Stoichiometric Inhibition of Reduced Xanthine Oxidase by Hydroxypyrazolo[3,4-d]pyrimidines

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SUMMARY

The inhibition of reduced bovine milk xanthine oxidase by 4-hydroxypyrazolo[3,4-d]pyrimidine (4-HPP) and 4,6-dihydroxypyrazolo[3,4-d]pyrimidine (4,6-diHPP) is "stoichiometric" or "titrating" in type, i.e. the inhibition is virtually complete (>90%) at concentrations of inhibitor equimolar with that of the enzyme. The dissociation constant of the reduced enzyme-4,6-diHPP complex, determined by the method of Easson and Stedman (Proc. Roy. Soc. Ser. B Biol. Sci., 121, 142 (1936)), is $5.4 \times 10^{-10}$ M. No decrease in the fractional inhibition is detectable on increasing the substrate concentration from 0.04 to 0.33 mM, or on 10-fold dilution of the enzyme-inhibitor complex. The inhibitory properties of 4-HPP are virtually identical with those of 4,6-diHPP; this identity in behavior is the result of the conversion of 4-HPP to 4,6-diHPP by the enzyme. The rate of development of 4,6-diHPP inhibition is markedly temperature-dependent, more than 2 hours being required to reach maximal inhibition at 2°, but less than 1 min at 40°. The time required to reach maximal inhibition is not decreased by increasing the 4,6-diHPP level 100-fold, from 28 μM to 2.8 μM; the inhibition appears, therefore, to be caused, not by the enzyme-inhibitor complex initially formed, but to a subsequent internal rearrangement of the latter to a less readily dissociable form.

4-Hydroxypyrazolo[3,4-d]pyrimidine and 4,6-dihydroxypyrazolo[3,4-d]pyrimidine, isomers of hypoxanthine and xanthine, respectively, are potent inhibitors of the metalloflavoprotein xanthine oxidase both in vitro and in vivo, and have been employed in biochemical, pharmacological, and clinical studies for almost a decade (1, 2). Despite the frequent and continuing use of these compounds as xanthine oxidase inhibitors, however, their mode of inhibition of the enzyme is not fully established. Elion (3), in studying the inhibitory action of these compounds in a conventional aerobic assay system with xanthine as substrate and molecular oxygen as electron acceptor, noted that 4-HPP and 4,6 diHPP differed in their effects on the enzyme, and that the mode of action of both inhibitors was complex. When substrate and inhibitor were added to xanthine oxidase simultaneously, 4-HPP appeared to act as a competitive inhibitor with a dissociation constant of $7 \times 10^{-7}$ M; when 4-HPP was initially incubated with the enzyme, however, the inhibition increased and was no longer competitive with substrate. With 4,6-diHPP, on the other hand, no increase in inhibition on preliminary incubation with enzyme was seen, although inactivation of the enzyme was noted after the addition of substrate. Inhibition by both compounds could be reversed by prolonged dialysis.

Another important early observation concerning these inhibitors was the finding of Lorz and Hitchings (4) that 4-HPP can act as a substrate for xanthine oxidase, undergoing extremely slow oxidation under conventional aerobic assay conditions to 4,6-diHPP; whether this conversion was of significance in the activity of 4-HPP and 4,6-diHPP as inhibitors was, however, unknown.

Our earlier studies with the 4-HPP-xanthine oxidase system concerned the use of this compound in studying the routes of electron transfer from the internal electron transport chain of the enzyme to external acceptors (5, 6). These studies served to establish that the site of inhibition of the enzyme by 4-HPP preceded the flavin and iron components of the internal electron transport chain of the enzyme; and also showed that the site of electron transfer from the internal electron transport chain to certain artificial acceptors (e.g. phenazine methosulfate, 2,6-dichlorophenol) differed from, and preceded, the site of electron transfer to the physiological acceptor, molecular oxygen.

