600 KCN would probably be more than sufficient to inhibit the reaction completely, as this concentration is effective with other systems dependent on traces of iron. In this respect too the plant is analogous to the animal aldehydase, which, as Bernheim and Dixon [1928] have shown, is unaffected by the addition of cyanide. Both reduction of nitrate and of methylene blue are inhibited to about the same extent, suggesting that the reductions are carried out by the same system.

Exp. 5. 3 cc. buffer p_H 7·3, 1 cc. enzyme, 0·5 cc. 1 % acetaldehyde were placed in each of six tubes with 1 cc. methylene blue or 1 cc. 5 % sodium nitrate. *M*/300 and *M*/600 KCN were then added and the reduction time of the methylene blue measured. The nitrate reduction was allowed to proceed 2 hours, and the nitrite formed determined as in the above experiments. Reductions were carried out anaerobically at 37°.

KCN added	Methylene blue reduction time	Nitrate reduction (1/colorimetric reading) × 100
None	80 mins.	50
M/300	200	Slight
M/600	90	45

Precipitants other than ammonium sulphate were tried with potato juice. The juice was precipitated by salts of copper, mercury, lead, iodine, molybdenum, and by phosphotungstic acid. The enzyme is destroyed by all except lead salts. The lead precipitate can be filtered off and obtained in a dry powder which will keep indefinitely. Like the original juice it reduces nitrate alone to a certain extent and very powerfully in the presence of aldehydes. The fact that the lead precipitate to some extent and the ammonium sulphate precipitate to a much less extent reduce nitrate alone is probably attributable to aldehydic substances in the juice which are carried down with the precipitates. One treatment with charcoal in the case of the ammonium sulphate precipitate completely abolishes this reduction. The lead salt being insoluble, it was impossible to treat it with

charcoal. It is used as a suspension in the reduction experiments. It reduces methylene blue alone slowly, and more rapidly in the presence of aldehydes. Treatment with H_2S to break up the lead salt destroys the enzyme.

To prepare the powder, neutral lead acetate is added to the expressed juice until no further precipitation occurs. It is then filtered with suction and washed thoroughly with water. The precipitate is then transferred to an evaporating dish and dried in a vacuum desiccator.

Exp. 6. 1 cc. lead salt suspension and 2 cc. buffer p_H 7·3 were placed in each of four tubes. Tube *A* contained 1 cc. 5 % sodium nitrate, tube *B* the same plus 0·5 cc. 1 % acetaldehyde. Tube *C* contained 1 cc. methylene blue instead of nitrate, and tube *D* the same plus 0·5 cc. acetaldehyde. The reductions were carried out anaerobically at 20°.

Tube	Reduction
A	+ +
B	+ + +
C	270 mins.
D	240

In this preparation of the enzyme also there is a definite correlation between the reduction of nitrate and methylene blue.

Summary.

(1) A method of preparation of the potato aldehyde oxidase is described and the properties of the enzyme are studied.

(2) The aldehyde oxidase will oxidise all aldehydes, but none of the other hydrogen donators tried.

(3) Beside nitrate, methylene blue, Clark's dyes, and quinone are reduced by the enzyme-aldehyde system. The failure of Bach and Michlin to obtain the reduction of methylene blue is explained.

(4) The p_H-activity curves for the nitrate and methylene blue reductions have been obtained.

(5) The action of the enzyme is not dependent on traces of iron.

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