Kinetics and Equilibria in Flavoprotein Systems

V. The Effects of pH, Anions and Partial Structural Analogues of the Coenzyme on the Activity of D-Amino Acid Oxidase

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The effect of pH, anions and partial structural analogues of flavin-adeninedinucleotide (FAD) on the activity of D-amino acid oxidase has been studied. The results suggest that the phosphoryl residues in FAD are linked to the apoenzyme. Furthermore it appears that the adenin residue as well as the ribosyl moiety of FAD takes part in the binding of the coenzyme to the surface of the protein. In contrast to some other flavin enzymes, the NH(3) group in *iso*alloxazine does not appear to be bound, because the D-amino acid oxidase is fluorescent.

The dissociation constant of the enzyme is lower at pH 9.2 ($K_M = 6 \times 10^{-8}$ M) than at pH 8.3 ($K_M = 1.2 \times 10^{-7}$ M). The experiments indicate a closer binding of coenzyme-protein at pH 9.2 than at 8.3. This may be a general phenomenon where flavin enzymes are concerned since the velocity of the association reaction between flavinmononucleotide (FMN) and the protein in the "old yellow enzyme" also increases in the pH region around 9 [1].

FMN and adenin nucleotides exhibit a pronounced inhibition of a competitive type. The inhibition is also shown by anions, the anions of strongly dissociated acids being the most effective inhibitors. The importance of these results on the physiological regulation of the enzyme activity is discussed.

Investigations carried out by Theorell and co-workers have shown that a number of anions affect the binding of coenzyme-apoenzyme, and thereby the velocity of the enzyme reactions. This has been demonstrated in the case of flavin enzymes such as the "old yellow enzyme"[1] and D-amino acid oxidase[2]. The same applies in the case of DPN- or TPN-linked enzymes such as alcohol dehydrogenase[3] and glucose-6-phosphate dehydrogenase[4]. The fact that enzymes are inhibited by partial structural analogues of the coenzyme has been shown in D-amino acid oxidase[5,2] and dehydrogenases[6,7].

A more thorough examination of the inhibitory effect of anions and substances structurally related to the coenzyme FAD on the D-amino acid oxidase has been carried out in the present work. The qualitative and quantitative nature of the inhibition has been characterised. The importance of these facts for the binding of coenzyme-apoenzyme is discussed.

METHODS

A highly purified apoenzyme of the D-amino acid oxidase from pig kidneys was prepared according to the method of Negelein and Brömel [8]. The lyophilized product has retained its activity unchanged for more than one year stored in a desiccator at + 1° C.

FAD was extracted from yeast as reported by Warburg and Christian [9] and purified until the cresol extraction stage. The further purification was carried out by paper electrophoresis [10] as shown in a previous paper. A pure preparation of FAD free from other nucleotides was obtained.

Activity measurements were carried out with D,L-alanin as substrate in the Warburg apparatus. The reaction mixture contained: 20 μg apoenzyme dissolved in 0.02 ml buffer, FAD in varying concentrations, most frequently 0.14 μM, 1 ml buffer, either M/10 pyrophosphate buffer pH 8.3 or M/10 glycine buffer pH 9.2. The reaction was started by introducing 0.2 ml 0.5 M D,L-alanin from the sidearm. Total volume was 3.6 ml. The enzyme activity was measured at 38° C. The O_2-consumption exhibited linear correlation with time for 30 min.

The inhibitors were dissolved in M/20 pyrophosphate buffer pH 8.3 and the pH of the solution adjusted to pH 8.30 when necessary. The final concentration of buffer in the incubation mixture was 0.028 M. A similar procedure was used with glycine buffer at pH 9.2 and other pH values. The inhibition was examined at two or more concentrations of inhibitor and with three or more concentrations of FAD in each experiment.

The following substances were used: FMN, synthetically prepared by "Sigma" Chemical Company. The product was purified by paper electrophoresis. The following preparations were also from "Sigma" Chemical Company: Adenylic acid (A-5-P), adenosine diphosphate (ADP), adenosine triphosphate (ATP), inosinic acid (I-5-P). The Ba-salts were converted to Na-salts by means of a cation-exchanger (Amberlite 120).

