# On the Interpretation of the Absorption Spectra of Flavoproteins with Special Reference to D-Amino Acid Oxidase*

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ABSTRACT: The formation of complexes between D-amino acid oxidase and a large number of carboxylic acids has been determined by effects on the visible absorption spectrum of the enzyme. In the case of twelve of these complexes possessing very low dissociation constants it has been shown that one molecule of the carboxylic acid anion is bound per molecule of enzyme flavin. With the remaining compounds the data are fully consistent also with the formation of such unit complexes.

The relevance of this property to the catalytic reaction mechanism is discussed. The effect of complex formation in producing marked spectral shifts in the 400- to 500-mμ region has been interpreted as being due to the abolition of hydrogen bonding from a charged amino group in the enzyme to the 2- or the 4-carbonyl oxygen of the isoalloxazine moiety of flavin–adenine dinucleotide (FAD). Evidence for this hypothesis has been drawn largely from a comparison of the effects produced by benzoate and a series of substituted benzoate derivatives. Supporting evidence has also been derived from a study of the ionization behavior of the 3-imino nitrogen of the isoalloxazine moiety in FAD, uncomplexed D-amino acid oxidase, and the benzoate complex of D-amino acid oxidase. The possible significance of these studies in the interpretation of the different absorption spectra found with different flavoproteins is discussed.

One of the many unexplained phenomena in the field of flavin chemistry is that of the alteration of the absorption spectrum on binding of the flavin prosthetic group to proteins. Whereas free FAD[1] in neutral aqueous solution has a simple spectrum with peaks at 375 and 448 mμ, in most flavoproteins these peaks are shifted; the 375-mμ peak is generally at shorter wavelengths and the 448-mμ peak is generally at longer wavelengths. In about one-third of the known flavoproteins, despite these shifts, the general form of the spectrum remains the same. With the remaining flavoproteins, however, the 448-mμ peak is partially resolved into a three-banded peak, with shoulders around 430 mμ and 480 mμ. Examples from the literature, illustrating this phenomenon, are shown in Table I. The enzyme D-amino acid oxidase provides an especially favorable opportunity for investigating this phenomenon, since it has been shown by Yagi and Ozawa (1962a,b,1963) that its spectrum can be changed from the simple type to the partially resolved type by the addition of compounds such as benzoate. In this paper are reported the results of studies of the effects of over fifty different compounds on the spectrum of D-amino acid oxidase. These studies indicate that hydrogen bonding to the isoalloxazine ring structure of flavins has a pronounced effect on absorption spectrum.

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[1] Abbreviation used in this work: FAD, flavin-adenine dinucleotide.

## Materials and Methods

*Enzyme.* Crystalline D-amino acid oxidase was prepared from pig kidneys by the method previously described (Massey *et al.*, 1961). Traces of benzoate were removed by repeated precipitation with 50% saturated $(NH_4)_2SO_4$ in the presence of 0.1 M DL-alanine and 0.1 M pyrophosphate, *p*H 8.5, essentially as described by Yagi and Ozawa (1962a,b). After five such precipitations the benzoate-free enzyme was dialyzed against several changes of 0.1 M pyrophosphate, *p*H 8.5, to remove $(NH_4)_2SO_4$ and alanine, and stored at −20°. Enzyme prepared in this way was used in all experiments.

*Chemicals.* $\Delta^1$-Pyrroline-2-carboxylic acid was prepared from DL-proline by reaction with D-amino acid oxidase and $O_2$. When the D-proline was exhausted, as judged by the return of yellow color to the D-amino acid oxidase, the latter was denatured by boiling or by precipitation with 10% (w/v) trichloroacetic acid followed by ether extraction to remove the trichloroacetic acid. $\Delta^1$-Piperideine-2-carboxylic acid was a gift from Dr. W. G. Robinson. All other chemicals used were obtained from the California Corp. for Biochemical Research, the Aldrich Chemical Co., or the Eastman Co. With the exception of the aminobenzoic acid and the hydroxybenzoic acid series (which were recrystallized), the remaining compounds were used without further purification.

*Spectrophotometric Analysis.* All of the spectra reported here, with the exception of those shown in Figures 10 and 12, were obtained in 0.1 M pyrophosphate buffer, *p*H 8.5, at a temperature of 16–17°. Compounds added were made up in the same buffer and the *p*H was adjusted to 8.5. Equal volumes of the compound were

TABLE I: Classification of Simple Flavoproteins into Two Classes Depending on Their Spectral Properties.

| Enzymes with an Unresolved 450-mμ Band | Enzymes with a Resolved 450-mμ Band[a] |
|---|---|
| Uncomplexed D-amino acid oxidase[b] | D-Amino acid oxidase benzoate complex[b] |
| Glucose oxidase[c,d,e] | Electron-transferring flavoprotein[f] |
| Butyryl CoA dehydrogenase[g] | Old Yellow enzyme[h] |
| Palmityl CoA dehydrogenase[i] | Vitamin K reductase[j] |
| The general fatty acyl CoA dehydrogenase[k] | Lipoyl dehydrogenase[l] |
| *Crotalus adamanteus* L-amino acid oxidase[m] | Glutathione reductase[n] |
| | Cytochrome $b_5$ reductase[o] |
| | Thioredoxin reductase[p] |
| DPNH peroxidase[q] | *Agkistrodon piscivorus* L-amino acid oxidase[r] |
| Glycolic acid oxidase[s] | Microsomal TPNH cytochrome *c* reductase[t] |
| | L-α-Hydroxy acid oxidase[u] |

[a] Shoulders at *ca.* 430 mμ and *ca.* 480 mμ. [b] Yagi and Ozawa (1962a,b). [c] Keilin and Hartree (1948). [d] Kusai (1960). [e] Swoboda and Massey (1965). [f] Crane and Beinert (1956). [g] Steyn-Parvee and Beinert (1958). [h] Theorell and Åkeson (1956). [i] Hauge *et al.* (1956). [j] Märki and Martius (1960). [k] Crane *et al.* (1956). [l] Massey (1960). [m] Wellner and Meister (1960). [n] Black and Hudson (1961). [o] Dolin (1957). [p] Strittmatter (1961). [q] Frigerio and Harbury (1958). [r] Moore *et al.* (1964). [s] Singer and Kearney (1950). [t] Masters *et al.* (1965). [u] Robinson *et al.* (1962).

added to the enzyme and reference cuvets, and spectra were determined using the 0–0.5 optical density slide wire of the Cary Model 14 recording spectrophotometer. At least five concentrations of each compound were tested and additions were continued until saturation was achieved. This was detected by the absence of further spectral changes on adding more of the compound. All spectra reported are corrected for volume changes.

## Results

*Binding Constant of FAD.* Many observations have led to the conclusion that, although FAD is fairly tightly bound to the protein of D-amino acid oxidase, it is nevertheless considerably more weakly bound than in most other flavoproteins. Thus, although the enzyme as isolated contains 12–16 μmoles FAD/mg protein, the content may be increased to 21–22 μmoles/mg by dialysis against $3 \times 10^{-4}$ M FAD (Massey *et al.*, 1961). Furthermore it was noted that whereas little FAD is lost on $(NH_4)_2SO_4$ precipitation of a concentrated solution of enzyme, proportionally greater losses were obtained as the concentration of enzyme was lowered. Quantitative evaluation of the binding constant of FAD proved to be somewhat difficult, owing to the fact that the apoprotein formed on loss of FAD is easily denatured. Table II shows the results of dialysis equilibrium experiments carried out at 0–4°. As can be seen, there is a reasonable scatter of values, independent of the protein concentration. The average value of $K$ = ([E][FAD]/[E·FAD] is $2.2 \times 10^{-7}$ M. This compares reasonably with the "$K_m$" value of $5.5 \times 10^{-7}$ M found at 25° by manometric assay of activity when apoprotein is treated with different concentrations of FAD. The practical consequence of such a binding constant is that, in the absence of added FAD, at a concentration of 10 mg/ml 3% of the FAD is free, while at a concentration of 1 mg/ml 10% would be free, and at 0.1 mg/ml 27% would be free. Thus it is clearly desirable in the isolation of the intact flavoprotein to keep the enzyme as concentrated as possible and to dialyze against several changes of small volume rather than once against a large volume of buffer.

*Binding of Benzoate.* As described previously by Yagi and Ozawa (1962a,b), benzoate has a pronounced effect on the spectrum of the enzyme. Figure 1 shows the spectral shift obtained on titration of benzoate-free enzyme with benzoate. For the sake of clarity, intermediate spectra have been omitted. The pronounced decreases in absorbancy in the region 350–400 mμ and 420–450 mμ, as well as the increase in the region 480–520 mμ, are to a large extent completed by the addition of 1 molecule of benzoate per molecule enzyme-bound FAD.

This is illustrated further in Figure 2 where the increase in absorbancy at 497.5 mμ is plotted against the amount of benzoate added. No significant difference is obtained when the titration is carried out on enzyme of higher FAD content obtained by dialysis equilibrium against excess FAD. From the results of Figure 2 the binding constant of benzoate to the enzyme, $K$ = ([E·FAD][free benzoate])/[E·FAD·benzoate], is calculated to be $3–4 \times 10^{-6}$ M. The fact that this constant is unaffected by added FAD and that the initial slope of the titration curve intercepts the maximum value observed at 1.0 mole benzoate per mole enzyme bound FAD, even when significant quantities of apoprotein are present, suggests that benzoate binds weakly or not at all with the apoenzyme.

