BBA 65611

D-ASPARTATE OXIDASE OF KIDNEY

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(Received February 15th, 1967)

SUMMARY

1. The D-aspartate oxidase (D-aspartate:oxygen oxidoreductase (deaminating), EC 1.4.3.1) of rabbit kidney has been purified almost 100-fold. Although it is not yet homogeneous, it is apparently free from other oxidizing enzymes.

2. The enzyme is a flavoprotein. As prepared, it is in the inactive apo-form. The activity is restored by adding FAD, but not by FMN or riboflavin. The combination and dissociation of FAD are not instantaneous, but require about 15 min at 30° for approximate completion.

3. The enzyme oxidizes D-aspartate and D-glutamate, but has no action on any of the substrates of D-amino-acid oxidase or on any L-amino acids. D-Aspartate and D-glutamate are oxidized by the same enzyme. At low concentrations, using ferricyanide as acceptor, D-aspartate is oxidized more rapidly than D-glutamate, but at high concentrations the reverse is the case. With oxygen as acceptor, however, D-aspartate is always more rapidly oxidized than D-glutamate.

4. Unlike D-amino-acid oxidase, D-aspartate oxidase is strongly and competitively inhibited by dicarboxylic but not by monocarboxylic acids.

5. Only three acceptors have been found to work with the enzyme, namely ferricyanide, oxygen and dichlorophenolindophenol. Ferricyanide, which is inactive with D-amino-acid oxidase, is the best acceptor. With ferricyanide or oxygen, the rate decreases with increase of acceptor concentration; with indophenol it increases.

6. With ferricyanide as acceptor, the progress curve of the enzyme reaction is autocatalytic in form. The increase of the velocity with time is due to the fall in the ferricyanide concentration as the reaction proceeds, and the consequent reversal of the inhibition by ferricyanide.

7. The pH optimum is exceptionally sharp; the fall on the alkaline side is due to destruction of the enzyme. The position of the optimum depends on the order of addition of the reactants; if the ferricyanide is added last it is at about pH 9.6, if the substrate is added last it is at 8.7. The effect of pH on the K_m for D-aspartate is complex, but the curve is quite different from that of D-amino-acid oxidase.

Abbreviations: PCMB, *p*-chloromercuribenzoate; DCIP, 2,6-dichlorophenolindophenol.

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8. *p*-Chloromercuribenzoate (PCMB) does not inhibit the enzyme when incubated with it in the presence of FAD, but in the absence of FAD it inhibits it strongly, suggesting that the FAD is combined with a thiol group, which is thereby protected from attack. Incubation of the enzyme with PCMB in the presence of FAD produces a progressive increase in the enzyme activity, so long as the enzyme reaction has not started. When once the reaction has started and proceeded to about 30% of completion, a rapid inhibition begins and the reaction stops completely when it has reached about half-way. The activation is not observed when oxygen is the acceptor.

9. The relation of the enzyme to others of the same class is discussed.

INTRODUCTION

The well known D-amino-acid oxidase (D-amino-acid:oxygen oxidoreductase (deaminating), EC 1.4.3.3, a flavoprotein which occurs especially in kidneys) oxidizes many D-amino acids, but it has no action on the dicarboxylic amino acids D-aspartate and D-glutamate[1,2]. These two amino acids, however, are oxidized by kidney extracts[3,4], indicating the presence of another enzyme (or enzymes), distinct from the D-amino-acid oxidase. The enzyme responsible for the first of these oxidations has been named D-aspartate oxidase (D-aspartate:oxygen oxidoreductase (deaminating), EC 1.4.3.1), but it is not clear from the literature whether this enzyme is also responsible for the oxidation of D-glutamate or whether there is a separate D-glutamate oxidase. In fact the literature on the enzyme is very meagre; it has been relatively little studied and, as far as we are aware, has not been purified, previous workers using either crude kidney extracts or extracts of acetone powder of kidneys.

D-Aspartate oxidase was first studied in kidney extracts by STILL *et al.*[3] in 1949; they stated that it oxidized D-glutamate at about one-third of the rate obtained with aspartate but had not much action on other amino acids tested. They showed that (as is the case with flavoprotein oxidases generally) H_2O_2 was produced in the reaction, but that the enzyme differed from D-amino-acid oxidase in not being inhibited by benzoate. In 1950 STILL AND SPERLING[4] confirmed these results (using DL-aspartate throughout as substrate), but they considered that D-aspartate and D-glutamate were oxidized by different enzymes, because they found some variations in the ratio of the rates with the two substrates. They demonstrated that the D-aspartate oxidase could easily be inactivated by treatment with $(NH_4)_2SO_4$, presumably by removal of a flavin prosthetic group, and could then be reactivated by the addition of FAD (FMN was not tested), giving clear evidence of the flavoprotein nature of the enzyme.

More recently ROCCA AND GHIRETTI[5] have reported, under the name 'D-glutamate oxidase', an 1100-fold purification of an enzyme from the hepatopancreas of Octopus which oxidizes both D-glutamate and D-aspartate with a constant ratio of velocities. This enzyme, however, is different from the D-aspartate oxidase in at least two respects. Its oxidation of aspartate is slower than that of glutamate. Moreover it was much more difficult to obtain evidence of flavoprotein nature; the spectroscopic analysis is stated to be 'unsatisfactory', and the usual methods for resolving flavoproteins into flavin and apo-enzyme failed to inactivate the enzyme,

although more drastic methods produced a partial inactivation which was reversed by FAD.

More recently still ROCCA[6], using quite a different method, has effected a 180-fold purification of a D-aspartate oxidase from the hepatopancreas of Octopus. This enzyme shows a strong flavoprotein spectrum, and the flavin bands disappear on reduction with D-aspartate.

We have undertaken a purification of the D-aspartate oxidase of kidney, partly in order to investigate whether the oxidation of D-glutamate is due to a separate enzyme, and partly in order to carry out a study of its properties for comparison with the study of D-amino-acid oxidase already reported from this laboratory[1,2,7].

MATERIALS AND METHODS

Purification of the enzyme

Stage 1

About 5 lb of rabbit kidneys, frozen shortly after the death of the animals, were thawed and after removal of the outer membranes and fat, washed once with water. Portions of about 250 g were then homogenized for 150 sec in a Waring blendor, each with 700 ml of acetone at −10°. The united homogenates were poured into a further 12 l of cold acetone, and allowed to stand for 10 min, after which the supernatant was decanted and the powder filtered off with suction. After drying in a current of air, the acetone powder was desiccated *in vacuo* over paraffin wax and NaOH pellets. Yield about 420 g.

Stage 2

400 g of acetone powder was extracted with 2 l of water at 0° with constant stirring for 30 min and centrifuged at 0° for 30 min at 23 000 × *g*. The supernatant was poured through two layers of clean muslin and the volume made up to 2 l.

Stage 3

380 g of Analar $(NH_4)_2SO_4$ were added to the liquid at 0°. After 30 min the precipitate was centrifuged down for 30 min at 0° and 23 000 × *g*, and dissolved in cold 0.1 M phosphate buffer (pH 7.6) to a final volume of 1 l.

Stage 4

The slightly turbid solution was placed in a stainless-steel beaker immersed in a bath at −10°, and fractionated by adding cold acetone. The acetone was run in a thin stream down the cold metal side of the vessel while the solution was well stirred mechanically. The precipitate formed on bringing the acetone concentration to 38% (v/v) was allowed to stand for 10–15 min, and then spun off for 5 min at 23 000 × *g* and −10°, and discarded. The solution was brought to 51% with acetone, and after 10–15 min the enzyme-containing precipitate was centrifuged down for 15 min under the same conditions, and quickly taken up in a small volume of 0.1 M phosphate (pH 7.6).