Other products used were: Ribose phosphate (R-5-P) (Schwarz), adenine (Hoffman-La Roche), adenosine (Hoffman-La Roche), hypoxanthine (Hoffman-La Roche), cytosine (Dougherty Chemicals), uracil (Eastman Kodak Company), imidazole (Eastman Kodak Company). Riboflavin from Hoffman-La Roche was recrystallised before use. Diphosphopyridine nucleotide (DPN) was prepared by Dr. R. K. Bonnichsen and had a degree of purity of 0.75. The fluorescence measurements were carried out on the apparatus constructed and described by Theorell and Nygaard [11].

Calculations [12,13]. Plotting of the reciprocal value of the reaction velocity (O_2-consumption), v, against the reciprocal value of the molar concentration of FAD resulted in a straight line. "Slope" and "intercept" on the ($1/v$)-axis were thereby determined. When the inhibition is competitive, the two curves which represent the reaction in the presence and absence, respectively, of inhibitor have a common intercept, but different slopes [14]. The inhibition constant K_I was calculated from the equation

Slope = $(K_M / v_{Max}) (1 + [I] / K_I)$

where: K_M = Michaelis constant

$1/v_{Max}$ = Intercept of the ($1/v$)-axis

$[I]$ = The molar concentration of inhibitor.

RESULTS

Absorption spectrum and fluorescence of D-amino acid oxidase

The fluorescence of FAD at neutral pH is 20 % of that given by FMN [10]. This quenching of the fluorescence of FAD is assumed to be due to a binding between the NH(3)-group in the *iso*alloxazine ring and the adenine part of the molecule (Bessey, Lowry and Love, 1945) [15]. These authors showed that on titration of FAD to lower pH value, an increase in the fluorescence took place. As seen in Fig. 1 the fluorescence of FAD decreases on titration in the alkaline
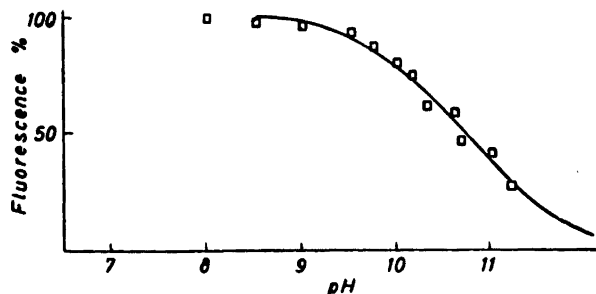
Fig. 1. The fluorescence of FAD at alkaline pH.

□ : The fluorescence (Expressed in per cent of fluorescence at pH 7)
————: Theoretical titration curve pK' = 10.5

region. The points fit approximately a titration curve with pK' 10.5. Thus the quenching of the fluorescence at alkaline pH is probably due to the dissociation of the NH(3)-group. Similar results are shown for FMN[1], where the fluorescence decreases according to a titration curve with pK' 10.2.

When combining FAD with the apoenzyme, no quenching of fluorescence in the pH interval 7—9.5 occurred. Thus the D-amino acid oxidase is a fluorescent enzyme. Straub's diaphorase [16] behaves in a similar manner, while the apoenzyme in the "old yellow enzyme" produces quenching of the fluorescence in FMN, probably while the NH(3)-group in the isoalloxazine ring is bound [17]. This also applies to most other flavin enzymes [18]. Thus there is no evidence in the case of D-amino acid oxidase to prove that any binding of apoenzyme to free NH(3)-groups takes place.

Under aerobic conditions, no definite changes in the absorption spectrum either at 375 mμ or 450 mμ of FAD were found when combining with the apoenzyme. On the other hand, in accordance with previous investigations [8] a reduction in the absorption at 450 mμ of the reduced enzyme was found under anaerobic conditions.

Dissociation constant for FAD-apoenzyme

The dissociation constant for FAD-apoenzyme has previously been measured by activity determinations and is found [9] at 38° C to be 2.7 $\times$ 10^{-7} M at pH 8.3 in pyrophosphate buffer. It has been recalculated [19] to 1.4 $\times$ 10^{-7} M by taking into account the free amount of FAD which is present at equilibrium. In cases where the apoenzymes are of a greater degree of impurity, values quoted for K_M are [19,12] 4.7 $\times$ 10^{-7} M and 4.6 $\times$ 10^{-7} M, under the same conditions.