*Binding of Other Carboxylic Acids Which Produce Benzoate-Type Spectra.* Aromatic compounds that cause spectral shifts similar in form to that caused by benzoate (i.e., development of shoulders in the spectrum at approximately 435 and 485 mμ and shift of the 455-mμ peak to longer wavelengths) include many of the substituted benzoates, β-nahpthoate, and the salts of various heterocyclic carboxylic acids such as nicotinic, orotic, and furoic acids. Simple nonaromatic carboxylic acids such as crotonic, methacrylic, β,β-dimethylacrylic acids, as well as cyclohexanecarboxylic acid, also produce spectral shifts similar to that of benzoate. All the compounds tested appear to be bound in the same way as

TABLE II: Determination of FAD-binding Constant.[a]

| Protein Concentration (M) | Volumes of Dialysate (ml) | FAD Bound (E·FAD) (M) | Free FAD (M) | Free Enzyme (M) | $K = \frac{[E][FAD]}{[E \cdot FAD]}$ (M) |
|---|---|---|---|---|---|
| $2.14 \times 10^{-4}$ | 100 | $1.50 \times 10^{-4}$ | $1.0 \times 10^{-6}$ | $6.4 \times 10^{-5}$ | $4.3 \times 10^{-7}$ |
| $2.38 \times 10^{-4}$ | 50 | $1.52 \times 10^{-4}$ | $4.0 \times 10^{-6}$ | $8.6 \times 10^{-5}$ | $2.3 \times 10^{-7}$ |
| $2.38 \times 10^{-4}$ | 250 | $1.45 \times 10^{-4}$ | $1.3 \times 10^{-7}$ | $9.3 \times 10^{-5}$ | $8.5 \times 10^{-8}$ |
| $7.95 \times 10^{-5}$ | 50 | $4.76 \times 10^{-5}$ | $3.8 \times 10^{-7}$ | $3.19 \times 10^{-5}$ | $2.6 \times 10^{-7}$ |
| $3.97 \times 10^{-5}$ | 50 | $2.27 \times 10^{-5}$ | $2.6 \times 10^{-7}$ | $1.70 \times 10^{-5}$ | $1.9 \times 10^{-7}$ |
| Average | | | | | $2.2 \times 10^{-7}$ |

[a] Benzoate-free enzyme was dialyzed with vigorous stirring at 0–4° for 28 hours against the volumes shown of 0.1 M pyrophosphate, *p*H 8.5. Protein concentration was measured and molarity was calculated assuming a unit molecular weight of 45,500. Free and bound FAD were estimated spectrophotometrically at 450 and 455 mμ, respectively, assuming in each case an extinction coefficient of $1.13 \times 10^4$ liter mole$^{-1}$ cm$^{-1}$.

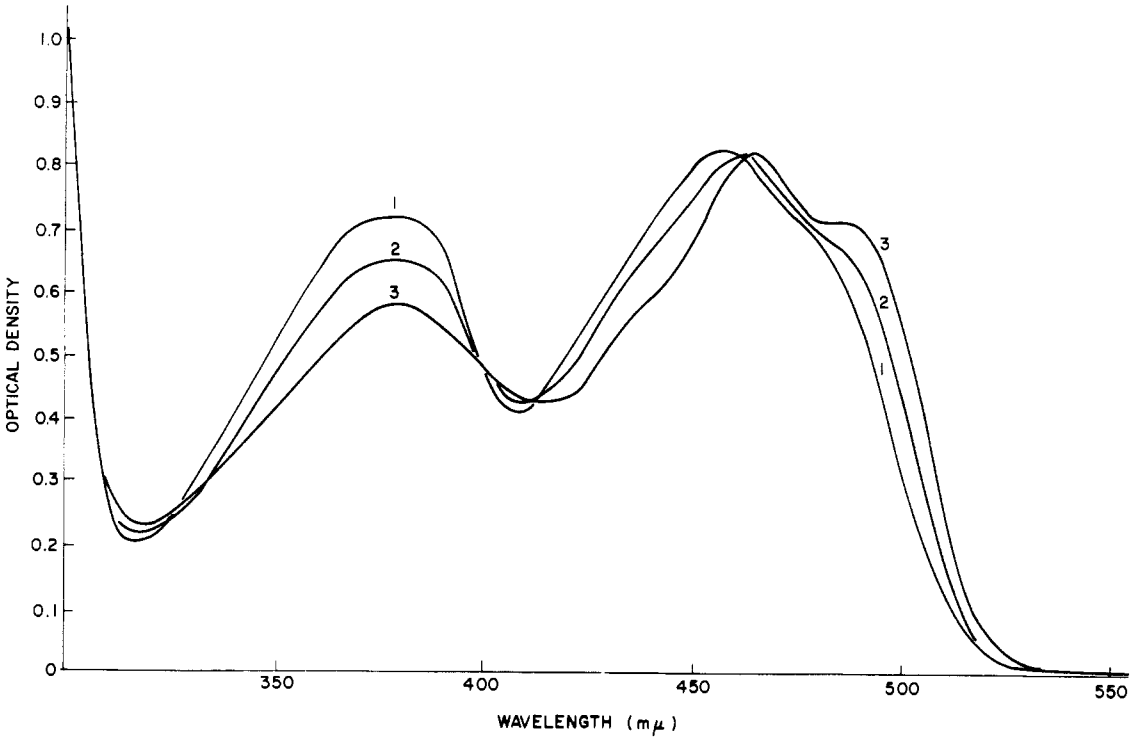


FIGURE 1: Effect of benzoate on the spectrum of D-amino acid oxidase. Curve 1, benzoate-free enzyme (4.85 mg/ml) in 0.1 M pyrophosphate, *p*H 8.5; curve 2, after 0.46 mole benzoate per mole enzyme FAD; curve 3, after 4.33 moles benzoate.

benzoate, since those that have a very strong binding affinity yield titration curves of the type shown in Figure 2. In every single case the data are consistent with the binding of 1 molecule of the added acid anion per molecule enzyme-bound FAD, permitting the calculation of the dissociation constant. Table III summarizes the spectral characteristics of the various complexes, together with the respective dissociation constants. This table also includes data for compounds which complex with the enzyme but give spectral shifts different in form from the benzoate-enzyme complex. These will be considered separately in a later section. While many compounds produce the same type of spectral shifts of the 455-mμ peak as those of benzoate, it should be pointed out that there is a considerable variation in the extent of the changes occurring in the 380-mμ region.

TABLE III: Effect of Carboxylates on the Spectrum of D-Amino Acid Oxidase.[a]