Stage 5

The solution was made 0.4 saturated with solid $(NH_4)_2SO_4$ at 0°, and after 15 min it was centrifuged for 30 min at 38 000 × *g* and 0°. The supernatant was discarded and the precipitate dispersed in about 10 times its volume of 0.1 M phosphate 0.4 saturated with $(NH_4)_2SO_4$. It was necessary to carry out the whole of this stage rapidly, using a Potter hand homogenizer to disperse the precipitate com-

pletely, in order to avoid denaturation by residual acetone, but the suspension can then be left overnight at 4°.

Stage 6

The suspension was spun at 38 000 × *g* for 30 min, and the precipitate dissolved in a small volume of 0.01 M phosphate buffer (pH 7.6). Any undissolved protein was centrifuged off, and the clear solution was desalted by passing through a 250-ml column of Sephadex G-25, previously equilibrated with the 0.01 M phosphate buffer. To the solution 0.87 mg of alumina Cγ gel was added for every 1 mg of protein, and the suspension centrifuged. The enzyme was eluted from the gel with three successive 25-ml portions of 0.1 M phosphate buffer (pH 7.6) and the eluates combined. The third eluate was discarded if of low activity.

Most of the work was carried out with the enzyme at this stage, suitably diluted. It is convenient to keep it as a suspension after adding solid $(NH_4)_2SO_4$ up to 0.45 saturation *(Stage 6a)*; it can then be left for months without appreciable loss of activity, and can be centrifuged down and redissolved as required.

Some additional increase in specific activity can be achieved by a further fractionation of *Stage 6* on a column of Sephadex G-200 followed by $(NH_4)_2SO_4$ fractionation, but at the expense of such a large drop in yield that it is hardly worth while for ordinary purposes *(Stage 7)*. At *Stage 6* the preparation is completely free from D-amino-acid oxidase and other enzymes which might interfere; in fact the only enzyme which we have been able to detect, apart from D-aspartate oxidase, is a small amount of catalase.

The oxidase is still not pure and there are some indications that a further purification of several fold may be necessary before it is homogeneous, but we have not found it easy to proceed beyond this point. Fractionation on various columns leads to considerable loss of activity, as does precipitation with alcohol, and calcium phosphate gel adsorbs enzyme and total protein to about the same extent.

The enzyme is somewhat unstable in the early stages, but the instability disappears as purification proceeds. It should be noted that the above method of preparation yields the apoenzyme, which is inactive until FAD is added.

TABLE I

PURIFICATION OF D-ASPARTATE OXIDASE

From 400 g acetone powder.

Stage	*Vol. (ml)*	*Units/ml*	*Protein (mg/ml)*	*Spec. act. (units/mg)*	*Total units*	*Degree of purification*	*Yield (%)*
2. H_2O extract	2000	0.83	33.5	0.0248	1658	(1)	(100)
3. $(NH_4)_2SO_4$ precipitate redissolved	1000	1.43	16.2	0.0886	1430	3.6	86
5. Suspension in $(NH_4)_2SO_4$ of 38–51% acetone fraction	125	7.43	15.0	0.497	929	20	56
5a. After desalting	220	3.74	8.6	0.437	823	18	50
6. Alumina eluate	175	2.91	1.97	1.48	509	60	31
6a. $(NH_4)_2SO_4$ suspension	15.5	29.7	17.8	1.67	460	67	28
7. Sephadex and $(NH_4)_2SO_4$ fractionation of *6*	16.7	2.97	1.23	2.43	50	98	3

Assay of activity

D-Aspartate oxidase utilizes O_2, and the reaction may be followed either manometrically[3,4], or preferably by the oxygen electrode in the same way as D-amino-acid oxidase[1]. The earlier workers added ethanol in order to double the O_2 absorption in their tests; the H_2O_2 formed in the oxidase reaction is then used up in the oxidation of ethanol by catalase[8], so that the oxidation of 1 molecule of aspartate uses up 2 atoms of oxygen. In our experiments with O_2, however, we have not added ethanol, preferring to avoid complications as far as possible, so that the H_2O_2 is decomposed by the catalase present and the oxidation of 1 molecule of aspartate requires only 1 atom of oxygen.

The most convenient technique, however, is with ferricyanide instead of O_2 (ref. 6). D-Aspartate oxidase readily reduces ferricyanide, in complete contrast to D-amino-acid oxidase, which has no action on it[2]. The reduction can be followed spectrophotometrically at 420 mμ; a Beckman DB recording spectrophotometer with a cell holder kept at 30° was used.

The routine test was as follows: 0.2 ml of 0.1 M D-aspartate (neutralized) (20 μmoles); 0.1 ml of FAD (0.47 mg/ml disodium salt, Sigma '96%') (about 0.05 μmole); 0.05 ml of enzyme solution; 0.04 ml of 0.057 M potassium ferricyanide (2.28 μmoles); 0.1 M phosphate buffer (pH 7.6) to 3.0 ml total volume. The reaction was carried out in a 10-mm Beckman cell which was fused to the upper part of a Thunberg tube, so that it could be evacuated (Fig. 2, but without the cup). The ferricyanide was placed in the hollow stopper and the remaining solutions in the cuvette, and the tube was then evacuated to remove O_2. After a preliminary incubation of 15 min at 30°, the reaction was started by tipping in the ferricyanide and the change of light absorption at 420 mμ was recorded as a function of time. The reference cell contained buffer and FAD only, and a special cover was constructed to exclude external light from the tubes during the reaction. The amount of ferricyanide was chosen to give an initial absorbance of 0.8, which is a convenient starting point for the Beckman DB.

The preliminary incubation of the enzyme with FAD is necessary because, as is shown below, the combination of FAD with the apoenzyme is relatively slow and time must be allowed for the excess of FAD to saturate the apoenzyme before the reaction is started.

As will be seen later, the reaction with ferricyanide involves certain complications, but when the test is carried out in this way the velocity initially is exactly proportional to the enzyme concentration over a wide range.

An alternative method is to use 2,6-dichlorophenolindophenol (DCIP) as acceptor instead of ferricyanide, following the reaction in the Beckman DB at 600 mμ, using the same type of evacuated cell.

Unit of enzyme

In accordance with the 1964 recommendations of the International Union of Biochemistry* we define 1 unit of enzyme as the amount which catalyses the oxidation of 1 μmole of D-aspartate per min at 30° under the above conditions. We have

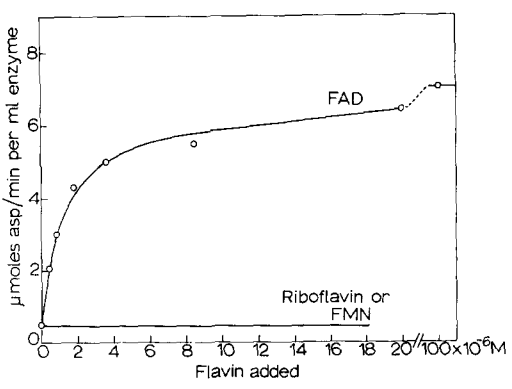
* *Enzyme Nomenclature, Recommendations 1964 of the International Union of Biochemistry* Elsevier, Amsterdam, 1965.

taken ferricyanide as the standard acceptor, but the rate depends on the nature of the acceptor, and if any other acceptor is used it is necessary to specify the unit adopted. In converting scale readings into units of enzyme it must be remembered that 1 molecule of aspartate is oxidized by 2 molecules of ferricyanide, or by 1 molecule of indophenol, or by 0.5 molecule of O_2 (in the presence of catalase). Taking our determination of the molar extinction coefficient of ferricyanide at 420 mμ as $1.05 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ (WILLIAMS AND KAMIN[9] give $1.02 \cdot 10^3\ M^{-1} \cdot cm^{-1}$), we calculate that 1 unit of enzyme in the above test mixture gives an initial absorbance change of 0.7 per min.