A reexamination of K_M at different pH's and in different buffers is carried out in this work. It appears from Fig. 2 that when the apoenzyme is approximately half saturated with FAD the pH-activity curve differs in pyrophosphate buffer and glycine buffer. The statement [20] that the enzyme in pyrophosphate buffer exhibits optimum activity between pH 8 and 9 has been
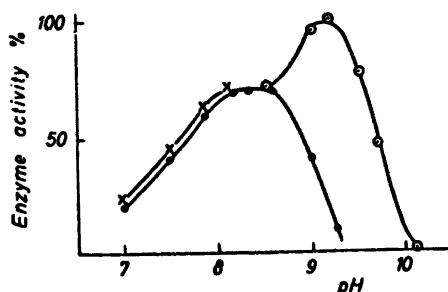
Fig. 2. The effect of pH on D-amino acid oxidase activity.
———•———•———: In pyrophosphate buffer.
———⊙———⊙———: In glycine buffer.
———×———×———: In glycyl-glycine buffer.
The concentration of FAD was 1.4 × 10⁻⁷ M.

confirmed. In glycine buffer however there occurs another optimum in the activity at pH 9.2. The explanation of this difference is that the pyrophosphate anion produces strong inhibition at pH > 9 similar to other anions.

As shown in Fig. 3 by plotting [14] the values for activity in glycine buffer, the curves at pH 8.3 and 9.2 have different slopes but the same intercept. This suggests that the binding between FAD and apoenzyme is stronger at pH 9.2, but probably without new points on the surface of the protein participating in the formation of the holoenzyme. Calculation of K_M gave 1.2 × 10⁻⁷ M at pH 8.3 while a lower value 6 × 10⁻⁸ M was found at pH 9.2.

It is very interesting to note that the association reaction for FMN and the apoenzyme in the "old yellow enzyme" exhibits a similar pH-dependence in the alkaline region as shown in the present work for the activity of the D-amino acid oxidase in glycine buffer. It is very likely that this is due to a beginning of the dissociation of the imino group in FAD (pK' = 10.5) and of the imino
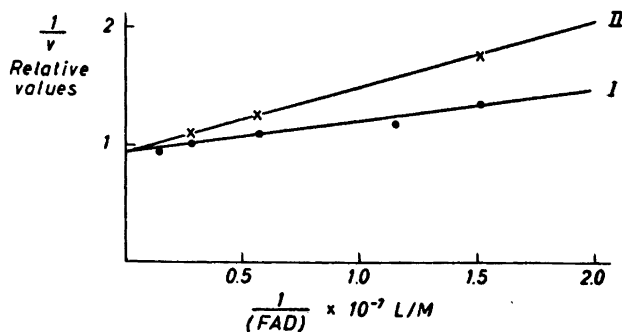

Fig. 3. The activity of D-amino acid oxidase at different pH and with variation of FAD concentration.
Curve I: pH 9.2.
Curve II: pH 8.3.

Table 1. Inhibition of D-amino acid oxidase by anions.

Inhibitor	pH	Conc. of Inhibitor	Slope	Intercept	K_I
None *	8.3		0.30	0.90×10^6	
Na acetate	8.3	2.0×10^{-1} M	0.44	—»—	4.2×10^{-1} M
» »	—»—	4.0×10^{-1} M	0.64	—»—	3.5×10^{-1} M
Na$_2$SO$_4$	8.3	8.0×10^{-2} M	0.42	—»—	2.0×10^{-1} M
»	—»—	1.6×10^{-1} M	0.55	—»—	1.9×10^{-1} M
NaBr	8.3	2.0×10^{-2} M	0.40	—»—	6×10^{-2} M
»	—»—	4.0×10^{-2} M	0.52	—»—	5.5×10^{-2} M
NaCl	8.3	8.6×10^{-2} M	1.12	—»—	3.2×10^{-2} M
Na phosphate	8.3	8.0×10^{-2} M	0.88	—»—	2.7×10^{-2} M
NaNO$_3$	8.3	3.0×10^{-2} M	0.70	—»—	2.3×10^{-2} M
»	—»—	6.0×10^{-2} M	1.25	—»—	1.9×10^{-2} M
None **	9.2		0.15	—»—	
NaCl	9.2	6.0×10^{-2} M	0.72	—»—	1.6×10^{-2} M

* $K_M = 1.2 \times 10^{-7}$ M.
** $K_M = 6.0 \times 10^{-8}$ M.

group in FMN ($pK^1 = 10.2$)[1] at pH$>$ 9, which results in an increased association velocity with the apoproteins in this pH region. The strong reduction in the activity of the D-amino acid oxidase that occurs at pH$>$ 9.5 may be due to an abolishment of the association reaction when the number of positively charged groups on the protein is reduced considerably.