In the course of these studies, however, it became evident that the form of the enzyme susceptible to pyrazolo[3,4-d]pyrimidine inhibition was not the fully oxidized form present under aerobic conditions in the absence of substrate, but rather a reduced or otherwise altered form of the enzyme generated during the catalytic cycle (7). The present paper describes this interaction between these pyrazolo[3,4-d]pyrimidine inhibitors and reduced xanthine oxidase, and presents evidence that the inhibition of reduced xanthine oxidase is "stoichiometric" or "titrating" in unknown.

1 The abbreviations used are: 4-HPP, 4-hydroxypyrazolo[3,4-d]pyrimidine; 4,6-diHPP, 4,6-dihydroxypyrazolo[3,4-d]pyrimidine.
type. Similar conclusions have also been reached in a parallel and independent study by Massey et al. (8).

A preliminary account of the present studies has appeared (9).

EXPERIMENTAL PROCEDURE

Bovine milk xanthine oxidase was prepared and stored as previously described (7); the specific activity of the preparation used in the present experiments was 10.0 units per ml per A₄₈₀ mp, as defined by Fridovich (10), and the "immediate" phase of 4-HPP-induced anaerobic reduction of the enzyme flavin (7), determined at 450 nm, amounted to 45% of the total reduction observed on the addition of sodium dithionite. Assays were performed in potassium phosphate buffer, pH 7.8, 0.16 M, containing EDTA, 33 μM, by methods previously described (7), except that the aerobic conversion of 4-HPP to 4,6-diHPP was monitored by measuring the rate of cytochrome c reduction at 550 nm, rather than of 4,6-diHPP formation at 285 nm. As previously shown, the rate of reduction of cytochrome c in the 4-HPP-xanthine oxidase system accurately parallels the rate of 4,6-diHPP formation, although the total molar equivalent of cytochrome c reduced is only approximately 32% of the total 4,6-diHPP generated (7). The sources of the reagents used have previously been indicated (11), with the exception of 14C-labeled 4-HPP, which was synthesized from 14C-formate and 2-oxobutyrate by the method of Elion et al. (12), and recrystallized to constant activity from dilute hydrochloric acid; the specific activity was 78 μCi per mmole.

Spectrophotometric assays at a single wave length were carried out with a Gilford model 2000 equipped with a thermostatted cell compartment; complete absorption spectra were obtained with an Aminco-Chance dual wave length split beam spectrophotometer. Details of the individual assay methods are given in the legends for figures and tables; the molar extinction coefficients used in the spectrophotometric assay methods have previously been indicated (11).

RESULTS

Stoichiometric Inhibition of Dithionite-reduced Xanthine Oxidase by 4-HPP and 4,6-diHPP—Xanthine oxidase, 1.35 mmoles, in 0.5 ml of potassium phosphate buffer, 0.05 M, containing EDTA, 10⁻⁴ M, was reduced by the addition of powdered sodium dithionite, 1 mg, and the solution incubated at 4° under nitrogen for 1 hour in order to insure complete reduction of the enzyme. To the members of a series of reduced enzyme solutions prepared in this way were added varying concentrations of either 4-HPP or 4,6-diHPP in dithionite-containing phosphate buffer; the final incubation mixture contained reduced enzyme, 4-HPP or 4,6-diHPP, potassium phosphate buffer, pH 7.8, 1 mmole, EDTA; 0.2 μmole, and sodium dithionite, 5.8 μmoles, in a total volume of 3 ml. These solutions were incubated at 4° for 12 hours.

To determine residual enzyme activity after this treatment, 0.1-ml portions of the solutions were transferred to cuvettes containing potassium phosphate buffer, pH 7.8, 0.5 mmole, EDTA, 0.1 μmole, and xanthine, 0.25 μmole, in a total volume of 3 ml; and uric acid formation was monitored under aerobic conditions at 295 nm at 25° (10). The concentration of enzyme in the final assay mixture was 15 μM.