RESULTS

Activation by flavin

The enzyme as prepared by the above method contains only a small trace of flavin and is almost inactive in the absence of added flavin. As Fig. 1 shows, it is not activated by either riboflavin or FMN, but is activated by FAD. Half the maximum activity is given by a total FAD concentration of $1.3 \cdot 10^{-6}$ M. STILL AND SPERLING[4],



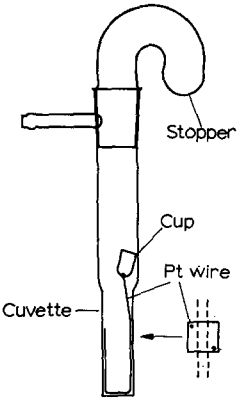


Fig. 1. Effect of flavin addition on velocity. Conditions as for routine test, but with variable amount of flavin. For most points, 15 min preincubation with flavin, but prolonged as necessary with the smaller amounts, to allow the combination to reach equilibrium.

Fig. 2. Thunberg–Beckman cell with removable cup. Made by fusing a 1-cm quartz Beckman cuvette to the upper part of a Thunberg tube. In the cross section the broken lines show the path of the light beam.

using a crude extract, found a value of about $3 \cdot 10^{-6}$ M. However, there is a time factor involved which would tend to make their value too high. It will be shown that with FAD concentrations of the order of $2 \cdot 10^{-6}$ M it takes about 15 min at 30° for the FAD to come into equilibrium with the apoenzyme. The rate of combination depends on the FAD concentration, and as this is further reduced it is necessary to prolong the preincubation more and more to obtain the full effect of the FAD when determining a curve such as Fig. 1. If this is not done the early part of the curve will be depressed, resulting in an apparent increase of the concentration for half-maximal activation.

The true Michaelis constant for FAD (K_m) must be considerably smaller than the apparent value of $1.3 \cdot 10^{-6}$ M obtained from the curve (which we will call 'K_m'), for 'K_m' is the total added FAD concentration for half activation, whereas K_m is the concentration of free FAD at this point. With such a high affinity, the free FAD is much less than the total since much of the FAD added is here bound to the enzyme. A rough estimate from the curve by the graphical method of DIXON[10] suggests that K_m is of the order of $8 \cdot 10^{-7}$ M, but as the enzyme is not yet pure, we do not give this as a precise value.

DIXON AND KLEPPE[1] showed that in the case of D-amino-acid oxidase the combination of the apoenzyme with FAD is far from being instantaneous, but takes some minutes, as also does the dissociation of the enzyme into apoenzyme and FAD. This effect is marked with D-aspartate oxidase. In order to measure the rate of combination, the apoenzyme was incubated for various times with FAD before the reaction was started by adding the ferricyanide. For this purpose we used the device shown in Fig. 2, by which the FAD and ferricyanide could be added independently in the absence of oxygen. The FAD was placed in a small glass cup supported above the main solution by a bent platinum wire fused to it. The wire was of such a shape that it would slide into the diagonally-opposite corners of the cuvette sufficiently rigidly while remaining well out of the path of the light beam. For convenience in mixing, the cup was placed off the axis so that its lip rested on one side of the tube; by tilting, the main solution could then readily be caused to wash out the contents of the cup. The ferricyanide was placed in the stopper and could be added subsequently by tilting the tube in the opposite direction. The apoenzyme, buffer and substrate were placed in the cuvette. After evacuation, the tube was placed in the temperature-controlled cell-holder of the spectrophotometer and allowed to reach the correct temperature. The FAD was then mixed in and allowed to combine for the desired time, after which the ferricyanide was mixed in and the enzyme reaction recorded.

For convenience, in the experiment shown in Fig. 3 a concentration of FAD about one-tenth of that used in the routine assay was chosen, as with the excess

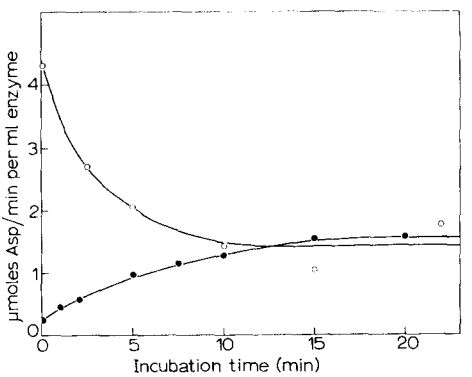


Fig. 3. Combination and dissociation of FAD and apoenzyme. Quantities as for routine test, except that only 5.15 mμmoles FAD were added (about one-tenth of normal). FAD in cup during 15 min preincubation, then mixed in at zero time and incubated for various times before starting reaction. ○—○, enzyme in cup during preincubation; ●—●, enzyme in cuvette. pH 7.6. 30°.

normally used the combination is considerably more rapid. The amount used here gave about one-third of complete activation at infinite time.

In order to measure the rate of dissociation of the FAD from the enzyme it was first necessary to get it combined with the apoenzyme. For this purpose the apoenzyme and FAD were incubated together in a small volume and therefore at much higher concentration, and after they had combined the mixture was diluted suddenly and the dissociation followed by the fall in activity with time. This was achieved merely by repeating the previous experiment with the same quantities, but with the enzyme placed with the FAD in the cup instead of in the cuvette, so that the combination took place during the initial 15-min preincubation. As the total volume placed in the cup was 0.06 ml, there was a 50-fold dilution on mixing the contents of the cup and cuvette at zero time. The dissociation then began and was followed by tipping in the ferricyanide at various times to measure the fall in activity. Each point on the curves of Fig. 3 represents a separate repetition of the experiment, since of course the ferricyanide can only be added at one time for each filling of the tube.

The resulting curve may be compared with the similar curve given by D-amino-acid oxidase (ref. 1, Fig. 3). DIXON AND KLEPPE[1] estimated the rate constants of combination and dissociation for D-amino-acid oxidase, but this cannot be done here as the concentration of the apoenzyme is unknown. In Fig. 3 the same final equilibrium point is naturally reached from opposite directions, since the composition of the system is the same in both cases.

These curves clearly show the necessity for the preincubation with FAD in assaying the activity of the enzyme.

Reaction product

The nature of the product formed in the D-aspartate oxidase reaction has not previously been determined, although by analogy with D-amino-acid oxidase one would expect it to be oxaloacetate. This was confirmed as follows.

Into the tube shown in Fig. 2 (without the cup) was placed the standard test mixture as given above, except that the amount of ferricyanide was 1.43 μmole, tipped in from the stopper after evacuation; this gives an initial absorbance of 0.5 at 420 mμ, measured against a control cuvette containing the same concentration of FAD. The ferricyanide was allowed to reduce completely, and then the tube was opened and 0.1 ml of 30% $HClO_4$ added immediately for deproteinization. The mixture was cooled to 0°, neutralized with 1 M K_2CO_3 and centrifuged.

0.5 ml of the supernatant was mixed with 0.1 ml of 30 mM NADH (excess) in a cuvette, together with 0.1 ml phosphate buffer (pH 7.1) and water to 2 ml; and the absorbance change at 340 mμ on adding purified malate dehydrogenase was read against a control cuvette containing the same dilution of supernatant in buffer. After the oxidation of NADH by oxaloacetate had ceased, some purified lactate dehydrogenase was added, in case some of the oxaloacetate had been converted into pyruvate.

The absorbance change found was 0.27 which, taking the molar absorbance of NADH as 6200, corresponds to 0.097 μmole of oxaloacetate in 0.5 ml of supernatant or 0.582 μmole in the original reaction. The theoretical amount, taking 2 molecules of ferricyanide as equivalent to 1 molecule of oxaloacetate, was

0.715 μmole, so that the yield of oxaloacetate from D-aspartate was 81% of theoretical. A control determination on a standard solution of oxaloacetate gave 87%. It is therefore established that the product of the oxidase reaction is oxaloacetate. ROCCA AND GHIRETTI[5] showed that the product of oxidation of D-aspartate by the D-glutamate oxidase of Octopus is oxaloacetate.