Inhibition of D-amino acid oxidase by anions

Table 1 shows the inhibition of enzyme activity by anions. As also indicated in Table 2, salts of strongly dissociated acids produced the strongest inhibition, while the inhibition by fluoride and acetate was weak. Thus the inhibition can most probably be ascribed to the anions. Furthermore the inhibition was of the same magnitude when potassium salts were used. Ammonium salts

Table 2. Relative inhibitory activity of anions. Pyrophosphate buffer, pH 8.3, concentration of FAD 1.4 $\times$ 10^{-7} M.

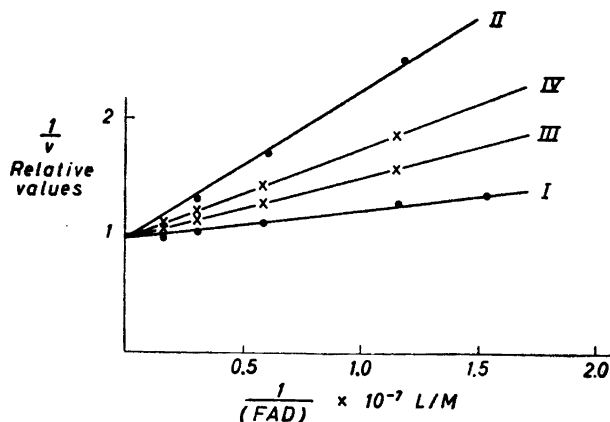| Inhibitor	50 % inhibition at	Relative inhibition
NaI	2×10^{-2} M	4.2
NaBr	3×10^{-2} M	2.8
NaNO$_3$	4.5×10^{-2} M	2.0
NaCl	8.5×10^{-2} M	1.0
Na$_2$SO$_4$	12×10^{-2} M	0.7
Na phosphate	15×10^{-2} M	0.6
Na acetate	25×10^{-2} M	0.3
NaF	$>30 \times 10^{-2}$ M	<0.3

Fig. 4. Inhibition by NaCl.

Curve   I: *No inhibitor, pH 9.2.*
Curve   II: *0.06 M NaCl, pH 9.2.*
Curve III: *No inhibitor, pH 8.3.*
Curve IV: *0.06 M NaCl, pH 8.3.*

on the other hand gave a stronger inhibition, indicating an effect of the ammonium ion on the protein.

In the experiments with NaI, catalase was added to decompose produced H_2O_2. Without catalase NaI gave complete inhibition in very low concentrations, probably because of the liberation of I_2 by H_2O_2. The competitive nature of the inhibition by anions indicates a competition with the dissociated phosphoryl groups in FAD. In Fig. 4 the inhibition with chloride is given as an example. It is reasonable to assume that negatively charged anions are bound to positively charged groups in the apoenzyme. The inhibition by anions was reversed when increasing the FAD concentration. The quoted experiments were all carried out in pyrophosphate buffer at pH 8.3. In experiments carried out at higher pH values the inhibition was stronger, as shown in Fig. 5.
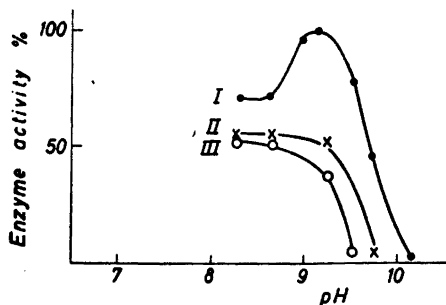

Fig. 5. Inhibition by chloride and phosphate.

Curve   I: *No inhibitor.*
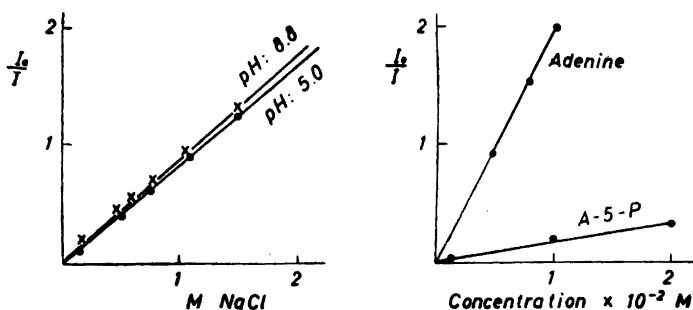Curve II: *0.06 M NaCl.*
Curve III: *0.075 M Na-phosphate.*

Fig. 6. Quenching effect of NaCl, adenine and A-5-P on the fluorescence of FAD.