| Addition | $K$ ($\mu$M) | $\lambda_{max}$ (m$\mu$) | $\lambda_{min}$ (m$\mu$) | Millimolar 330 | 340 | 350 | 360 | 370 | 380 | 390 | 400 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| None | | 370–380, 455 | 407 | 4.10 | 5.55 | 7.16 | 8.60 | 9.65 | 9.70 | 8.92 | 6.40 |
| Benzoate | 3 | 379, 462 | 413 | 3.98 | 4.93 | 6.50 | 7.48 | 7.95 | 8.25 | 7.75 | 6.86 |
| 2-Fluorobenzoate | 111 | 388, 463 | 418 | 4.00 | 4.64 | 5.39 | 6.30 | 7.30 | 7.94 | 8.03 | 7.69 |
| 3-Fluorobenzoate | 0.4 | 381, 464 | 416 | 3.95 | 4.71 | 5.56 | 6.67 | 7.63 | 8.14 | 7.85 | 6.95 |
| 4-Fluorobenzoate | 0.5 | 380, 463 | 415 | 4.10 | 4.88 | 5.80 | 6.69 | 7.53 | 8.00 | 7.70 | 6.79 |
| 2-Chlorobenzoate | 800 | 382, 462 | 414 | 3.96 | 4.90 | 6.03 | 7.10 | 8.16 | 8.75 | 8.52 | 7.15 |
| 3-Chlorobenzoate | 0.36 | 383, 465 | 415 | 3.90 | 4.72 | 5.76 | 6.90 | 7.96 | 8.59 | 8.30 | 7.18 |
| 4-Chlorobenzoate | 1.6 | 383, 463 | 414 | 3.90 | 4.88 | 6.08 | 7.10 | 8.08 | 8.76 | 8.48 | 7.28 |
| 2-Iodobenzoate | ~40,000 | Could not saturate, incomplete spectral shifts observed in same direction as rest of | | | | | | | | | |
| 3-Iodobenzoate | 2.7 | 387, 466 | 414 | 3.45 | 4.26 | 5.50 | 6.91 | 8.25 | 8.98 | 9.05 | 7.60 |
| 4-Iodobenzoate | 100 | 383, 462 | 412 | 3.76 | 4.85 | 6.30 | 7.02 | 8.71 | 9.16 | 8.80 | 7.27 |
| 2-Methylbenzoate | 3,000 | 380, 464 | 412 | 4.00 | 4.97 | 6.06 | 7.17 | 8.25 | 8.79 | 8.28 | 6.87 |
| 3-Methylbenzoate | 0.43 | 383, 467 | 414 | 3.50 | 4.40 | 5.51 | 6.10 | 8.08 | 8.95 | 8.55 | 6.80 |
| 4-Methylbenzoate | 2.6 | 383, 463 | 414 | 3.48 | 4.54 | 5.83 | 7.16 | 8.45 | 9.11 | 8.62 | 7.00 |
| 3,5-Dimethylbenzoate | 4.8 | 387, 465 | 415 | 3.30 | 4.20 | 5.45 | 6.80 | 8.21 | 9.25 | 9.35 | 7.50 |
| 3,4-Dimethylbenzoate | 0.8 | 384, 463 | 412 | 3.50 | 4.56 | 5.95 | 7.51 | 8.85 | 9.75 | 9.45 | 7.28 |
| 4-Methoxybenzoate | 43 | 380, 461 | 411 | 3.35 | 4.62 | 6.09 | 7.40 | 8.52 | 8.82 | 8.48 | 7.03 |
| 4-Nitrobenzoate | 8 | 390, 464 | 416 | 3.95 | 4.75 | 5.68 | 6.55 | 7.34 | 7.85 | 8.24 | 7.47 |
| 3-Hydroxybenzoate | 46 | 377, 465 | 412 | | | 5.75 | 6.52 | 7.25 | 7.38 | 7.11 | 6.52 |
| 4-Hydroxybenzoate | 190 | 385, 462 | 415 | 3.30 | 4.36 | 5.73 | 6.90 | 8.20 | 8.80 | 8.65 | 7.00 |
| 2-Hydroxybenzoate | 5.1 | 382, 457 | 407 | | 4.55 | 5.57 | 6.82 | 7.74 | 8.20 | 7.78 | 6.30 |
| 2,6-Dihydroxybenzoate | ~6,000 | 380, 454 | 405 | | | 6.56 | 7.65 | 8.50 | 8.81 | 8.18 | 7.35 |
| 2,3-Dihydroxybenzoate | 60 | 384, 455 | 407 | | 5.06 | 6.36 | 7.76 | 8.85 | 9.48 | 8.91 | 6.95 |
| 2,4-Dihydroxybenzoate | 3,700 | 377, 455 | 405 | | | 6.91 | 8.24 | 9.08 | 9.28 | 8.10 | 6.28 |
| 2,5-Dihydroxybenzoate | 300 | 382, 455 | 405 | | | | 6.65 | 7.48 | 8.05 | 7.70 | 6.34 |
| 4-Aminobenzoate | 340 | 382, 460 | 414 | 3.68 | 4.56 | 5.85 | 7.12 | 8.35 | 8.90 | 8.52 | 7.16 |
| 3-Aminobenzoate | 32 | 378, 460 | 410 | | | 5.90 | 6.90 | 7.85 | 8.15 | 7.32 | 6.22 |
| 2-Aminobenzoate | 20 | 381, 441, 565 | 402 | | | | 6.44 | 7.68 | 8.40 | 7.95 | 6.95 |
| 3-Hydroxy-2-aminobenzoate | 100 | 378, 450, 590 | 405 | | | 7.07 | 8.24 | 9.13 | 9.36 | 8.45 | 6.74 |
| 2-Hydroxy-4-aminobenzoate | 2,200 | 377, 454 | 405 | | | 7.05 | 8.33 | 9.25 | 9.35 | 8.15 | 6.35 |
| 3,4-Diaminobenzoate | 730 | 385, 460, 600 | 409 | | | 7.03 | 8.30 | 9.37 | 10.15 | 9.80 | 7.40 |
| 3,5-Diaminobenzoate | 600 | 385, 457, 650 | 411 | | | | 7.90 | 8.95 | 9.86 | 9.94 | 7.68 |
| $\beta$-Naphthoate | 32 | 382, 465 | 410 | | | 5.65 | 7.00 | 8.15 | 8.75 | 8.35 | 6.51 |
| Thioproline | 1,000 | 373, 458 | 402 | 5.30 | 6.28 | 7.18 | 8.00 | 8.56 | 8.31 | 7.16 | 6.23 |
| $\Delta^1$-Pyrroline-2-carboxylate | | 382, 459, 615 | 410 | 5.20 | 6.16 | 7.13 | 8.15 | 8.92 | 9.25 | 8.90 | 7.66 |
| $\Delta^1$-Piperideine-2-carboxylate | 2 | 385, 444, 640 | 405 | 4.52 | 5.34 | 6.35 | 7.60 | 8.66 | 9.40 | 9.12 | 7.45 |
| Indole-2-carboxylate | 0.6 | 385, 458 | 410 | 4.33 | 4.70 | 5.66 | 6.80 | 7.95 | 8.60 | 8.40 | 6.18 |
| Pyrrole-2-carboxylate | | 381, 459 | 410 | 3.46 | 4.50 | 6.00 | 7.60 | 8.86 | 9.55 | 8.80 | 6.91 |
| Fumarate monomethyl ester | 21 | 390, 462 | 415 | 3.00 | 3.83 | 5.13 | 6.57 | 8.20 | 9.17 | 9.75 | 8.06 |
| $\beta,\beta$-Dimethylacrylate | 38 | 380, 464 | 410 | 3.78 | 4.72 | 5.77 | 6.68 | 7.48 | 7.74 | 7.35 | 6.49 |
| Cinnamate | 55 | 385, 462 | 414 | 3.18 | 4.14 | 5.45 | 6.73 | 8.00 | 8.65 | 8.60 | 6.90 |
| Sorbate | 180 | 386, 460 | 412 | 3.25 | 4.25 | 5.51 | 6.90 | 8.36 | 9.16 | 9.22 | 7.30 |
| Methacrylate | 400 | 380, 463 | 414 | 3.98 | 4.85 | 5.73 | 6.53 | 7.22 | 7.48 | 7.30 | 6.65 |
| Crotonate | 70 | 390, 461 | 415 | 3.60 | 4.18 | 5.08 | 6.28 | 7.52 | 8.35 | 8.79 | 8.14 |
| Orotate | 100 | 384, 462 | 409 | | 4.45 | 6.16 | 7.80 | 9.15 | 9.61 | 9.28 | 6.10 |
| L-Dihydroorotate | 600 | 386, 464 | 412 | 3.45 | 4.35 | 5.59 | 6.95 | 8.30 | 8.85 | 8.90 | 6.88 |
| Nicotinate | 27 | 387, 465 | 415 | 3.53 | 4.08 | 4.96 | 5.92 | 6.95 | 7.53 | 7.72 | 6.68 |
| Picolinate | 28 | 393, 462 | 418 | 3.95 | 4.60 | 5.43 | 6.38 | 7.38 | 8.11 | 8.45 | 8.29 |
| Furoate | 30 | 385, 461 | 410 | 3.36 | 4.38 | 5.79 | 7.18 | 8.36 | 9.18 | 9.00 | 6.52 |
| Homocycloleucine[b] | 1,850 | 380, 464 | 415 | 4.16 | 5.34 | 6.80 | 8.14 | 9.42 | 9.82 | 9.64 | 8.15 |
| Cyclohexanecarboxylate | 400 | 390, 462 | 417 | 3.43 | 4.20 | 5.42 | 6.70 | 8.14 | 9.26 | 9.86 | 9.06 |

[a] The dissociation constant, $K$, is defined by the expression $K$ = ([free enzyme][free carboxylate])/[enzyme-carboxylate complex] in 0.1 M pyrophosphate, *p*H 8.5, 16–17°. The millimolar extinction coefficients quoted are calculated assuming that the millimolar extinction coefficient of the uncomplexed enzyme at 455 m$\mu$ is the same as

This is illustrated in Figures 3 and 4. Figure 3 shows the effect of the addition of $\beta,\beta$-dimethylacrylate which produces changes throughout the whole visible spectrum almost identical with those of benzoate, whereas Figure 4 shows the effect of 3,5-dimethylbenzoate. This compound differs in effect from benzoate in the 380-m$\mu$ region by producing only a shift in the maximum to longer wavelengths but no decrease in extinction coefficient. Other examples of this phenomenon are apparent from an inspection of Table III.

*Effect of Hydroxybenzoates.* The effects of binding of various hydroxybenzoates to D-amino acid oxidase provides an interesting comparison with the halogen, methyl, methoxy, and nitro-substituted benzoates studied. Whereas the latter compounds uniformly produce a benzoatelike effect on the 455-m$\mu$ peak, this is not