If either dithionite or inhibitor was omitted from the incubation mixture, no significant loss of catalytic activity was observed on subsequent assay; dithionite-reduced xanthine oxidase to which either 4-HPP or 4,6-diHPP had been added, however, showed significant inhibition at levels of inhibitor which were less than equimolar with that of enzyme. On plotting the fractional activity against inhibitor concentration (Figs. 1 and 2), it was found that a tangent to the straight line portion of the inhibition curve intersected the abscissa at a value for inhibitor concentration equimolar with that of enzyme concentration (15 μM). Since marked inhibition was seen at levels of inhibitor which were less than the concentration of enzyme present, and since the inhibition was almost complete at a concentration of inhibitor equimolar with that of enzyme, it is evident that the 4-HPP and 4,6-diHPP-reduced xanthine oxidase systems fall within the classification of "mutual depletion" systems, i.e., systems in which, as a consequence of the low dissociation constant of the enzyme-inhibitor complex, the concentration of free inhibitor cannot be assumed to be independent of enzyme concentration (13).

Effect of Substrate Concentration on Fractional Inhibition—In order to determine the effect of substrate concentration on the fractional inhibition of xanthine oxidase by 4-HPP and 4,6-diHPP, experiments were carried out in which reduced enzyme was initially incubated with inhibitor at a concentration of 0.88

![Fig. 1](http://www.jbc.org/) Fractional activity of xanthine oxidase as a function of 4-HPP concentration. Dithionite-reduced xanthine oxidase was incubated with varying concentrations of 4-HPP as described in the text, and 0.1 ml aliquots removed for determination of fractional activity in the conversion of xanthine, 0.083 mm, to uric acid (10). α = v/v. The concentration of xanthine oxidase was 0.42 μmol in the incubation mixture, and 15 μM in the final assay mixture.

![Fig. 2](http://www.jbc.org/) Fractional activity of xanthine oxidase as a function of 4,6-diHPP concentration. The incubation and assay conditions were identical with those described for Fig. 1.
Dithionite-reduced xanthine oxidase was incubated with 4-HPP or 4,6-diHPP for 12 hours, as described in the text, and 0.1-ml aliquots removed for determination of initial fractional inhibition of the catalytic activity of the enzyme in the aerobic oxidation of xanthine. The enzyme, inhibitor, and substrate concentrations shown are those present in the final assay mixture. $t = \frac{(v - vi)}{v}$.

**Table I**

<table>
<thead>
<tr>
<th>Enzyme and inhibitor</th>
<th>Concentration (μM)</th>
<th>Xanthine concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.04</td>
<td>0.08</td>
</tr>
<tr>
<td>Xanthine oxidase...</td>
<td>16</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>4,6-diHPP.............</td>
<td>55</td>
<td></td>
</tr>
<tr>
<td>Xanthine oxidase...</td>
<td>15</td>
<td>0.83</td>
</tr>
<tr>
<td>4-HPP..................</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Xanthine oxidase...</td>
<td>15</td>
<td>0.78</td>
</tr>
<tr>
<td>4,6-diHPP.............</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

μM as described above. Enzyme activity was then assayed at initial substrate concentrations ranging from 0.04 to 0.3 μM, and final inhibitor concentrations of 14 and 55 μM; assay conditions are described in the legend for Table I. As indicated in Table I, no decrease in initial fractional inhibition was seen when the substrate concentration was raised.

As discussed in a later section, such an inability to detect competition between substrate and inhibitor is frequently observed in mutual depletion systems, and cannot necessarily be interpreted as indicating that the inhibition is not of a competitive type. Very slow substrate-dependent partial reversal of the fractional inhibition was, in fact, observed when xanthine was allowed to continue over extremely long periods (Table II). These findings do indicate, however, that the inhibition is "apparently noncompetitive" as defined by Easson and Stedman (14), i.e., the inhibitor is not displaced by substrate from its complex with the enzyme to an extent which appreciably affects the experimental determination of the initial fractional inhibition. Utilizing the relationship developed by these authors for cases of this type,

$$\frac{I}{i} = \frac{Ke}{1 - i} + E$$

where

$$i = \frac{v - vi}{v} = \frac{EI}{E}$$

fractional inhibition data obtained by the method described above was plotted as shown in Fig. 3. $EI$ is the enzyme-inhibitor complex. From the slopes of the latter plots, inhibition constants of $6.3 \times 10^{-18}$ and $5.4 \times 10^{-18}$ M can be estimated for 4-HPP and 4,6-diHPP, respectively.