$\dfrac{I_0}{I}$: $\dfrac{\text{Fluorescence without quencher}}{\text{Fluorescence with addition of quencher}}$

After the addition of chloride or phosphate in concentrations which at a pH of 8.3 gives moderate inhibition, the inhibition is almost complete at pH 9.2 and the pH optimum of the enzyme activity at this pH almost disappears. Pyrophosphate is without effect at pH 8.3 to 8.6 but gives strong inhibition at higher pH. This explains why the enzyme activity is lost in pyrophosphate buffer at pH > 9. The inhibition by chloride and phosphate is competitive with FAD also at pH 9.2. This suggests that the mechanism of the inhibition is similar to that assumed for the inhibition at pH 8.3.

The activity of the enzyme drops rapidly at pH values above 9.5. This could be due to the fact that the number of positively charged groups in the protein is reduced. In the case of chloride and phosphate the strongly sloping part of the activity curve is shifted towards lower pH. The reason for this may be the well known fact that salts reduce the pK' values of proteins towards the isoionic point.

It is shown that NaCl at a relatively high concentration produces quenching of the fluorescence in FAD (Fig. 6). Provided that a non-fluorescent complex is formed: $FAD + Cl^- \rightarrow (FAD)Cl$, the dissociation constant K can be calculated from the equation

$$K = \frac{I}{I_0-I} \times C$$

C = concentration of NaCl.
I_0 = Fluorescence without NaCl
I = Fluorescence with NaCl

In acetate buffer at pH 5.0 K was 2.5 M and in glycin buffer at pH 8.8 2.2 M. NaCl, however, inhibits the enzyme activity in a concentration interval considerably lower than that producing the quenching effect on the fluorescence of FAD. The fact that the chloride inhibition of D-amino acid oxidase is strongly increased at a pH > 9, could not be explained by assuming that chloride inhibits the dissociation of the NH(3)-group in FAD. Any definite change in the pK of FAD by chloride in the alkaline region could not be detected.

Table 3. Inhibition of D-amino acid oxidase by partial structural analogues of FAD. Experiments performed at pH 8.3.

Inhibitor	Conc. of inhibitor	Slope	Intercept	K_I
FMN	2.5×10^{-4} M	2.86	0.90×10^6	3.0×10^{-5} M
A-5-P	4.0×10^{-4} M	0.49	—»—	6.4×10^{-4} M
—»—	8.0×10^{-4} M	1.34	—»—	2.3×10^{-4} M
ATP	8.0×10^{-4} M	0.44	—»—	1.8×10^{-3} M
Riboflavin	5.6×10^{-4} M	0.43	0.98×10^6	Non competitive
Adenosine	5.0×10^{-3} M	0.42	1.05×10^6	—»—
Hypoxanthine	5.0×10^{-3} M	0.42	1.11×10^6	—»—
Adenine	5.0×10^{-3} M	0.45	1.06×10^6	—»—
Cytosine	1.5×10^{-2} M	0.34	1.02×10^6	—»—
Uracil	1.5×10^{-2} M	0.36	1.09×10^6	—»—

Inhibition by partial structural analogues of FAD

Table 3 shows the inhibition by these compounds. Further the relative inhibition by this group of substances is shown in Table 4. The strongest inhibition was produced by FMN, A-5-P and other nucleotides. This group of substances with dissociated phosphoryl groups, all exhibited a competitive, reversible inhibition with FAD. One example is given in Fig. 7. Because of the competitive nature of inhibition, it is probable that the groups in the apoenzyme involved in the formation of the complex with the inhibitor, are the

Table 4. Relative inhibitory activity of partial structural analogues of FAD. Pyrophosphate buffer, pH 8.3, concentration of FAD 1.4×10^{-7} M/L.