| Extinction Coefficients at the Wavelengths Specified (mμ) | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 410 | 420 | 430 | 440 | 450 | 460 | 470 | 480 | 490 | 500 | 510 | 530 | 550 | 600 | 650 | 700 |
| 5.79 | 7.05 | 8.60 | 9.90 | 11.00 | 11.15 | 10.25 | 9.25 | 7.30 | 4.24 | 1.54 | 0.13 | 0 | 0 | 0 | 0 |
| 6.24 | 6.36 | 7.66 | 8.71 | 10.00 | 11.65 | 11.00 | 10.15 | 10.15 | 7.75 | 3.16 | 0.13 | 0 | 0 | 0 | 0 |
| 6.96 | 6.96 | 7.75 | 8.75 | 9.90 | 11.50 | 11.10 | 10.08 | 9.90 | 7.80 | 3.52 | 0.16 | 0 | 0 | 0 | 0 |
| 6.12 | 6.07 | 7.20 | 8.30 | 9.50 | 11.10 | 10.80 | 9.70 | 9.60 | 7.68 | 3.38 | 0.20 | 0 | 0 | 0 | 0 |
| 6.02 | 6.08 | 7.25 | 8.35 | 9.52 | 11.10 | 10.63 | 9.68 | 9.55 | 7.45 | 3.35 | 0.26 | 0 | 0 | 0 | 0 |
| 6.20 | 6.31 | 7.55 | 8.60 | 9.81 | 11.10 | 10.56 | 9.70 | 9.35 | 6.91 | 3.00 | 0.21 | 0 | 0 | 0 | 0 |
| 6.20 | 6.20 | 7.20 | 8.38 | 9.45 | 11.30 | 11.24 | 10.05 | 10.05 | 8.60 | 4.36 | 0.42 | 0 | 0 | 0 | 0 |
| 6.15 | 6.25 | 7.50 | 8.56 | 9.78 | 11.16 | 10.65 | 9.70 | 9.40 | 7.20 | 3.20 | 0.28 | 0 | 0 | 0 | 0 |
| halogen series | | | | | | | | | | | | | | | |
| 6.12 | 6.10 | 7.20 | 8.35 | 9.25 | 10.80 | 11.10 | 9.80 | 9.72 | 8.60 | 4.75 | 0.41 | 0 | 0 | 0 | 0 |
| 6.09 | 6.56 | 7.90 | 9.05 | 10.24 | 11.30 | 10.58 | 9.63 | 9.00 | 6.50 | 2.93 | 0.20 | 0 | 0 | 0 | 0 |
| 6.05 | 6.33 | 7.48 | 8.60 | 9.67 | 11.10 | 10.94 | 9.80 | 9.56 | 7.75 | 3.92 | 0.32 | 0 | 0 | 0 | 0 |
| 5.83 | 5.95 | 7.05 | 8.05 | 9.00 | 10.62 | 10.90 | 9.75 | 9.73 | 8.75 | 5.00 | 0.39 | 0 | 0 | 0 | 0 |
| 5.94 | 6.13 | 7.42 | 8.52 | 9.70 | 11.25 | 10.70 | 9.75 | 9.68 | 7.36 | 3.18 | 0.20 | 0 | 0 | 0 | 0 |
| 6.05 | 6.11 | 7.39 | 8.37 | 9.45 | 10.90 | 10.85 | 9.88 | 9.55 | 7.90 | 4.50 | 0.50 | 0 | 0 | 0 | 0 |
| 5.95 | 6.31 | 7.55 | 8.53 | 9.60 | 10.85 | 10.55 | 9.65 | 9.26 | 7.58 | 4.02 | 0.47 | 0.13 | 0 | 0 | 0 |
| 5.94 | 6.28 | 7.65 | 8.75 | 10.00 | 11.12 | 10.42 | 9.65 | 8.96 | 6.38 | 2.93 | 0.47 | 0.22 | 0 | 0 | 0 |
| 6.03 | 5.79 | 6.97 | 8.30 | 9.34 | 10.85 | 10.58 | 9.50 | 9.11 | 7.20 | 3.32 | 0.23 | 0 | 0 | 0 | 0 |
| 5.95 | 6.25 | 7.56 | 8.80 | 9.95 | 11.60 | 11.50 | 10.45 | 10.40 | 8.50 | 4.30 | 0.71 | 0.23 | 0 | 0 | 0 |
| 5.90 | 5.87 | 7.22 | 8.45 | 9.75 | 11.02 | 10.52 | 9.70 | 9.35 | 6.90 | 3.23 | 0.39 | 0.13 | 0 | 0 | 0 |
| 5.91 | 6.95 | 8.51 | 9.60 | 10.88 | 11.38 | 10.35 | 9.68 | 8.32 | 5.53 | 2.80 | 0.89 | 0.45 | 0 | 0 | 0 |
| 7.28 | 8.01 | 9.05 | 10.01 | 10.70 | 10.52 | 9.65 | 8.40 | 6.36 | 3.71 | 1.60 | 0.25 | 0 | 0 | 0 | 0 |
| 6.40 | 7.50 | 8.90 | 10.10 | 11.10 | 11.10 | 10.20 | 9.34 | 7.35 | 4.40 | 2.40 | 1.28 | 1.00 | 0.64 | 0.35 | 0 |
| 6.08 | 7.26 | 8.73 | 9.91 | 11.02 | 11.11 | 10.20 | 9.45 | 7.75 | 4.83 | 2.31 | 0.84 | 0.63 | 0.35 | 0.14 | 0 |
| 6.07 | 7.21 | 8.69 | 10.00 | 11.02 | 11.02 | 10.30 | 9.30 | 7.36 | 4.51 | 2.56 | 1.57 | 1.44 | 0.97 | 0.54 | 0.14 |
| 6.04 | 6.25 | 7.55 | 8.71 | 10.11 | 11.30 | 10.51 | 9.95 | 9.40 | 6.36 | 2.58 | 0.56 | 0.43 | 0.38 | 0.25 | 0.10 |
| 5.75 | 6.28 | 7.55 | 8.55 | 10.10 | 11.05 | 10.18 | 9.95 | 9.40 | 6.20 | 2.95 | 1.28 | 1.18 | 1.05 | 0.79 | 0.55 |
| 7.30 | 8.35 | 9.56 | 10.30 | 10.05 | 9.40 | 8.21 | 6.06 | 4.15 | 3.05 | 2.78 | 2.92 | 3.13 | 2.95 | 2.15 | 1.20 |
| 6.65 | 7.85 | 9.24 | 10.28 | 10.59 | 10.20 | 9.04 | 7.42 | 5.40 | 3.31 | 1.85 | 1.25 | 1.30 | 1.41 | 1.19 | 0.90 |
| 6.28 | 7.48 | 8.94 | 10.10 | 11.17 | 11.05 | 10.20 | 9.30 | 7.49 | 4.64 | 2.24 | 1.13 | 0.97 | 0.72 | 0.53 | 0.40 |
| 6.35 | 7.20 | 8.52 | 9.60 | 10.65 | 11.30 | 10.56 | 9.95 | 8.88 | 6.46 | 3.80 | 1.69 | 1.41 | 1.35 | 1.24 | 1.10 |
| 6.18 | 6.78 | 8.03 | 9.10 | 10.15 | 10.50 | 9.80 | 8.95 | 7.54 | 4.73 | 2.61 | 1.40 | 1.31 | 1.48 | 1.54 | 1.30 |
| 5.47 | 6.04 | 7.30 | 8.61 | 9.65 | 10.90 | 10.73 | 9.88 | 9.28 | 7.70 | 4.92 | 1.70 | 0.96 | 0 | 0 | 0 |
| 6.49 | 7.50 | 8.95 | 10.05 | 11.40 | 11.92 | 11.14 | 10.58 | 9.50 | 6.44 | 3.40 | 1.24 | 0.69 | 0.35 | 0 | 0 |
| 7.26 | 7.81 | 8.93 | 9.98 | 10.67 | 11.02 | 9.98 | 8.50 | 7.20 | 4.93 | 2.60 | 1.07 | 1.07 | 1.23 | 1.16 | 0.95 |
| 7.24 | 8.15 | 9.43 | 10.47 | 10.39 | 9.75 | 8.93 | 6.66 | 3.95 | 2.58 | 2.14 | 2.13 | 2.39 | 2.96 | 3.03 | 2.69 |
| 5.00 | 5.85 | 7.30 | 8.39 | 9.80 | 10.45 | 9.70 | 9.40 | 8.48 | 5.59 | 2.83 | 1.34 | 1.25 | 1.12 | 0.81 | 0.46 |
| 5.85 | 6.44 | 7.80 | 8.86 | 10.36 | 11.19 | 10.18 | 10.00 | 9.15 | 5.80 | 2.58 | 1.11 | 0.98 | 0.74 | 0.43 | 0.25 |
| 5.85 | 5.79 | 7.27 | 8.39 | 9.70 | 10.91 | 10.20 | 9.35 | 8.88 | 6.18 | 2.60 | 0.13 | 0 | 0 | 0 | 0 |
| 5.76 | 6.16 | 7.54 | 8.72 | 9.90 | 11.35 | 11.00 | 9.91 | 9.70 | 7.74 | 3.60 | 0.26 | 0 | 0 | 0 | 0 |
| 5.40 | 5.65 | 7.03 | 8.22 | 9.35 | 10.60 | 10.19 | 9.21 | 8.75 | 6.76 | 3.28 | 0.26 | 0 | 0 | 0 | 0 |
| 5.71 | 6.04 | 7.45 | 8.70 | 9.76 | 10.85 | 10.30 | 9.35 | 8.69 | 6.31 | 2.92 | 0.40 | 0.15 | 0 | 0 | 0 |
| 6.04 | 6.19 | 7.37 | 8.60 | 9.71 | 11.10 | 10.72 | 9.60 | 9.15 | 7.24 | 3.50 | 0.25 | 0 | 0 | 0 | 0 |
| 6.76 | 6.55 | 7.76 | 8.80 | 10.10 | 11.32 | 10.60 | 9.70 | 9.21 | 6.60 | 2.65 | 0.15 | 0 | 0 | 0 | 0 |
| 5.04 | 5.78 | 7.34 | 8.50 | 9.55 | 10.94 | 10.40 | 9.30 | 8.90 | 6.90 | 3.01 | 0.26 | 0 | 0 | 0 | 0 |
| 5.12 | 5.44 | 7.14 | 8.45 | 9.55 | 10.98 | 10.98 | 9.49 | 9.17 | 7.61 | 4.09 | 0.28 | 0 | 0 | 0 | 0 |
| 5.28 | 5.30 | 6.55 | 7.80 | 8.88 | 10.46 | 10.46 | 9.32 | 9.12 | 7.73 | 4.10 | 0.26 | 0 | 0 | 0 | 0 |
| 7.29 | 6.75 | 7.58 | 8.58 | 9.75 | 10.98 | 10.45 | 9.59 | 9.06 | 6.79 | 3.18 | 0.27 | 0 | 0 | 0 | 0 |
| 5.41 | 6.12 | 7.57 | 8.70 | 9.86 | 10.83 | 10.21 | 9.38 | 8.75 | 6.47 | 3.16 | 0.43 | 0.13 | 0 | 0 | 0 |
| 6.23 | 6.31 | 7.65 | 9.02 | 10.00 | 10.87 | 10.69 | 9.45 | 8.30 | 6.45 | 3.60 | 0.42 | 0 | 0 | 0 | 0 |
| 7.25 | 6.75 | 7.70 | 8.71 | 9.86 | 11.11 | 10.59 | 9.64 | 9.14 | 6.88 | 3.15 | 0.26 | 0 | 0 | 0 | 0 |

that of free FAD at 448 mμ (11.3 liters mmole$^{-1}$ cm$^{-1}$). The concentration of enzyme used in these experiments was in the range 3.5–4.0 × $10^{-5}$ M with respect to flavin. [b] Cyclohexane-1-amino-1-carboxylate.