Specificity for substrate

The specificity of D-aspartate oxidase is very clean-cut. It can be regarded as being complementary to that of D-amino-acid oxidase: neither enzyme acts on L-amino acids, but whereas D-amino-acid oxidase acts on the majority of D-amino acids but not on D-aspartate or D-glutamate (ref. 2, Fig. 1), D-aspartate oxidase acts only on D-aspartate and D-glutamate (see Table II). Thus D-aspartate oxidase

TABLE II

SUBSTRATE SPECIFICITY OF D-ASPARTATE OXIDASE

Relative rate of oxidation (D-Asp = 100). pH 7.6, 30°. Ferricyanide as acceptor. Amino acid concn. 6.67 mM. Conditions as for standard test.

Amino acid	*Relative rate with*	
	D-*form*	L-*form*
Ala	0.0	0.0
β-Ala	0.0	
Arg	0.0	0.0
Asn	0.0	0.0
Asp	100	0.0
Glu	15.9	0.0
Gly	0.0	
His	0.0	0.0
Ile	0.0	0.0
Leu	0.0	0.0
Lys	0.0	0.0
Met	0.0	0.0
Norvaline	0.0	0.0
Orn	0.0	0.0
Phe	0.0	0.0
Pro	0.0	0.0
Ser	0.0	0.0
Thr	0.0	0.0
Trp	0.0	0.0
Val	0.0	0.0

acts only on dicarboxylic amino acids, but D-amino-acid oxidase only on monocarboxylic amino acids.

In comparing aspartate and glutamate as substrates for D-aspartate oxidase the concentration must be taken into account. From Table II it would appear that D-aspartate is much the better substrate, but with ferricyanide as acceptor this is only true at low concentrations where the enzyme is not saturated. With substrate concentrations high enough to saturate the enzyme, the maximum velocity V is more than twice as great with D-glutamate as with D-aspartate (Table III). The comparatively low rate with D-glutamate at the smaller concentration is due to the fact that the K_m is much higher for D-glutamate than for D-aspartate, so that the

enzyme is much less saturated with glutamate than with aspartate under such conditions.

The rate at saturation with D-glutamate raises the question whether the oxidase ought not to be called 'D-glutamate oxidase'. We believe, however, that it should not, for the following reasons. If it were so called, it would be confused with the different enzyme from Octopus which has been given that name by ROCCA AND GHIRETTI[5]. The name 'D-aspartate oxidase' has been used for the present enzyme for many years, and has been approved by the International Union of Biochemistry. The superiority of D-glutamate as substrate is very evident with ferricyanide as acceptor, but is not seen with oxygen (Table III); and since the name 'oxidase' implies a reaction with oxygen, it would seem that the name of the enzyme should be based on the results with oxygen.

TABLE III

COMPARISON OF D-GLUTAMATE WITH D-ASPARTATE

K_m and v_{max} determined from Lineweaver–Burk plots, which were straight lines. Values are means of several concordant determinations. Velocities are given as μmoles substrate oxidized per min per ml of enzyme solution. pH 7.6. 30°.

Substrate	*Ferricyanide as acceptor*			O_2 *as acceptor*		
	K_m *(mM)*	*V*	*v at 6.67 mM*	K_m *(mM)*	*V*	*v at 6.67 mM*
D-Aspartate	5.2	2.49	1.37	0.86	1.6	1.43
D-Glutamate	166	7.45	0.34	5.68	0.22	0.12

It will be noted that with both substrates the value of K_m depends very much on the nature of the acceptor used, from which it may be concluded that the K_m is partly kinetic in nature and is not merely determined by the enzyme–substrate affinity. If the Michaelis constant is represented by an expression of the Briggs–Haldane form, it would be expected that for each substrate the higher value of K_m would be associated with the higher V, as is actually the case.

Specificity for competitive inhibitors

As in the case of D-amino-acid oxidase, the study of substances competing with the substrate has thrown light on the nature of the enzyme–substrate combination.

Of the D-amino acids, none produces any inhibition of the oxidation of D-aspartate in a concentration of about 10 mM, except for D-glutamate, which has a small competitive effect.

The results with L-amino acids are shown in Table IV. None of them, not even the dicarboxylic amino acids, has any effect, except for L-valine and L-leucine, which inhibit slightly. It is interesting to compare this with D-amino-acid oxidase (ref. 2, Table III), which is inhibited much more strongly by L-norvaline (but not by L-valine), by L-leucine and by L-methionine, but by no other L-amino acids.

The two enzymes are sharply contrasted with respect to inhibition by mono- and dicarboxylic acids: D-amino-acid oxidase is quite strongly inhibited by fatty

TABLE IV

INHIBITION OF OXIDATION OF D-ASPARTATE BY L-AMINO ACIDS

Ferricyanide as acceptor. D-Aspartate concn. 6.67 mM. pH 7.6. 30°. Conditions as for standard test.

Amino acid	*% inhibition by 6.67 mM*
L-Ala	0
L-Arg	0
DL-Asn	0
L-Asp	0
L-Glu	0
L-His	0
L-Ile	0
L-Leu	6.3
L-Lys	0
L-Met	0
L-Norvaline	0
L-Orn	0
L-Phe	0
L-Pro	0
L-Ser	0
L-Thr	0
L-Trp	0
L-Val	6.3

acids and benzoic acid, but not by dicarboxylic acids, while D-aspartate oxidase is very slightly, if at all, inhibited by fatty acids or benzoic acid, but quite strongly by dicarboxylic acids. Table V shows the effect of monocarboxylic acids at 6.67 mM on the oxidation of D-aspartate. Table VI shows the inhibition by dicarboxylic acids at various concentrations, using both substrates. The high and specific affinity of the enzyme for dicarboxylic acids is obvious, and it is evidently connected with its action as an oxidase for dicarboxylic amino acids.

One enzyme or two?

In spite of the suggestion by STILL AND SPERLING[4] mentioned earlier, we have been assuming that both substrates are oxidized by the same enzyme, or in other words that there is no specific D-glutamate oxidase. This is already strongly suggested

TABLE V

INHIBITION OF OXIDATION OF D-ASPARTATE BY SODIUM SALTS OF FATTY ACIDS

Conditions as for Table IV.

Fatty acid	*% inhibition by 6.67 mM*
Formic	0
Acetic	2
Propionic	0
Butyric	3
Valeric	0
Caproic	7

TABLE VI

INHIBITION OF OXIDATION OF D-ASPARTATE AND D-GLUTAMATE BY SODIUM SALTS OF DICARBOXYLIC ACIDS

Conditions as for Table IV, but dicarboxylic acids tested at three concentrations and with both substrates.

Dicarboxylic acid	*% inhibition of oxidation of*					
	D-*aspartate by (mM)*			D-*glutamate by (mM)*		
	6.66	*0.66*	*0.33*	*6.66*	*0.66*	*0.33*
Oxalic	40			33		
Malonic	100	78		100		80
Succinic	66			80		
Glutaric	62			74		
Adipic	15			23		
Fumaric	78			100		
Maleic	100	87		100	100	
D-Malic	100	100	90	100	100	100
L-Malic	79			100		
Meso-tartaric	100	76		100	100	90
D-Tartaric	77	19		100	50	
L-Tartaric	24			29		
Citric	29			43		
Oxaloacetic	90			100	36	
2-Oxoglutaric	64			79		
Pyrophosphoric	0			0		

by the striking similarity shown in Table VI, allowing for a lower affinity for D-glutamate than for D-aspartate. We have, however, sought more conclusive evidence of identity.