**Effects of Dilution on Fractional Inhibition**—A characteristic property of "titrating" or "stoichiometric" inhibitors is the lack of significant reversal in the fractional inhibition on dilution of the reaction mixture (14). As shown in Table III, 10-fold dilution of the enzyme-inhibitor complex from a concentration in

**Table II**

<table>
<thead>
<tr>
<th>Initial concentration of xanthine (μM)</th>
<th>Time after addition of substrate (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
</tr>
<tr>
<td>0.04</td>
<td>&gt;0.3</td>
</tr>
<tr>
<td>0.08</td>
<td>&gt;0.3</td>
</tr>
<tr>
<td>0.10</td>
<td>&gt;0.3</td>
</tr>
<tr>
<td>0.30</td>
<td>&gt;0.3</td>
</tr>
</tbody>
</table>

the final assay mixture of 14 to 1.4 μM, did not result in significant decrease of the inhibition observed.

**Temperature and Concentration Dependence of Rate of Development of Fractional Inhibition**—A frequent although not invariably observed characteristic of mutual depletion systems is the extreme slowness with which equilibrium is attained between enzyme and inhibitor, as indicated by the necessity for prolonged incubation between enzyme and inhibitor before maximal inhibition is observed (14, 15). With either 4-HPP or 4,6-diHPP, inhibition of xanthine oxidase was found, with the experimental protocol described above, to reach maximal levels in less than 1 min of incubation of enzyme and inhibitor at 40°, but to require 25 min to reach comparable levels of inhibition at 11°, and more than 2 hours at 2° (Figs. 4 and 5).

At temperatures above 11°, no significant difference could be detected in the fractional inhibition exerted by 4-HPP and by 4,6-diHPP (Fig. 4). When these experiments were repeated at 2°, however, a difference in the rate of development of inhibition was detectable, with inhibition developing more rapidly with 4,6-diHPP than with 4-HPP, although the final maximal inhibition observed with the two compounds was the same (Fig. 5).

Of interest also is the observation that a 100-fold increase in the concentration of 4,6-diHPP, from 28 μM to 2.8 μM, did not
result in a detectable increase in the rate of development of inhibition (Fig. 5).

Identity of Active Inhibitor in Dithionite Reduction Experiments—It is evident from the experiments described above that, by solely kinetic criteria, there are only slight quantitative differences in the fractional inhibition of reduced xanthine oxidase exerted by 4-HPP and by its oxidation product, 4,6-diHPP. There would appear to be two possible explanations for this behavior: either 4-HPP and 4,6-diHPP are almost equally active as inhibitors of the reduced enzyme, or alternatively, 4-HPP undergoes enzyme-catalyzed conversion to 4,6-diHPP, with the consequence that identical fractional inhibition is seen irrespective of whether 4,6-diHPP or its precursor is added to the reaction mixture. Such conversion would necessarily be rapid since, with the exception of the experiments carried out at 2° (Fig. 5), no detectable time lag is seen in the development of inhibition (Fig. 5).

TABLE III

Effect of dilution on initial fractional inhibition (i) of xanthine oxidase by 4-HPP and 4,6-diHPP

<table>
<thead>
<tr>
<th>Dilution</th>
<th>4-HPP, 14 μM</th>
<th>4,6-diHPP, 14 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted assay mixture</td>
<td>0.83</td>
<td>0.78</td>
</tr>
<tr>
<td>Diluted 1:2</td>
<td>0.87</td>
<td>0.83</td>
</tr>
<tr>
<td>Diluted 1:10</td>
<td>0.84</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Fig. 4. Time and temperature dependence of fractional inhibition of xanthine oxidase by 4-HPP and by 4,6-diHPP. Dithionite-reduced xanthine oxidase, 0.45 μM, was incubated with either 4-HPP or 4,6-diHPP, 0.83 μM, for the times indicated on the abscissa, and fractional inhibition then determined at 25° by the same assay method in Figs. 1 to 3, at a final enzyme concentration of 15 μM. Incubation temperature: A, 40°; B, 25°; C, 11°. Inhibitor: ●, 4-HPP; O, 4,6-diHPP.