the case with the hydroxybenzoate series. Thus while 3-hydroxybenzoate (Figure 5) produces the usual benzoate effect, 2-hydroxybenzoate (Figure 6) has little effect in forming a 480-mμ shoulder. In fact, the small shoulder seen may be due to the short wavelength edge of a weak charge-transfer absorption with a maximum centered around 500 mμ, and evidenced by the extended absorption to beyond 550 mμ. The lack of a pronounced effect of 2-hydroxybenzoate is also seen in the small shift in maximum (455–457 mμ). Another striking feature of the results with this compound is its binding strength compared with the 3- and 4-substituted compounds. With the halogen and the methyl series, the 2-substituted derivatives are bound very much more weakly than the 3- and 4-substituted derivatives (see Table III). In the case of the hydroxy derivatives, however,

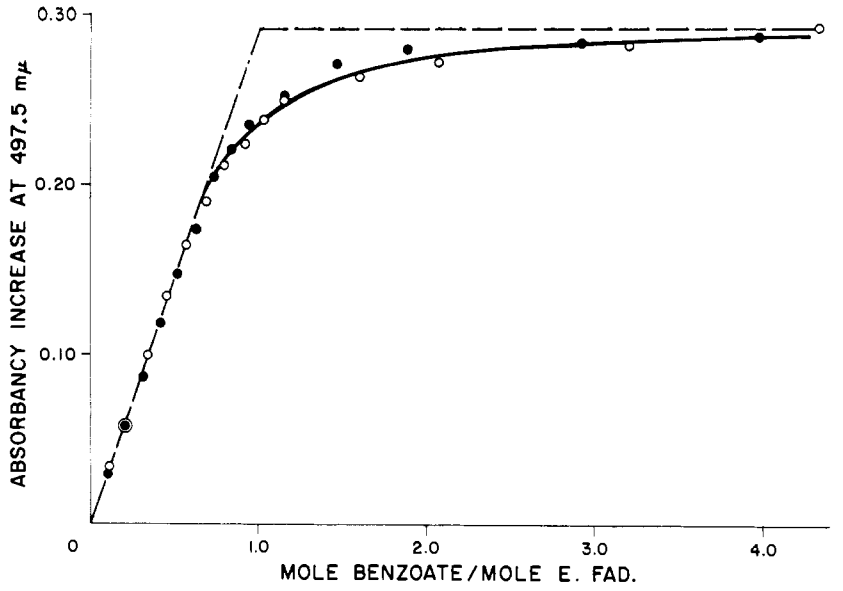


FIGURE 2: Increase in absorbancy at 497.5 mμ on titration of D-amino acid oxidase with benzoate. Conditions as in Figure 1. (○), Results obtained in the absence of added FAD (FAD content of the preparation used was 15.4 mμmoles/mg protein); (●), results obtained on enzyme dialyzed to equilibrium against $2 \times 10^{-4}$ M FAD (bound FAD content 20 mμmoles/mg protein). The concentration of enzyme-bound FAD in each experiment was $7.3 \times 10^{-5}$ M.

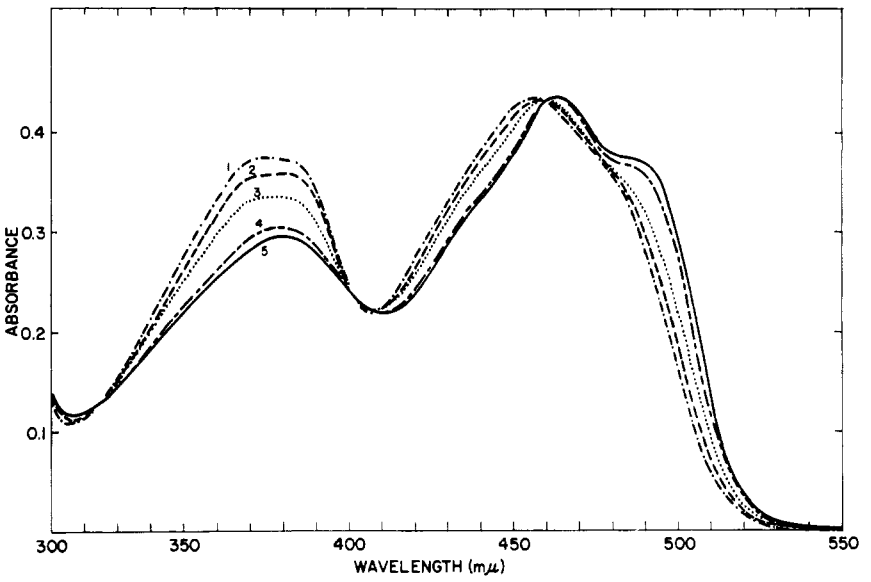


FIGURE 3: Effect of β,β-dimethylacrylate on the spectrum of D-amino acid oxidase. Curve 1, no addition; curves 2–4, with 0.5, 1.5, 6.5, and 16.5 moles dimethylacrylate per mole enzyme-bound FAD.

the reverse is true; the 2-hydroxy compound is bound more strongly than either the 3- or 4-substituted derivatives. In the hydroxy-substituted salicylate series studied very much weaker binding is observed, and long wavelength absorption is observed except in the case of 2,6-dihydroxybenzoate. With none of these substances is the typical benzoate-type spectral shift observed.

*Effect of Aminobenzoates.* The spectral effects of amino-substituted benzoates are of two types. Each of the seven derivatives tested produced charge-transfer-like absorption bands extending to 700 mμ or to even longer wavelengths. This effect is particularly marked with 2-aminobenzoate (anthranilate) with an extinction coefficient at 550 mμ of greater than $3 \times 10^3$ liter mole$^{-1}$ cm$^{-1}$. The phenomenon is illustrated in Figure 7, which shows for comparison the spectra of the complexes formed with the 2- and 3-amino-substituted benzoates.

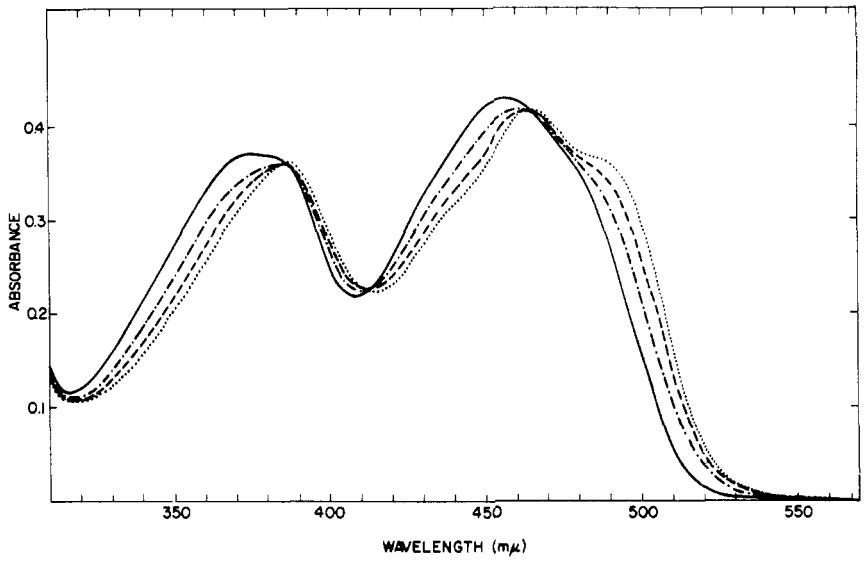


FIGURE 4: Effect of 3,5-dimethylbenzoate. ———, no additions; -.-.-., 0.5 mole; ---------, 1.0 mole; ·····, 11 moles 3,5-dimethylbenzoate per mole enzyme-bound FAD.

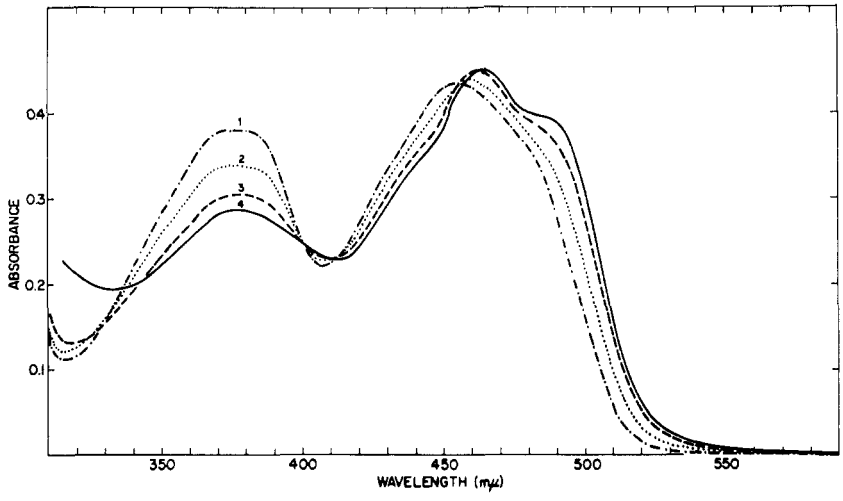


FIGURE 5: Effect of 3-hydroxybenzoate on the spectrum of D-amino acid oxidase. Curve 1, no additions; curves 2–4, 1.0, 3.5, and 18.5 moles 3-hydroxybenzoate per mole enzyme-bound FAD.