The inhibitor constant K_i of a reversible competitive inhibitor is equal to the reciprocal of the affinity of the inhibitor for the enzyme; it is therefore a characteristic property of the particular enzyme and inhibitor, and not of the substrate. The degree of inhibition produced by a certain concentration of inhibitor may depend on the nature of the substrate, because of the competition between them, but the value of K_i should be independent of the substrate. If therefore an inhibitor is found to give exactly the same value of K_i using two different substrates, it is good evidence that the same enzyme is acting on both, for there is no reason why two different enzymes should give the same value of K_i. If the same thing is found with more than one in-

TABLE VII

K_i FOR D-MALATE AND MALONATE WITH BOTH SUBSTRATES

K_i determined from plots of 1/rate against inhibitor concentration with different substrate concentrations.

Substrate	K_i *(μM) for*	
	D-*malate*	*malonate*
D-Aspartate	10.5	60
D-Glutamate	10.0	65

hibitor, the evidence of identity is greatly reinforced. Accordingly the K_i's for D-malate and for malonate were determined for both substrates, using the method of DIXON[11], and the values are shown in Table VII.

A different method, which also provides strong evidence, is to produce a partial destruction of the enzyme by controlled heating, and to determine the percentage loss of activity with the two substrates. Since the process of denaturation has a very high temperature coefficient, it would be extremely unlikely that two different enzymes would suffer the same degree of inactivation. Heating at 55° for a short period produced a loss of activity of 22% with D-aspartate and 25% with D-glutamate, while rather longer heating gave a loss of 54% with aspartate and 58% with glutamate.

It may therefore be taken as established that one enzyme is responsible for both oxidations.

Specificity for acceptors

D-Aspartate oxidase shows some interesting and unusual features in relation to acceptors. The only acceptors found to act were oxygen, ferricyanide and 2,6-dichlorophenolindophenol; surprisingly, no action was found with methylene blue or phenazine methosulphate. As with D-amino-acid oxidase, DCIP is the least active of the acceptors that work. At the same molarity, ferricyanide is even better than oxygen, in marked contrast to D-amino-acid oxidase, which does not use ferricyanide at all. The rates given by the three acceptors in equal concentrations are shown in Table VIII, with D-aspartate as substrate. Here the molarities of all the acceptors

TABLE VIII

RATES WITH DIFFERENT ACCEPTORS

Relative rates of oxidation of D-aspartate (ferricyanide = 100). Concentrations of all acceptors $2.3 \cdot 10^{-4}$ M. pH 7.6. 30°. D-Aspartate concn. 6.7 mM.

Acceptor	*Relative rate*
Ferricyanide	100
Oxygen	33
DCIP	6.7

were made equal to that of oxygen in air-saturated water. The apparent discrepancy between this table and Table III is due to the fact that in the routine tests used for that table the concentrations of ferricyanide and oxygen were different.

On attempting to determine the K_m's for these acceptors, we found to our surprise that, whereas the rate increases with DCIP concentration as expected, it decreases with increase of ferricyanide or oxygen concentration. With 100% oxygen the rate was only about 50% of the rate obtained with air (see Fig. 4). This was not due to destruction of enzyme by the higher concentration of oxygen, for on adding more enzyme after the reaction had proceeded linearly for some time, the rate was increased in proportion to the amount of enzyme and then remained constant.

The dependence of the rate on ferricyanide concentration is shown in Table IX, which indicates that ferricyanide inhibits its own reduction by the enzyme. The

TABLE IX

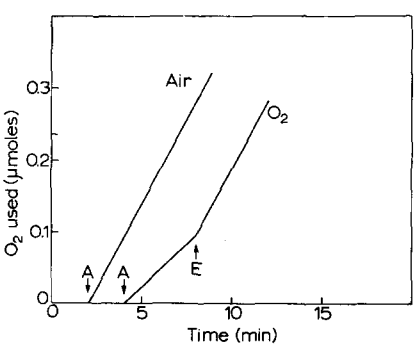
EFFECT OF FERRICYANIDE CONCENTRATION

Conditions as for routine test, but with different ferricyanide concentrations. Rates expressed as μmoles D-aspartate per min per ml enzyme.

Initial ferricyanide concn. (mM)	*Initial rate*
0.73	11.4
0.35	15.6
0.22	24.8

inhibition is not due to the ferrocyanide formed in the reaction, for the addition of ferrocyanide initially has no effect. Moreover the inhibition by ferricyanide is reversible; the reaction speeds up as the ferricyanide concentration falls during its progress, resulting in a progress curve of autocatalytic form, as shown in Fig. 5, Curve A. This is the case even when plenty of time is allowed for the combination of FAD with apoenzyme before the reaction is started. Although the effect complicates the kinetics somewhat, it does not interfere with the measurement of the initial velocity which, as mentioned above, is an accurate measure of the enzyme activity. It will be noted that the rate increases up to very nearly the end of the reaction; this indicates that the affinity for ferricyanide must be high, otherwise there would be some falling off as its concentration approaches zero.

Clear evidence that the speeding up of the reaction is due simply to the fall in ferricyanide concentration is given by the fact that progress curves obtained with different initial concentrations of ferricyanide are found to run parallel (Fig. 5). This



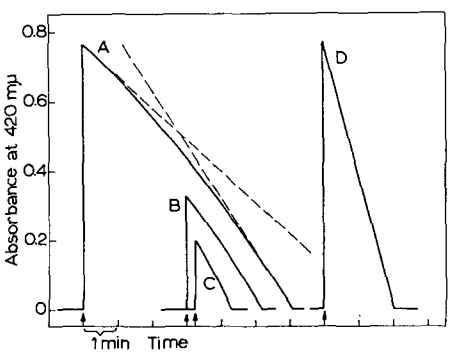


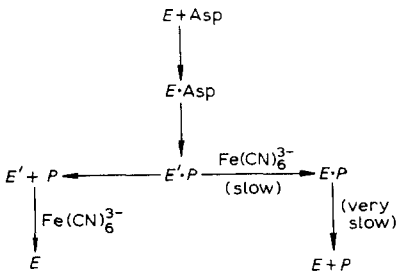
Fig. 4. Effect of concentration of oxygen. Oxygen uptake measured by electrode. Quantities of components as in routine test, but without ferricyanide and in total volume of 4 ml. Enzyme (0.05 ml) and FAD preincubated 15 min in electrode vessel. At A, reaction started by adding D-aspartate. At E, a further 0.05 ml of enzyme added (doubling the concentration). Total oxygen initially present: for air, 0.92 μmole; for oxygen, 4.4 μmoles.

Fig. 5. Effect of ferricyanide concentration on rate. Curves A, B and C at pH 7.6; Curve D at pH 9.25. Conditions as for routine test, except for ferricyanide concentration added: A and D, 2.28 μmoles (normal); B, 1.0 μmole; C, 0.57 μmole. Ferricyanide added at arrows to start reaction. The broken lines are tangents drawn to indicate the initial and final velocities.

means that at any given ferricyanide concentration the rate will be the same no matter whether the concentration is produced by direct addition or whether it is reached from a higher concentration by reduction during the course of the reaction. Since the only difference between the initial parts of the different curves is the ferricyanide concentration, it must be concluded that it is this which determines the rate throughout Curve A.

While the mechanism of the inhibition has not been definitely determined, the fact that it is shown by two such different substances as ferricyanide and oxygen suggests that it may be due to a reversible oxidation of the enzyme to a less active form. The inability of DCIP to produce such an inhibition might be connected with its lower position on the redox scale, which might make it incapable of bringing about certain oxidations that are easily accomplished by oxygen or ferricyanide by virtue of their position near the oxidizing end of the scale. Other cases are known of the inactivation of flavoproteins by oxidation or reduction to forms which do not take part in the catalytic cycle[12,13].