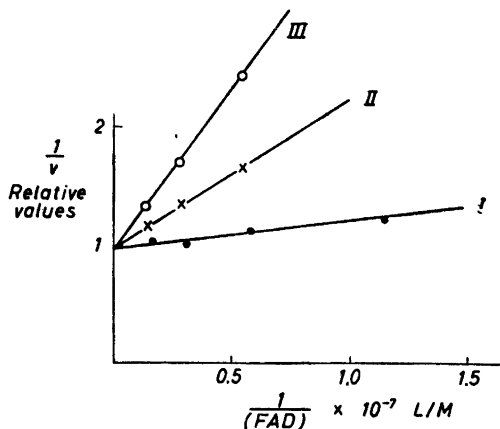| Inhibitor	50 % inhibition at	Relative inhibition
FMN	4×10^{-4} M	1.2
A-5-P	5×10^{-4} M	1
ADP	5×10^{-4} M	1
ATP	6×10^{-4} M	0.8
Inosinic acid	5×10^{-4} M	1
DPN	2.5×10^{-3} M	0.2
Adenosine	6×10^{-3} M	0.08
Hypoxanthine	6×10^{-3} M	0.08
Adenine	6×10^{-3} M	0.08
Ribose-5-phosphate	2×10^{-2} M	0.0025
Cytosine	1.5×10^{-2} M	0.003
Uracil	2×10^{-2} M	0.0025
Riboflavin	25 % inhibition at 5.4×10^{-4} M	

Fig. 7. Inhibition given by FMN and A-5-P.
Curve   I: No inhibitor.
Curve   II: 0.0008 M A-5-P.
Curve III: 0.00025 M FMN.
The experiments are performed in pyrophosphate buffer, pH 8.3.

same as in the FAD-apoenzyme complex. The experiments indicate that in addition to a binding of the dissociated phosphoryl group, the riboflavin and adenosine part are also linked to the protein.

The inhibition by riboflavin and adenosine was considerably less than in the case of FMN and A-5-P. Other purine and pyrimidine derivatives also produced a weaker inhibition. Furthermore the inhibition in this group was non-competitive with FAD, as shown by an example in Fig. 8. It has previously been shown [21] that the riboflavin analogues isoriboflavin, dichloroflavin
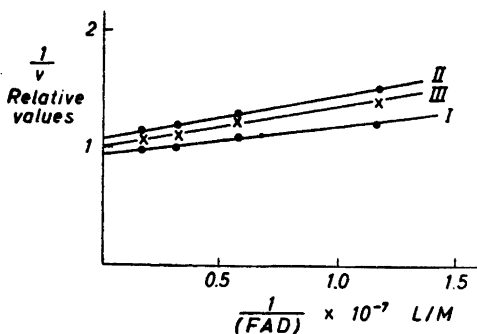

Fig. 8. The inhibition given by riboflavin and adenine.
Curve   I: No inhibitor.
Curve   II: 0.005 M adenine.
Curve III: 0.00054 M riboflavin.
The experiments are performed in pyrophosphate buffer, pH 8.3.
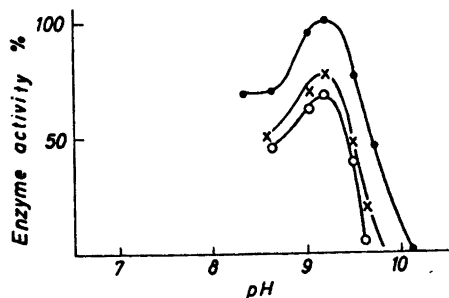
Fig. 9. Inhibition given by FMN and A-5-P.

—●—●—: No inhibitor.
—⊙—⊙—: 0.00025 M FMN.
—×—×—: 0.0004 M A-5-P.
The concentration of FAD was 1.4 × 10⁻⁷ M.

and galactoflavin exhibit a competitive inhibition which is, however, not truly reversible. There is some evidence for believing that the ribose part of the molecule is of specific importance in the binding with the protein, since the inhibition with ribose-5-phosphate was considerably stronger than with phosphate. Adenine and adenosine, however, produced the same inhibition. The (6)-NH_2 group in the purine ring was without specific importance, since K_I for adenine and hypoxanthine was of the same magnitude. Likewise there was no difference in K_I for adenylic acid and inosinic acid.

It is known that adenine, adenosine and purines usually produce a quenching of the fluorescence in fluorescent molecules [22]. Quenching of FAD by purines has been demonstrated [23,5]. As shown in Fig. 6 adenine and to a lesser degree adenylic acid produce a quenching of the fluorescence in FAD. At the concentrations used a formation of a complex between adenine and FAD would make the latter inactive as a coenzyme. This might explain the inhibition found in enzyme-activity experiments. Such an explanation however, is not possible in the case of adenylic acid which inhibits at considerably lower concentrations. The inhibition by FMN, A-5-P and ATP were of the same degree at different pH (Fig. 9). This was also the case for the inhibition by riboflavin and adenine.