This figure also illustrates the second type of effect: that the benzoatelike shoulder at 480–490 mμ is not observed when the 2 position is substituted with either an amino or a hydroxyl group, but is observed when the 3 or 4 positions are substituted. The possible significance of this observation will be considered in the discussion section.

*Charge-Transfer Absorption with Heterocyclic Compounds.* Besides the hydroxy- and aminobenzoates, five other compounds have been found which produce charge-transferlike absorption bands. These all contain either nitrogen or sulfur in a heterocyclic structure, and β to the carboxyl group. These species are thioproline, indole-2-carboxylate, pyrrole-2-carboxylate, $\Delta^1$-pyrroline-2-carboxylate, and $\Delta^1$-piperideine-2-carboxylate. Figure 8 shows the effect of indole-2-carboxylate, which has a very pronounced affinity for binding to D-amino acid oxidase (dissociation constant 0.6 μM). It should be noted that not all these compounds produce in addition the benzoate-type shoulder at 480–490 mμ. For example, the complexes with $\Delta^1$-piperideine-2-carboxylate and $\Delta^1$-pyrroline-2-carboxylate both lack a shoulder at 480 mμ.

*Compounds That Are either Weakly Bound or Apparently Not Bound at All.* While the list of compounds that bind to D-amino acid oxidase is impressively long, many compounds fail to give any spectral evidence of binding, and a number are bound so weakly that spectra of the complexes could not be determined with certainty. These compounds are listed in Tables IV and V. The lack or paucity of effect of the compounds listed in these

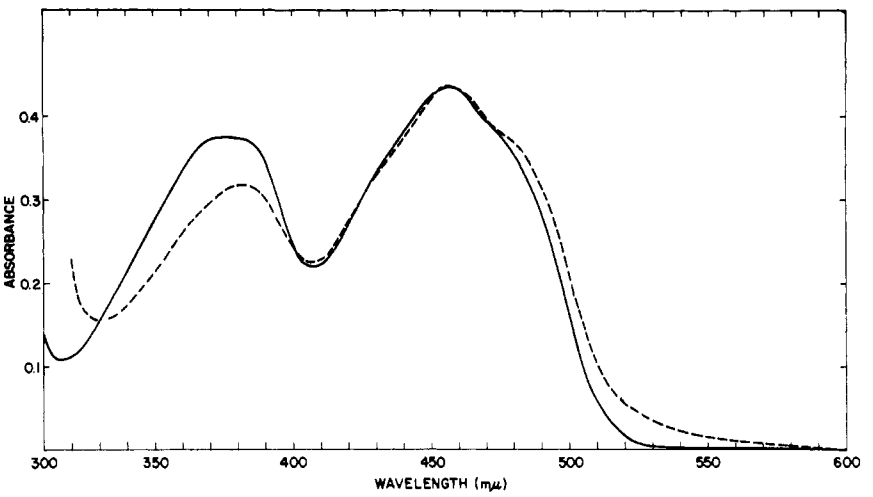


FIGURE 6: Effect of 2-hydroxybenzoate. ———, no additions; - - - -, 6 or 206 moles 2-hydroxybenzoate per mole enzyme-bound FAD.

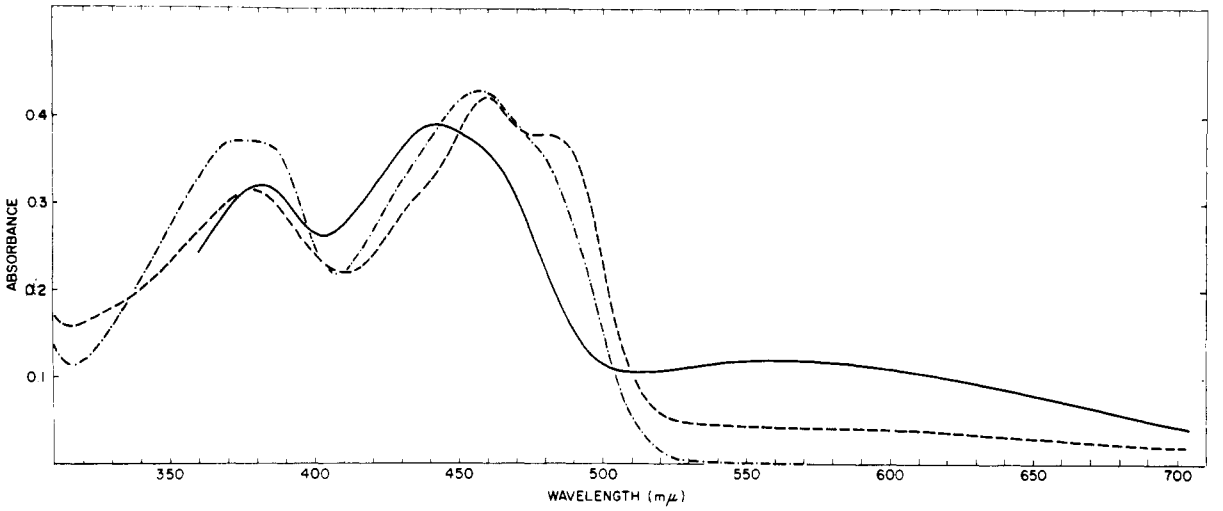


FIGURE 7: Comparison of the spectra of the complexes of D-amino acid oxidase with 2-aminobenzoate and 3-aminobenzoate. -·-·-, D-amino acid oxidase, no additions; ———, plus 300 moles 2-aminobenzoate; - - - -, plus 300 moles 3-amino benzoate.

tables is very informative in a consideration of the nature of complex formation with the enzyme; this will be considered in detail in the discussion.

*Effect of Benzoate on the Ionization of the Flavin of* D-*Amino Acid Oxidase.* It is well known that the isoalloxazine ring structure of the flavin coenzymes can exist in cationic, neutral, or anionic forms depending on the *p*H. Figure 9 shows this relationship (after Hemmerich and Muller, 1965). From variation of $E_h$ with *p*H, Lowe and Clark (1956) determined the *pK* value for the ionization of the 3-imino residue of FAD to be 10.4. By fluorescence-intensity studies, Walaas and Walaas (1956) determined a *pK* of 10.5. Figure 10 shows the effect on the visible spectrum of FAD of change in $[H^+]$ in the range *p*H 7–12. The marked change in the 375-mμ peak with *p*H provides a sensitive method for the determination of the *pK* of ionization of the 3-imino nitrogen. From a plot of the change in absorbance at 350 mμ the *pK* is determined to be 10.4 (Figure 11). This value is in excellent agreement with the earlier estimations using other methods. When the same technique is applied to D-amino acid oxidase, the *pK* is found to be at the considerably lower *p*H of 9.4. Figure 12 shows the spectral shifts accompanying the titration of D-amino acid oxidase. When the titration is carried out in the presence of a large excess of benzoate the *pK* is raised to such a high value that it is difficult to determine accurately because of denaturation of the enzyme and subsequent liberation of the free FAD at the high *p*H values necessary to produce complete ionization. A

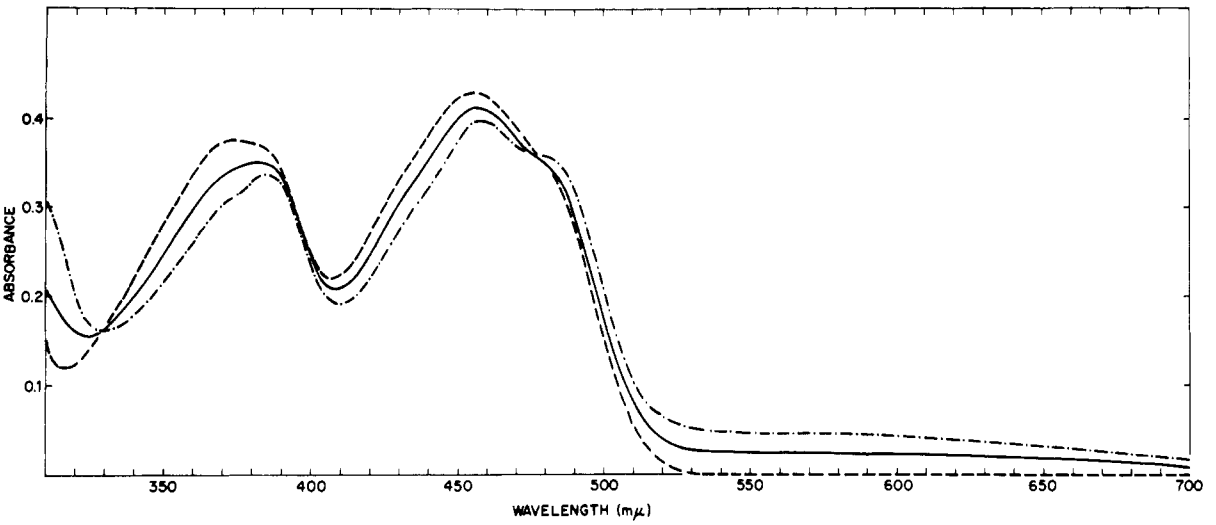


FIGURE 8: Effect of indole-2-carboxylate on the spectrum of D-amino acid oxidase. – – – –, no additions; ———, plus 0.5 mole indole-2-carboxylate; –·–·–, 1.5 or 115 moles indole-2-carboxylate per mole enzyme-bound FAD.