Fig. 5. Time and temperature dependence of fractional inhibition of xanthine oxidase by 4-HPP and by 4,6-diHPP. The experimental conditions were identical with those of Fig. 4, except that incubation was carried out at 2°. Enzyme concentration in incubation mixture: 0.45 μM; inhibitor concentration: ○, 4-HPP, 0.83 μM; △, 4-HPP, 83 μM; Δ, 4,6-diHPP, 0.83 μM; ●, 4,6-diHPP, 83 μM. The incubation mixture was diluted 30-fold for assay.
4-HPP (Fig. 6). It would appear therefore that the identity in inhibitory activity between 4-HPP and 4,6-diHPP noted in the dithionite reduction experiments was caused by the rapid enzyme-catalyzed conversion of 4-HPP to 4,6-diHPP, i.e. despite the fact that most of the enzyme is in a reduced form when dithionite is present in excess, since conditions were not completely anaerobic, aerobic reoxidation is still able to take place under these conditions at a rate sufficient to permit catalytic cycling of the enzyme when substrate is added to the system. Thus, irrespective of which of the two compounds was added, the inhibitory activity observed is that of 4,6-diHPP.

Temperature Dependence of Rate of Xanthine Oxidase-catalyzed Generation of 4,6-diHPP from 4-HPP—The experiments described above indicate that the inhibition of reduced xanthine oxidase by 4,6-diHPP is “stoichiometric” or “titrating” in type. In previous studies, we have observed, however, that when 4,6-diHPP is generated by the xanthine oxidase-catalyzed oxidation of 4-HPP, multiple catalytic cycles (approximately 73 to 77 cycles at 25°) can take place before maximal enzyme inactivation is observed (7). Since both the molecular species which participate in enzyme-inhibitor complex formation, 4,6-diHPP and reduced enzyme, are generated with each catalytic cycle, and since the inhibition is stoichiometric in type, the question arises as to the reason why under these reaction conditions, enzyme inactivation is not observed after a single turnover.

The studies described above would appear to offer a possible explanation for this inconsistency, since these experiments indicate that the attainment of equilibrium between 4,6-diHPP and reduced enzyme is a slow process with a marked temperature dependence (Figs. 4 and 5). Furthermore, variations in the level of 4,6-diHPP in the medium did not exert a detectable effect on the rate of development of inhibition, indicating that the latter did not depend on the rate of initial enzyme-inhibitor complex formation, but rather on an internal rearrangement of the latter.

In order to determine whether these considerations might permit multiple catalytic cycling before maximal inhibition when 4,6-diHPP is generated from 4-HPP, the temperature dependence of the latter reaction was examined. The conversion of 4-HPP to 4,6-diHPP was measured by following the rate of reduction of cytochrome c, 0.83 μM, at 550 μM; we have previously shown that the aerobic fast phase of conversion of 4-HPP to 4,6-diHPP as measured by the rate of cytochrome c reduction paralleled the duration and rate of the aerobic fast phase as measured by following 4,6-diHPP formation directly (7).

As shown in Fig. 7, the rate of xanthine oxidase-catalyzed electron transfer from 4-HPP to cytochrome c under aerobic conditions is characterized by an inactivation of the enzyme which proceeds by a process following first order kinetics; the rate of decline in enzyme activity is markedly temperature-dependent, with the half-time increasing from 3 min at 25° to 6.2 min at 5°. Furthermore, when the amount of cytochrome c reduced prior to development of maximal enzyme inhibition was determined, it was found that the total amount converted was not a constant value, but that it increased with decreasing temperature (Fig. 8). As had previously been noted in the experiments with dithionite reduced enzyme, the addition of 4,6-diHPP to the medium in excess of that generated from 4-HPP, did not accelerate the rate of development of inhibition.
(Fig. 9). It was concluded from these experiments that the multiple catalytic cycling before maximal inhibition observed when 4,6-diHPP is generated from 4-HPP, does not indicate a requirement for a fixed molar excess of 4,6-diHPP over enzyme before maximal inhibition is attained, but rather, as was seen in the dithionite reduction experiments described above, is a consequence of the fact that the stoichiometric inhibition of reduced enzyme by 4,6-diHPP, is a slow process, with the time required to reach maximal inhibition being markedly temperature-dependent.