While this paper was being written, an article by GAWRON *et al.*[14] appeared, describing an analogous inhibition by ferricyanide of succinate dehydrogenase (also a flavoprotein) and suggesting an alternative explanation. Translated into terms appropriate to the aspartate oxidase, this would be as follows. The enzyme–substrate complex E. Asp is first converted into a complex of reduced enzyme and product, $E'.P$. It is assumed that the product can dissociate rapidly from the reduced enzyme,



but only very slowly from the oxidized enzyme, and that the reduced enzyme can be oxidized by ferricyanide rapidly when it is in the free state or slowly while it is still combined with product. At the lower concentrations of ferricyanide little of the $E'.P$ complex is oxidized as such, and the reaction proceeds mainly down the left-hand path; but as the ferricyanide concentration is increased, more and more of the enzyme is diverted into the abortive complex $E.P$ and is therefore removed from active catalysis, since $E.P$ only decomposes very slowly. The kinetic equations derived by GAWRON show that under suitable conditions this mechanism is adequate to explain the observed inhibition.

The ferricyanide effect depends upon the pH and on the order of addition of the reactants, as illustrated in Table X. The results can be explained by a combination of the following factors. (i) As the solution is made more alkaline the rate of the enzyme reaction increases (compare Expts. 1a and b), and at the same time the autocatalysis becomes less marked, until at a pH of about 9.2 it has disappeared

TABLE X

EFFECT OF ORDER OF ADDITION OF REACTANTS

Ferricyanide as acceptor. Quantities as in routine test. Preincubation 15 min at 30°. Rates given as μmoles D-aspartate per min per ml enzyme.

Expt. No.	*Preincubated together 15 min in cuvette*	*Added from stopper after preincubation*	*Initial rate*	*Final rate*	*Final rate as % of initial*	*Initial rate as % of Expts. 1*
pH 7.55						
1a	E + FAD + Asp	$Fe(CN)_6^{3-}$	6.2	7.4	120	100
2a	E + FAD	$Fe(CN)_6^{3-}$ + Asp	5.5	5.5	100	89
3a	E + FAD + $Fe(CN)_6^{3-}$	Asp	4.8	4.8	100	77
pH 9.25						
1b	E + FAD + Asp	$Fe(CN)_6^{3-}$	44	44	100	100
2b	E + FAD	$Fe(CN)_6^{3-}$ + Asp	30.2	30	99	69
3b	E + FAD + $Fe(CN)_6^{3-}$	Asp	23.8	23.8	100	54

altogether and the progress curve has become accurately a straight line (*i.e.* zero order, see Expt. 1b and Curve D of Fig. 5). (ii) The enzyme is slightly unstable at 30° in the absence of aspartate, especially at the higher pH's. In the routine test the presence of aspartate in the cuvette along with enzyme and FAD during the preincubation protects the enzyme, but if the aspartate is placed in the stopper with the ferricyanide, and tipped in after the preincubation, there is an irreversible loss of activity of about 10% at pH 7.6 and 30% at pH 9.2 (see Expts. 2a and b). (iii) The irreversible loss during preincubation in the absence of substrate is somewhat increased if ferricyanide also is present, amounting to some 20% at pH 7.6 and 45% at pH 9.2 (Expts. 3a and b). When once the reaction is started, the presence of the substrate arrests further destruction, except in more alkaline solutions.

When oxygen and ferricyanide are present together, each acceptor inhibits the reduction of the other. Fig. 6 shows an experiment with the oxygen electrode in which the oxidation of D-aspartate by oxygen was allowed to proceed under the usual conditions until at the Point F 0.286 μmole of ferricyanide were added in the

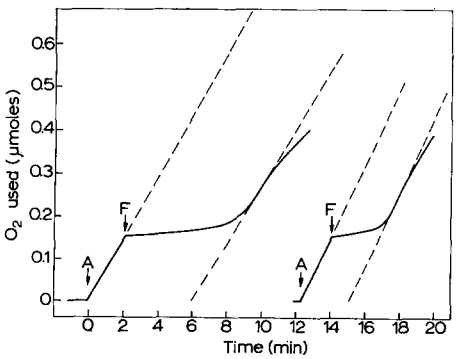


Fig. 6. Competition between ferricyanide and oxygen. Oxygen uptake measured by electrode. Conditions as for Fig. 4, with air. At F, 0.286 μmole ferricyanide was added in the first case (one-eighth of normal), and 0.143 μmole in the second.

first case and 0.143 μmole in the second. It will be seen that the uptake of oxygen is almost completely arrested for a time which is proportional to the amount of ferricyanide added. During this time the ferricyanide is being reduced, and when its reduction is nearly complete the oxygen uptake is resumed at its previous rate. The competition, however, is mutual, for in Curve C of Fig. 5 we see that in the absence of oxygen 0.57 μmole of ferricyanide are reduced in 1.3 min, so that 0.286 μmole (the amount used in the first curve of Fig. 6) would be reduced in 0.65 min instead of 6 min. Therefore the presence of oxygen has cut down the ferricyanide reduction to about one-tenth of its anaerobic rate. D-amino-acid oxidase shows no such effect.

Effect of pH

The stability of the apoenzyme in the absence of aspartate was tested by incubating for 10 min at 25° at different pH's, and then neutralizing and testing in the usual way. As Fig. 7 shows, it is largely destroyed at pH 4.5 on the one side and pH 11 on the other, but it is fairly stable between 6 and 8.5.

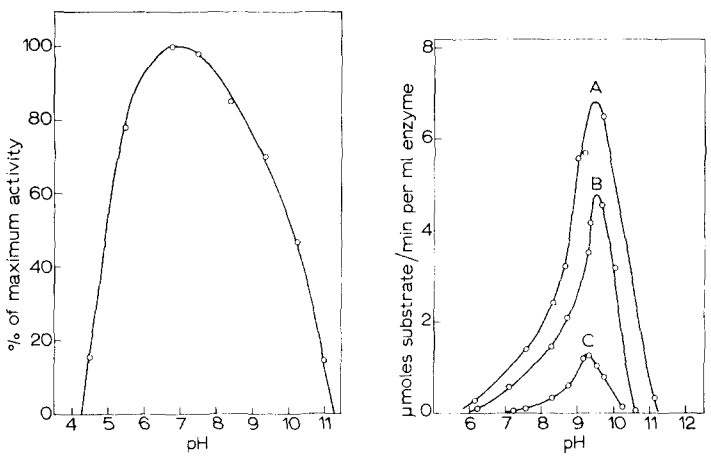


Fig. 7. Effect of pH on stability of apoenzyme. Apoenzyme incubated at pH's shown for 10 min at 25°; then brought to pH 7.6 for testing by routine assay.

Fig. 8. pH curves with the two substrates. Ferricyanide as acceptor. B and C with 6.67 mM substrate (routine amount), and A with 53.4 mM to saturate the enzyme. A and B with D-aspartate; C with D-glutamate. Normal procedure, with ferricyanide added last to start the reaction.

Fig. 8 shows the pH–activity curves obtained with the routine substrate concentration for D-aspartate (Curve B) and D-glutamate (Curve C), and also with a sufficient D-aspartate concentration to saturate the enzyme (Curve A). The pH optimum is exceptionally sharp; this, however, is partly due to the fact that the enzyme is being destroyed on the right-hand side, even in the presence of substrate. These results were obtained by the routine test, with ferricyanide in the stopper and aspartate in the cuvette with the enzyme during the preincubation.

On repeating the determinations with aspartate only in the stopper and ferricyanide in the cuvette with the enzyme during the preincubation, different curves were obtained (Fig. 9). The main effect is a shift of the pH optimum by a whole pH

unit towards the acid side. This is largely, but not entirely, due to a great increase in the destruction on the alkaline side of the optimum, resulting in the loss of nearly all of that part of the curves of Fig. 8 which shows the greatest activity.