TABLE IV: Compounds Producing Spectral Changes, but with Such Weak Binding that Saturation was not Achieved.

| Anion of: | Highest Concentration Tested (moles/mole enzyme-FAD) | 480 mμ Shoulder |
|---|---|---|
| Phenylacetic acid | 316 | + |
| Cycloleucine[a] | 511 | – |
| Traumatic acid | 86 | + |
| D-Lactic acid | 312 | + |
| L-Lactic acid | 116 | + |
| Glycolic acid | 471 | + |

[a] Cyclopentane-1-amino-1-carboxylic acid.

TABLE V: Compounds Producing no Significant Change in the 455-mμ Peak of D-Amino Acid Oxidase.

| Compound | Highest Concentration Tested (moles/mole enzyme-FAD) |
|---|---|
| Nicotinamide | 1000 |
| Pyruvate | 235 |
| Benzamide | 510 |
| Phthalimide | 83 |
| Fumarate | 501 |
| Maleate | 411 |
| *trans*-Aconitate | 211 |
| Kynurenate | 56 |
| Imidazole | 8300 |
| Toluenesulfonate | 655 |
| Phthalate | 760 |
| Terephthalate | 555 |
| Phthalamate | 325 |
| Shikemate | 211 |
| *p*-Mercuribenzoate | 39 |
| Glyoxylate | 1250 |
| 2,4,6-Trimethylbenzoate | 155 |

tentative value for the ionization constant of the benzoate enzyme complex is *p*H 10.9 (Figure 11). For the reason stated this may be lower than the actual value. Whatever the true value it is evident that the binding of benzoate has a very dramatic effect on the ionization of the 3-imino nitrogen of the isoalloxazine moiety.

## Discussion

Before considering the possible information from these studies that can be inferred regarding the forces involved between the FAD prosthetic group and the apoprotein of D-amino acid oxidase, it is important to reiterate that the complexes formed with benzoate and with all the other compounds listed in Table III are unit complexes. In other words, 1 molecule of benzoate, indole-2-carboxylate, and the like is bound per molecule of enzyme flavin. As most, if not all, of the compounds tested are competitive inhibitors of the enzyme (Frisell *et al.*, 1956), it can therefore be concluded that the specific binding studied here is with the active center of the enzyme. As 1 molecule of inhibitor is bound for each molecule of enzyme flavin, it is evident that the catalytic reaction mechanism of the enzyme must therefore involve only 1 molecule of flavin per active center. These results are fully consistent with the reaction mechanism

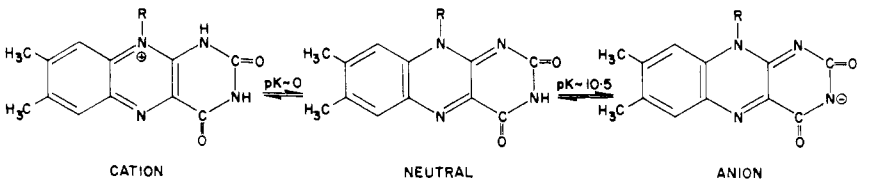


FIGURE 9: Ionic forms of the isoalloxazine moiety of flavins (after Hemmerich and Müller, 1965).

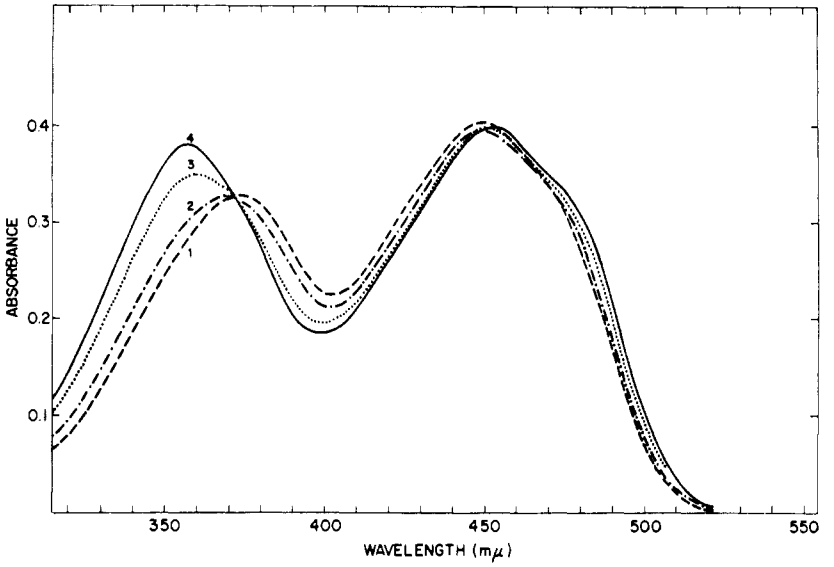


FIGURE 10: Effect of *p*H on the spectrum of FAD in 0.1 M pyrophosphate, 7°. Curve 1, *p*H 8.70; curve 2, *p*H 10.04; curve 3, *p*H 10.60; curve 4, *p*H 12.1.

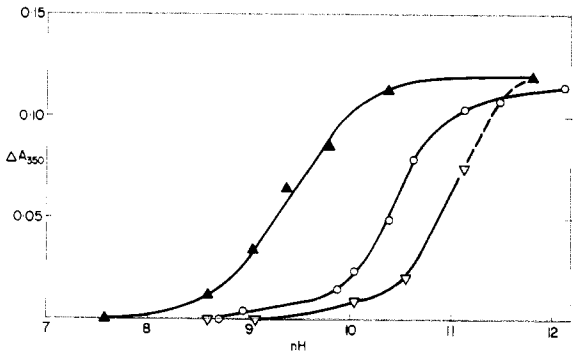


FIGURE 11: Ionization of the 3-imino group of the isoalloxazine moiety in FAD (○), D-amino acid oxidase (▲), and the benzoate complex of D-amino acid oxidase (△).

previously suggested (Massey and Gibson, 1964). This point is emphasized since the molecular weight of the enzyme is not known with certainty. Previous studies have shown that the unit molecular weight per flavin is 45,500 (Massey *et al.*, 1961). At concentrations of protein greater than 4 mg/ml, the molecular weight was shown by the Archibald technique to be four times this value (Charlwood *et al.*, 1961). As the protein concentration was decreased below 4 mg/ml the molecular weight dropped, but it was not possible to tell with certainty whether the limiting molecular weight at very low concentrations was 45,000 or 90,000. Thus from the ultracentrifuge data it was not possible to distinguish between the possibilities of the active site's possessing 1 or 2 molecules of flavin. The present data, especially in conjunction with the previous studies of mechanism (Massey and Gibson, 1964), can leave little doubt that there is only 1 flavin molecule per active center.

The essential characteristic for binding of inhibitors to D-amino acid oxidase has been found to be a carboxyl group. Whereas 4-methylbenzoate is bound very strongly (Table III), no binding whatsoever could be detected with *p*-toluenesulfonate (Table V). That an ionized carboxylate group is required is shown by the fact that neither benzamide nor nicotinamide form detectable complexes (Table V), whereas benzoate and nicotinate form strong complexes (Table III). It is also evident that the essential structure for the production of a benzoate-type spectrum is not an aromatic ring, since the anions of simple straight chain carboxylic acids such as crotonic, methacrylic, or β,β-dimethylacrylic acids all produce spectra similar to that found

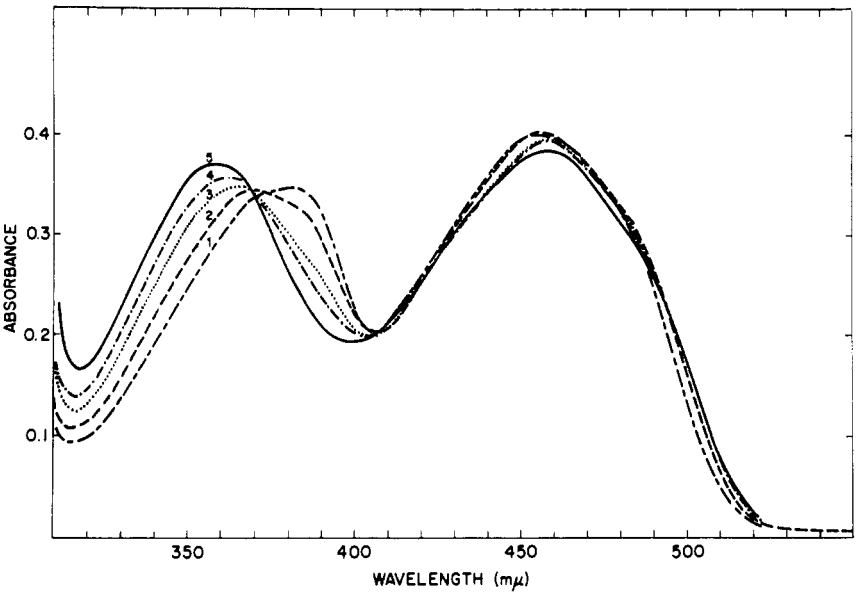


FIGURE 12: Effect of *p*H on the spectrum of D-amino acid oxidase in 0.1 M pyrophosphate, 7°. Curve 1, *p*H 7.58; curve 2, *p*H 9.04; curve 3, *p*H 9.38; curve 4, *p*H 9.80; curve 5, *p*H 10.38.

with benzoate in the 400-to 500-mμ region. The presence of an α,β-unsaturated bond in conjugation with the carboxyl group apparently facilitates binding of the compound. A good example of this is the pronounced difference found between benzoic and phenylacetic acids. Whereas the spectral changes produced by benzoate are almost complete with concentrations equimolar to that of the enzyme flavin (Figure 2), even the addition of a 300 molar excess of the anion of phenylacetic acid is not sufficient to produce saturation (Table IV). These spectral effects parallel the inhibition studies of Frisell *et al.* (1956), who concluded that an α,β-unsaturated anionic structure was the essential moiety for effective inhibition of D-amino acid oxidase. Their lists of inhibitors, in fact, were useful in screening compounds for our initial spectral studies because of the close correspondence discovered between relative inhibition potency and ability to form spectral complexes.