**Discussion**

These studies permit the addition of 4,6-diHPP to the list of pharmacologically active agents which function by virtue of their action as "stoichiometric" or "titrating" enzyme inhibitors. That both 4-HPP and 4,6-diHPP are potent inhibitors of xanthine oxidase has been known for some years (3); it was not recognized until recently, however, that the form of the enzyme which is susceptible to inhibition is a reduced form, rather than the fully oxidized form in which the noncycling enzyme exists under aerobic conditions (7, 8). Thus, inhibition constants for 4-HPP and 4,6-diHPP derived from early studies of the ability of these agents to inhibit the initial rate of aerobic oxidation of xanthine by the enzyme, have little quantitative significance when considered as indications of the tightness of binding of inhibitor to enzyme (9). These early studies were valuable, however, because of the observations previously cited concerning the nature of the inhibition exerted by 4-HPP and 4,6-diHPP, i.e. that the inhibition by 4-HPP increases with time of aerobic initial incubation of inhibitor with the enzyme before the addition of substrate, while with 4,6-diHPP, an increase in the degree of inhibition is noted only after the reaction is started by the addition of substrate. These observations served to indicate that the form of the enzyme susceptible to inhibition might be a reduced or otherwise altered form generated during the catalytic cycle.

The value of $6 \times 10^{-10}$ M for the dissociation constant of the reduced enzyme 4,6-diHPP complex is comparable to the inhibition constants observed with previously studied "stoichiometric" enzyme inhibitors such as methotrexate for dihydrofolate reductase (16, 17) and ethoxzolamide for carbonic anhydrase (15). Methods for the determination of the tightness of binding of such inhibitors have been developed by a number of authors (14, 18-20), and a comprehensive treatment, together with a summary of previous work, has recently appeared (21). Of critical importance in the determination of enzyme-inhibitor dissociation constants by such kinetic methods, however, is the assumption that the residual activity measurable at equimolar ratios of inhibitor and enzyme, or at inhibitor to enzyme ratios which exceed 1:1, is caused by that fraction of the enzyme which is present in the free rather than the complexed form. Otherwise stated, these methods assume that the departure from a direct linear relationship between fractional inhibition and inhibitor concentration in "titration" plots such as Figs. 1 and 2 of the present paper, represents the fact that measurable dissociation of the enzyme-inhibitor complex takes place. While this assumption is undoubtedly correct in the case of many such systems, and may be applicable to the present system also, such an interpretation is not easily susceptible of direct proof in the case of a complex enzyme such as xanthine oxidase. A possible alternate explanation is that such enzyme activity, rather than representing that fraction of the total enzyme available for catalysis because of spontaneous dissociation of the reduced enzyme-inhibitor complex, represents enzyme regaining catalytic activity because of the aerobic reoxidation of the reduced electron transfer component (probably molybdenum (6, 8)) to which the inhibitor binds, with such reoxidation occurring either directly or by way of the enzyme flavin. Another possible explanation is that, if it is assumed that the enzyme-bound 4,6-diHPP does not occupy the substrate site of the enzyme, the low rates of substrate oxidation observed with the inhibited enzyme may indicate that the enzyme present in the enzyme-inhibitor complex may retain a low rate of catalytic activity, with electron transfer to molecular oxygen bypassing the inhibited portion of the internal electron transport chain of the enzyme. It should be noted, however, that if such considerations apply, they would result in the apparent concentration of catalytically active enzyme resulting from dissociation of the enzyme-inhibitor complex being greater than is actually the case, i.e. if such factors are operative, the value for the dissociation constant obtained by a conventional kinetic method, such as that used here, would represent a maximal value and the enzyme-inhibitor binding would be tighter than the kinetic studies would indicate.