FMN, riboflavin and DPN could not operate as coenzymes for the D-amino acid oxidase when added in concentrations up to 5 × 10⁻⁴ M. Neither could FMN + adenylic acid when added in equimolecular concentrations function as a coenzyme.

DISCUSSION

The experiments have indicated that several linkages are involved in the formation of the slightly dissociated coenzyme-apoenzyme complex in the D-amino acid oxidase.

The competitive type of inhibition by anions with the coenzyme molecule, indicates that the phosphoryl residue in FAD is linked to the apoenzyme. The fact that anions of strongly dissociated acids produce the most pronounced

dissociation of the enzyme may be explained by assuming that the negatively charged phosphoryl groups in FAD are linked to positively charged groups in the apoenzyme. The strong inhibition produced by FMN and adenylic acid suggest attachment to the riboflavin part of the FAD molecule as well as to adenosine. There is some evidence to indicate the chemical groups which are of importance for the binding.

It is improbable that the free NH-(3) in *iso*alloxazine participates in a linkage, since the formation of the coenzyme-apoenzyme complex was not followed by a quenching of the fluorescence in FAD. The smaller dissociation constant of FAD at pH 9.2 than at 8.3, is probably due to the fact that the beginning of the dissociation of the NH-(3) group in *iso*alloxazine results in a closer binding to the apoenzyme. A direct binding of the NH-(3) group, however, does not appear to occur since the D-amino acid oxidase is also fluorescent at pH 9.2.

In this connection it should be emphasized, that the effect of pH in the alkaline region on the activity of the D-amino acid oxidase and on the association reaction of FMN and the protein in the old yellow enzyme is completely identical. In both cases the optimal peak of the reaction at pH 9.2 disappears when NaCl is added. Thus it is possible that the dissociation of the NH-(3) group in *iso*alloxazine is of general importance for the formation of the coenzyme-protein complex in flavin enzymes.

The (6)-NH$_2$ group in the adenin moiety was without specific importance since K_I for adenylic and inosinic acids were of the same magnitude. The importance of the ribosyl group was indicated by the fact that the inhibition by ribose-5-phosphate was considerably stronger than by phosphate. On the other hand K_I for adenine and adenosine were of the same magnitude. In the case of the latter two substances however, the inhibition can be explained by assuming the formation of non-fluorescent complexes with FAD.

In the reduced enzyme new tight bonds appear to occur since the reduced enzyme is almost undissociated [8]. By hydrogenation of *iso*alloxazine in FAD in the reduced enzyme, a reduction in the absorption at 450 mμ occurs. Preliminary investigations indicated that chloride did not dissociate the reduced enzyme. Thus, no detectable effect of NaCl on the formation of the complex coenzyme-apoenzyme occurred, as examined by absorption measurements at 450 mμ. Continued investigations on the reduced enzyme are in progress.

It has recently been shown that a number of flavin enzymes are metal enzymes with, e. g., Mo, Fe, or Cu as a component [24]. There is, however, no evidence to show that metal ions are of importance for the activity of the D-amino acid oxidase. Thus it has been shown that cyanide is without effect and in present experiments chelating agents such as versene (2 $\times$ 10^{-3} M) and 8-hydroxyquinoline (2 $\times$ 10^{-4} M) produced no inhibition.

The fact that anions produce dissociation of enzymes, which has been shown where flavin and pyridine nucleotides occur as coenzymes, may be a phenomenon of general importance. The practical result of the presence of anions in buffers has been clearly demonstrated by the fact that D-amino acid oxidase is completely inhibited at pH>9 when pyrophosphate buffer is employed. Furthermore it should be mentioned that tris(hydroxymethyl)aminomethane buffer produced complete inhibition at a concentration of 10^{-3} M.

It is very likely that inhibition of enzyme reactions by anions and by partial structural analogues of coenzymes is of physiological importance in the control of cellular metabolism. Thus it is well known that the optimal reaction velocity for enzyme reactions is greater in extracts and homogenates than in isolated tissue slices of the same organ [25]. It is therefore of interest to bear in mind that the chloride concentration in the extracellular fluid corresponds to a concentration which produces considerable inhibition in the D-amino acid oxidase system. The same applies in the case of the intracellular concentration of adenine nucleotides.

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