Although monocarboxylic acids having α,β-unsaturation complex readily with D-amino acid oxidase, the presence of additional carboxyl groups in analogous compounds such as fumarate, maleate, and *trans*-aconitate hinders or prevents complex formation (Table V). Phthalate and terephthalate also have negligible complexing ability. Although steric hindrance may be involved in some cases, the presence of a second negative charge is presumably the chief factor in preventing complex formation, since the monomethyl ester of fumarate, in spite of its greater bulk, is bound quite tightly (Table III). If the two carboxyls are separated sufficiently, binding capacity is regained. Traumatate, a higher homolog of fumarate in which the carboxyl groups are separated by ten carbon atoms instead of two, is bound weakly and produces a benzoate-type spectral shift (Table IV).

The finding that a single carboxyl group is the primary requirement in a compound for ability to bind to the active center of D-amino acid oxidase implies the existence in the enzyme of a positively charged group responsible for this binding, and presumably close to the isoalloxazine ring structure of the flavin. The lack of binding of dicarboxylic acids such as fumarate, maleate, and transaconitate implies also the existence of a negatively charged group in the vicinity of the active center. No evidence is presently available for the nature of the latter group; it could conceivably be one of the negatively charged phosphate groups of the FAD.

A possible clue to the interactions between the flavin and protein and the spectral effects reported in this paper is given by the work of Harbury *et al.* (1959), who studied the influence of solvents on the spectrum of 3-methyllumiflavin. These authors concluded that the hydrogen bonding between solvent and the 3-methyllumiflavin had a major effect on the position of the near-ultraviolet absorption band of this compound. However, it is also evident from their studies that the presence or absence of hydrogen bonding from the solvent can have a major influence on the spectrum of the 450-mμ band. In water, 3-methyllumiflavin has a simple characterless absorption peak in this region strongly resembling that of uncomplexed D-amino acid oxidase. However, in solvents such as benzene where hydrogen bonding is precluded, 3-methyllumiflavin has a resolved 450 mμ-peak remarkably similar in form to that of the benzoate complex of D-amino acid oxidase. Similar solvent effects to those observed by Harbury *et al.* (1959) have recently been observed also in this laboratory with tetraacetylriboflavin and 3-methyltetraacetylriboflavin (G. Greull and V. Massey, unpublished results).

It is suggested, therefore, that in uncomplexed D-amino acid oxidase some group within the protein is hydrogen bonded to the isoalloxazine moiety of the flavin, and that when compounds with a carboxyl grouping such as benzoate are bound to the enzyme this interaction is abolished and the spectrum of nonhydrogen-bonded FAD is produced. The possibilities of hydrogen bonding in the isoalloxazine structure are limited; the most likely acceptors are the carbonyl oxygen atoms at positions 2 and 4. A simplifying hypothesis that would explain both the spectral effects and the binding of compounds with carboxyl groups is that the protein grouping that contributes to hydrogen bonding with the flavin in the uncomplexed enzyme is the same grouping that combines with the carboxyl group of the inhibitors. This would be entirely feasible if the carboxyl-binding group were a charged amino group of the protein. Thus the native enzyme is envisaged as having a considerable degree of hydrogen bonding between a charged amino group at the active center and either of the two carbonyl oxygen atoms at positions 2 and 4 of the isoalloxazine ring system. When a molecule such as benzoate is bound at this amino group the hydrogen bonding is abolished with a resultant spectral change analogous to that found when model compounds are dissolved in non–hydrogen-bonding media compared with hydrogen-bonding media. Hydrogen bonding to other portions of the isoalloxazine ring is presumably left intact; the pronounced blue shift of the near-ultraviolet peak of 3-methyllumiflavin that occurs when all hydrogen bonding is precluded (Harbury *et al.*, 1959) was never seen in the present studies.

The foregoing hypothesis is supported by several lines of evidence. When the effects of substituted benzoates are considered (Table III) it is seen that fluoro, chloro, iodo, methyl, methoxy, and nitro derivatives all produce the same type of spectral effect as benzoate, whether they are substituted at the 2, 3, or 4 positions. In general, with the compounds tested, stronger acids such as the *m*- and *p*-fluoro- and chloro-substituted benzoates are bound more strongly than benzoate itself, and qualitatively follow the Hammett relationship (Hammett, 1940). With all the above-mentioned compounds the *ortho* derivatives have binding constants two to three orders of magnitude weaker than the *meta* compounds. With the hydroxy- and aminobenzoate series the effects are entirely different. Ignoring for the moment the charge-transfer phenomena also seen with some of the members of these series, it is evident from Table III that with these compounds the *ortho* derivatives are more tightly bound than the *meta* or *para* compounds. While some of these compounds produced a benzoatelike spectrum on binding to the enzyme (e.g., the 3-hydroxy and 4-hydroxy compounds), any compound tested which was substituted in the *ortho* position with either a hydroxyl or an amino group completely failed to produce the benzoate-type spectral shift. In fact with many of these compounds, such as 2,6-dihydroxybenzoate, 2-aminobenzoate, and 3-hydroxy-2-aminobenzoate, not only was there no shoulder produced in the neighborhood of 480–490 mμ, but the 455-mμ peak was shifted to shorter wavelengths. These compounds, while presumably eliminating the hydrogen bonding normally existing between the active-center amino group and the carbonyl oxygen atoms of the isoalloxazine structure, appear to substitute this interaction with one of their own, the hydrogen bonding now being from the hydroxyl hydrogen or the amino hydrogen of the bound derivative. In this interaction it would appear that the effect is maximal from an *ortho*-substituted hydroxyl or amino group.

Finally the ionization behavior of the 3-imino nitrogen of the isoalloxazine moiety of D-amino acid oxidase (Figures 11 and 12) can be explained readily by the foregoing hypothesis. If the isoalloxazine structure were hydrogen bonded to the protein as proposed, the 3-imino nitrogen would have to be within fairly close proximity to the charged amino group of the active center. Thus the *pK* of ionization of the 3-imino nitrogen, which in FAD is at *p*H 10.4, would be expected to be somewhat lower because of facilitation from an adjacent positively charged group, and also because of the inductive effect produced by hydrogen bonding to either of the adjacent carbonyl oxygen atoms. In full accord with the hypothesis, the *pK* is in fact found to be at *p*H 9.4, one whole *p*H unit below that of free FAD. When benzoate is complexed both of these facilitating effects on ionization would be expected to disappear. Again in keeping with prediction, the *pK* in the presence of benzoate is raised; in fact it is somewhat higher than that of free FAD. This may be a reflection of a lowered *pK* of free FAD in the hydrogen-bonding medium of water compared to a perhaps hydrophobic environment in the enzyme-benzoate complex.

The near-infrared absorption bands shown by many of the complexes are somewhat more difficult to interpret. In form they are reminiscent of the charge-transfer bands found under certain conditions with free flavins (Gibson *et al.*, 1962) and with lipoyl dehydrogenase (Massey and Palmer, 1962). One feature is common to all the compounds producing this type of spectral change: they all have a potentially electronegative atom (nitrogen, oxygen, or sulfur) two to four atoms removed from the carboxyl group. This distance could be great enough to bring the electronegative atom in proximity to either the $N_9$ or $N_{10}$ atom of the isoalloxazine ring system. A charge transfer to the potentially positively charged $N_9$ atom would therefore be an attractive hypothesis to explain the observed results. Interaction of the isoalloxazine ring and groups in the protein might therefore be the explanation of the long-wavelength bands previously observed with butyryl CoA dehydrogenase (Steyn-Parvee and Beinert, 1958) and with the "old yellow" enzyme (Rutter and Rolander, 1957).

While it is considered that the primary binding force in complex formation is a salt linkage involving a positively charged group of the protein and an ionized carboxyl group of the complexing agent, the binding strength of many derivatives appears too great for this to be the only linkage involved. In agreement with the inhibition studies of Frisell *et al.* (1956), the com-

pounds that are most strongly bound to the enzyme are those with an $\alpha,\beta$-unsaturated conjugation. It is perhaps some interaction between such a linkage and the conjugated ring structure of the flavin that is the cause of the enhanced binding strength of this group of compounds.

In conclusion we wish to reiterate the desirability of a much more extensive study of the chemistry of free flavins and model compounds. The solvent effects on the spectrum of 3-methyllumiflavin reported by Harbury *et al.* (1959) have suggested a very plausible explanation of the spectral properties of D-amino acid oxidase and the effects of complex formation with various carboxylic acids. It is tempting to believe that this may provide a general explanation of the two different types of spectrum of flavoproteins listed in Table I; that those which show a simple characterless absorption in the 400- to 500-m$\mu$ range do so because of hydrogen bonding to the isoalloxazine moiety from groups in the protein or because the flavin is exposed freely to hydrogen bonding from the aqueous solvent. On the other hand it is suggested that those which show a partially resolved three-banded absorption peak in this wavelength region do so because the flavin is located in a hydrophobic environment in the protein, and that the resultant spectrum is therefore of the non-hydrogen-bonded type. Work is in progress with a number of flavoproteins to test this hypothesis.

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