The effect of pH on the K_m for D-aspartate was studied in some detail for comparison with the corresponding curves obtained with D-amino-acid oxidase by DIXON AND KLEPPE[7]. Each point shown in Fig. 10 represents a separate Lineweaver–Burk plot, involving the recording of five progress curves. Since it was not possible to carry out all this work on the same day, and there might be slight variations in the activity of the enzyme solution from day to day, a determination of the activity at pH 7.6 was made each day and a small correction applied to all the activity values obtained on that day, so as to make all the points comparable. The range of pH is restricted to that shown by the instability of the enzyme in more acid or alkaline solutions.

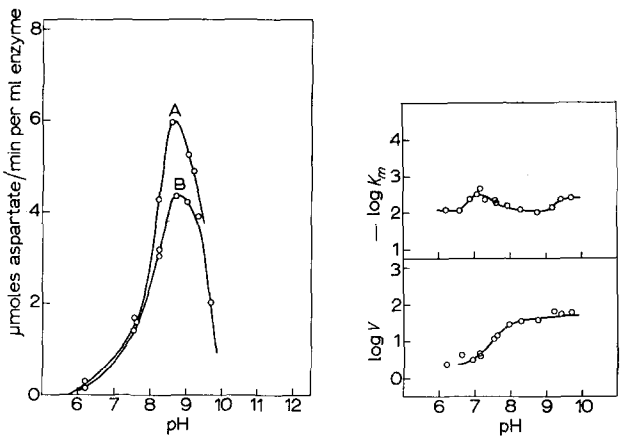


Fig. 9. pH curves by reversed procedure. Aspartate as substrate. A and B corresponding to A and B of Fig. 8, with high and normal substrate concentration, respectively, but with ferricyanide with the enzyme in the cuvette during preincubation and aspartate in the stopper, added last to start the reaction.

Fig. 10. Effect of pH on $-\log K_m$ and $\log V$. Aspartate as substrate and ferricyanide as acceptor. Each point obtained from a separate reciprocal plot.

The $-\log K_m$ curve is more complex than any obtained with D-amino-acid oxidase, probably involving six ionization constants, three for the enzyme–substrate complex and three for the free enzyme (or two if the pK at about 9.5 represents the amino group of the free aspartate). The pK's of the carboxyl groups of the free aspartate lie well outside the range of the curves. The pK's within the range lie too close together to permit actual values to be deduced, and with the available data it is not possible to give an interpretation of the results in chemical terms. It is interesting, however, that the curve is completely different from all those obtained with D-amino-acid oxidase. With most substrates the latter enzyme shows a marked hill in the pH 8–9 region, whereas with D-aspartate oxidase this region is occupied by a well-marked valley between two hills. The $\log V$ curves for the two enzymes, however, are not dissimilar.

Inhibitors

Apart from the dicarboxylic acids, mentioned above, no strong inhibitor of the D-aspartate oxidase reaction was found, except under special conditions. Cyanide (3.333 mM) had no effect, suggesting that the reaction was not dependent on a metal. Iodoacetate (3.333 M) likewise was without action, even when incubated with the enzyme at 30°.

p-Chloromercuribenzoate (PCMB), however, produced either acceleration or inhibition according to the method of treatment. Table XI is representative of the results obtained when ferricyanide is the acceptor; they have been confirmed repeatedly. The experiments were carried out in the vacuum cuvette with the cup shown in Fig. 2. Various combinations of the reactants were first preincubated together in the lower part before the PCMB was added from the cup. The PCMB (0.067 mM) was then allowed to act during a further period of incubation before the enzyme reaction was started by tipping in the remaining component from the stopper.

TABLE XI

EFFECTS OF PCMB

Quantities as for routine test. 0.1 ml of 2 mM PCMB (0.2 μmole) in cup where shown. pH 7.6. 30°. Mixture of components shown in Column 2 first incubated for 15 min; then contents of cup mixed in and a further incubation for time shown in Column 4; then reaction started by tipping in contents of stopper. Rate given as μmoles D-aspartate per min per ml of enzyme.

Expt. No.	*Preincubated together for 15 min in cuvette*	*In cup*	*Incubation time with PCMB (min)*	*In stopper*	*Initial rate*
1	*E* + FAD + Asp	—	None added	$Fe(CN)_6^{3-}$	3.32
2	*E* + FAD	PCMB	5	$Fe(CN)_6^{3-}$ + Asp	3.03
3	*E* + Asp	PCMB	5	$Fe(CN)_6^{3-}$ + FAD	0.74
4	*E* + FAD + $Fe(CN)_6^{3-}$	PCMB	5	Asp	1.09
5a	*E* + FAD + Asp	PCMB	0	$Fe(CN)_6^{3-}$	3.15
5b	*E* + FAD + Asp	PCMB	5	$Fe(CN)_6^{3-}$	3.26
5c	*E* + FAD + Asp	PCMB	10	$Fe(CN)_6^{3-}$	3.78
5d	*E* + FAD + Asp	PCMB	15	$Fe(CN)_6^{3-}$	4.18
5e	*E* + FAD + Asp	PCMB	20	$Fe(CN)_6^{3-}$	4.64
5f	*E* + FAD + Asp	PCMB	30	$Fe(CN)_6^{3-}$	4.40
5g	*E* + FAD + Asp	PCMB	45	$Fe(CN)_6^{3-}$	4.92

The most obvious effect is that, while PCMB has little inhibitory action on the complete enzyme in the presence of FAD (compare Expts. 1 and 5a), it strongly attacks the apoenzyme when FAD is not present (Expt. 3), producing an irreversible inhibition. This suggests that the group attacked by PCMB (possibly a thiol group) is one that binds the FAD to the enzyme protein; in the complete flavoprotein therefore the FAD bound to the group protects it from attack. The presence of ferricyanide during the incubation, however, appears to interfere with this protective action (Expt. 4) in some way not yet explained.

On longer incubation with PCMB the complete enzyme is not inhibited but progressively activated (Expts. 5 a–g). This effect is unexplained, but activations by PCMB have occasionally been observed with other enzymes. In the present case, however, it does not follow that there is a true activation of the enzyme itself, for

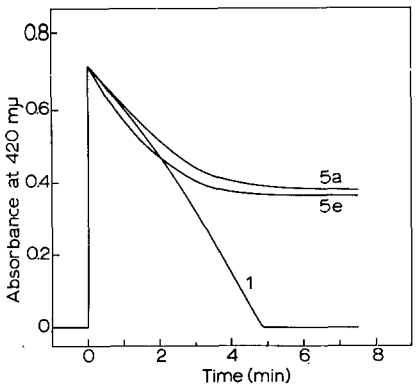


Fig. 11. Effect of PCMB. Progress curves of Expts. 1, 5a and 5e of Table XI.

any action of the PCMB which tended to abolish the inhibition by ferricyanide would produce a similar effect.

There is yet one further effect of PCMB that is very difficult to explain; this is illustrated in Fig. 11, in which the progress curves of Expts. 1, 5a and 5e are shown. Without PCMB, as already mentioned, the reaction proceeds right through to complete reduction of the ferricyanide without detectable slowing. After incubation of the complete enzyme with PCMB, even for 1 h or more, the reaction shows no initial inhibition and proceeds linearly until about 20% of the ferricyanide has been reduced. But after this point, which may be reached in 2 or 3 min from the start of the reaction, inhibition appears and develops very rapidly, so that the reaction stops when half the ferricyanide has been reduced. This occurs in all the seven curves of Expt. 5, independently of the period of incubation with PCMB, and in all cases the reaction stops fairly accurately when half completed, although this may be a coincidence. It cannot be due to exhaustion of substrate, which is in 10-fold excess over the ferricyanide, which itself is in 10-fold excess over the PCMB. The ferrocyanide which is formed in the enzyme reaction does not react with PCMB to form either a precipitate or a substance absorbing at 420 mμ, which might simulate ferricyanide remaining unreduced. It appears most likely that the inhibition is due to a combined action by ferricyanide and PCMB. Further work on the effect is required.