A feature of interest in the inhibition of reduced xanthine oxidase by 4,6-diHPP is the time required for maximal inhibition to develop. The inactivation of reduced xanthine oxidase by 4,6-diHPP follows first order kinetics, and appears to be dependent only on the reduced enzyme concentration and on temperature, and not on the concentration of inhibitor in the medium. Because of this lack of dependence on inhibitor concentration, the process would appear to represent not the initial enzyme-inhibitor complex formation, but rather an internal rearrangement of the latter. Such a time dependence has been observed with the xanthine oxidase inhibitors 2-amino-4-hydroxypteridine-6-aldehyde (22) and purine-6-aldehyde (23), and with the carbonic anhydrase inhibitors ethoxzolamide and dichlorphenamide (15), but its significance is little understood. In the case of the carbonic anhydrase inhibitors cited, there was no correlation within the series of eight inhibitors studied, between the time required for maximal inhibition to develop and the tightness of binding. Such factors render systems such as these unsuitable for kinetic treatment by methods such as that recently described by Morrison (21), in which the assumption is made that the equilibria between the enzyme, substrates, and inhibitor are attained at a sufficiently rapid rate as to enable true steady state initial velocities to be determined.

Studies with "stoichiometric" enzyme inhibitors have proved to be of value in determining the number of catalytically active sites per enzyme molecule. In the case of xanthine oxidase, such information would be of particular interest because it has not been definitely established whether there are one or two active sites per enzyme molecule (i.e. per 2 FAD molecules). Such interpretations are complicated however, by the circumstance first noted by Morell (24), and since confirmed by other workers (8, 25), that all preparations of bovine milk xanthine oxidase contain catalytically inactive enzyme which, however, contains the normal complement of FAD. Determination of such an interpretation is not easily susceptible of direct proof in the case of a complex enzyme such as xanthine oxidase. A possible alternate explanation is that such enzyme activity, rather than representing that fraction of the total enzyme available for catalysis because of spontaneous dissociation of the reduced enzyme-inhibitor complex, represents enzyme regaining catalytic activity because of the aerobic reoxidation of the reduced electron transfer component (probably molybdenum (6, 8)) to which the inhibitor binds, with such reoxidation occurring either directly or by way of the enzyme flavin. Another possible explanation is that, if it is assumed that the enzyme-bound 4,6-diHPP does not occupy the substrate site of the enzyme, the low rates of substrate oxidation observed with the inhibited enzyme may indicate that the enzyme present in the enzyme-inhibitor complex may retain a low rate of catalytic activity, with electron transfer to molecular oxygen bypassing the inhibited portion of the internal electron transport chain of the enzyme. It should be noted, however, that if such considerations apply, they would result in the apparent concentration of catalytically active enzyme resulting from dissociation of the enzyme-inhibitor complex being greater than is actually the case, i.e. if such factors are operative, the value for the dissociation constant obtained by a conventional kinetic method, such as that used here, would represent a maximal value and the enzyme-inhibitor binding would be tighter than the kinetic studies would indicate.
the number of active sites per FAD moiety from the present data would therefore necessitate knowing whether catalytically inactive reduced enzyme can bind 4,6-diHPP. If such considerations are not taken into account, i.e. if it is assumed that all the enzyme present can bind 4,6-diHPP, the number of active sites present can be calculated from Fig. 3 to be 0.8 per molecule of enzyme, or 0.4 per molecule of FAD; the possible presence of FAD-containing enzyme which lacks catalytic activity and also the ability to bind 4,6-diHPP would, however, result in a low value for this ratio.

Acknowledgments—We would like to thank Drs. V. Massey and G. B. Elion for making available to us a copy of their paper (8) prior to publication. The experiment described under “Identity of Active Inhibitor in Dithionite Reduction Experiments” of the present paper is a modification of a procedure described by these authors.

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T. Spector and D. G. Johns


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