TABLE XII

AEROBIC EFFECT OF PCMB

Oxygen as acceptor; rates measured with oxygen electrode. Solutions saturated with air. 30°. pH 7.6. Quantities of all components as before, but total vol. 4 ml. Enzyme and FAD preincubated together 15 min; then PCMB added and incubated as shown; then reaction started by adding D-aspartate. Rates as μmoles D-aspartate per min per ml enzyme.

Incubation time with PCMB (min)	*Initial rate*
None added	1.89
0	1.34
5	1.14
10	0.78
15	0.38

When oxygen is used as acceptor instead of ferricyanide, these effects are not obtained. There is no activation by PCMB, but simply a progressive inhibition, as shown in Table XII. Here of course the reaction was started by adding D-aspartate, so that the conditions were somewhat different from those of Table XI. Moreover oxygen was present during the incubations with FAD and with PCMB.

DISCUSSION

The two amino-acid oxidases of kidney, D-amino-acid oxidase and D-aspartate oxidase, clearly belong to the same class. Both are flavoproteins having FAD as prosthetic group; both use molecular oxygen to oxidize amino acids to keto acids; both are restricted in their action to the D-isomers of their substrates. The two enzymes, however, show some striking differences in behaviour. Their substrate specificities are mutually complementary, neither enzyme having any action on the substrates of the other. D-amino-acid oxidase oxidizes many monocarboxylic amino acids, but fails to act on the dicarboxylic amino acids glutamic and aspartic acid; D-aspartate oxidase oxidizes these two dicarboxylic amino acids, but has no action on the monocarboxylic amino acids.

Evidently connected with this difference in substrate specificity is the sharp difference in specificity for competitive inhibition, shown in Tables V and VI. D-Amino-acid oxidase is well inhibited by monocarboxylic fatty acids, but not by dicarboxylic acids; D-aspartate oxidase is strongly inhibited by dicarboxylic acids, but not by monocarboxylic acids. There is also one striking difference in the acceptor specificities: ferricyanide, which does not act at all with D-amino-acid oxidase, is the best of the acceptors for D-aspartate oxidase. Since the oxidations brought about by the two enzymes are so similar, there is evidently an important difference in their acceptor sites, which is shown also in their different behaviour towards methylene blue.

The active centre of D-aspartate oxidase evidently contains groups which combine with the two carboxyl groups of the substrate or inhibitor, giving two links which together are sufficient to hold the molecule to the enzyme, while one such link, formed with a monocarboxylic compound, is insufficient. The presence of an amino group in the wrong (L-) configuration, however, makes a negative contribution to the affinity sufficient to overcome the attraction of the two carboxyl groups, as shown by the fact that, while oxaloacetate and oxoglutarate compete with the substrate, L-aspartate and L-glutamate do not. This suggests that when the substrate is in position on the active centre the amino group is directed away from the enzyme surface, in complete analogy with D-amino-acid oxidase[2].

Three enzymes oxidizing both D-aspartate and D-glutamate have now been partially purified: one (a) from kidney (as reported here), and two from Octopus by ROCCA[5,6], of which one (b) is considered to be a D-glutamate oxidase and the other (c) a D-aspartate oxidase. These three enzymes probably differ in their properties sufficiently to be considered separate enzymes. They differ in physical properties (as shown by differences in the methods of isolation), in firmness of combination with their prosthetic groups, and in the ratio of the rates with the two substrates. Enzyme (a) holds its flavin group so loosely that it is lost during purification, so that the apoenzyme is obtained. Enzyme (c) is obtained as the complete flavoprotein, but

the flavin group is readily removed. In Enzyme (b) the prosthetic group is held so tightly that it is not removed by the usual methods, and even drastic methods only produce a partial inactivation, reversible by FAD. Enzyme (a) does not give a flavin spectrum, being obtained as the apoenzyme; Enzyme (c) gives a well-marked flavin spectrum, while for Enzyme (b) the spectrum is said not to be satisfactory.

At substrate concentrations of 10 mM, Enzyme (a) gives a velocity ratio $v_{Asp}/v_{Glu} = 10.8$ with oxygen as acceptor; Enzyme (b) gives $v_{Asp}/v_{Glu} = 0.7$ under the same conditions; and Dr. E. ROCCA has kindly informed us that for Enzyme (c) the ratio is about 1.2.

Enzyme (b) also differs from Enzyme (a) in the following respects: (1) it does not show the inhibition with increase of oxygen concentration (Fig. 4), the rate with 100% oxygen being 70% more than with air, (2) L-glutamate competes with the substrate D-glutamate, (3) it is inhibited by iodoacetate, and (4) no addition of FAD is required to obtain full activity. We may safely conclude that it is a different enzyme. Enzyme (a) differs from Enzyme (c) also in not being inhibited by benzoate.

In none of the three cases is there satisfactory evidence of purity, and it is probable that none of the enzymes is yet homogeneous. Unfortunately our Enzyme (a), being the apoenzyme, does not give a flavin spectrum, from which the purity could be estimated, and there are no data on the spectrum of Enzyme (b). The spectrum of Enzyme (c), however, is given by ROCCA[6], from which it appears that a solution containing 4.5 mg of the enzyme in 1 ml gives a flavin band at 450 mμ with a height of 0.15 absorbance units. From this it can be calculated that the preparation contains about 340 000 g per mole of flavin. Many flavoproteins have molecular weights of the order of 80 000 per flavin group, and if that were so for this enzyme it would suggest that a further 4-fold purification would make it homogeneous. From the spectrum and specific activity we calculate that Enzyme (c) has a catalytic centre activity ('turnover number') of nearly 16 000 per min, which is about the same as glucose oxidase.

It is of some interest to compare the specific activities of the three preparations, although if they are different enzymes this cannot be relied upon to give their relative purities. For Enzyme (b) figures are only available for oxygen as acceptor, and for Enzyme (c) only for ferricyanide as acceptor; but for our Enzyme (a) both figures are available. With oxygen at pH 8.3 and with 10 mM D-aspartate at 35° in presence of catalase, we estimate that our enzyme as purified 98-fold oxidizes about 3 μmoles of substrate per min per mg; whereas under the same conditions Enzyme (b) as purified 1100-fold oxidizes 6.7 μmoles per min per mg. With this substrate, therefore, the two enzymes as finally obtained have activities not differing greatly, although with D-glutamate Enzyme (b) would be about 15 times as active as Enzyme (a).

Comparing Enzyme (a) with Enzyme (c), using ferricyanide as acceptor but otherwise under the same conditions, Enzyme (a) at 98-fold purification oxidizes about 9 μmoles of aspartate per min per mg, whereas Enzyme (c) at 184-fold purification is stated to oxidize 46 μmoles of aspartate per min per mg at 25°, equivalent possibly to twice this amount at 35°. It is therefore about 10 times as active as our preparation, although of course this does not tell us the purity of our enzyme*.

* Dr. E. ROCCA has kindly informed us that although there is a misprint in the value of the extinction coefficient for ferricyanide given in his paper, the correct value was used in his calculations.

The D-aspartate oxidase of kidney has a number of interesting properties which deserve further investigation, but we are not now in a position to continue the work ourselves.

ACKNOWLEDGEMENTS

We are indebted to the Manager of Messrs J. Sainsbury's Poultry Packing Station, Bury St. Edmunds, for his cooperation in making it possible for us to obtain rabbit kidneys in quantity. The oxygen electrode outfit was purchased with a grant from the Royal Society to one of the authors (M.D.).

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