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The Inhibition of Mammalian D-Amino Acid Oxidase by Metabolites and Drugs. Inferences concerning Physiological Function[1]

GORDON A. HAMILTON AND DAVID J. BUCKTHAL

Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania 16802

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The inhibition of D-amino acid oxidase (EC 1.4.3.3) by a large number of metabolites and drugs is reported. When the substrate is an adduct (thiazolidine-2-carboxylate) formed from cysteamine and glyoxylate, at least five different mechanisms of inhibition are possible, and examples of each are given. Effective inhibitors include (a) some adenosine containing nucleotides, in particular ADP, AMP, ADP ribose, NADH, NADPH and dephospho-CoA; (b) diuretics, especially furosemide, ethacrynic acid, and mersalyl; (c) anti-inflammatory agents, such as salicylate, indomethacin, phenylbutazone, acetylsalicylate, mefenamic, and flufenamic acids; (d) hypoglycemic, hypocalcemic, and hypolipidemic compounds, including 5-methylpyrazole-3-carboxylate, pyrrole-2-carboxylate, 5-methylthiophene-2-carboxylate, thiophene-2-carboxylate, benzoate, and nicotinate; (e) aldehydes, for example, formaldehyde, acetaldehyde, and succinate semialdehyde; (f) thiols, especially β-aminothiols such as cysteine and penicillamine; (g) tropolone, and (h) hydrogen peroxide. The rate of the reaction is also very sensitive to oxygen pressure; at pH 7.4 and 25°C the K_m for O_2 is 1.1 mM.

These data, in conjunction with literature information concerning the biological affects of such compounds, are used to suggest possible physiological processes in which the D-amino acid oxidase reaction may be involved. Although such correlations based on circumstantial evidence are not conclusive, they suggest that D-amino acid oxidase may play a major role in the control of metabolism in animals. Some processes in which it may participate include maintenance of ion and water balance in the kidney, inflammatory response, transmission of nerve impulses, sensing of O_2 concentration, control of cell growth, and as part of an intracellular messenger system for some hormones, especially insulin.

D-Amino acid oxidase (EC 1.4.3.3; D-AA oxidase[2]) was initially characterized in the early 1930s by Krebs (*1, 2*), who demonstrated its presence in the kidneys and livers of a large number of animals. However, it is now known to also be a constituent of several other animal tissues as well, including brain (*3*), nerve (*4*), leukocytes (*5*), adrenal cortex (*6*), preputial gland (*6*), intestine (*7*), heart (*8*), tongue (*9*), skin (*10*) stomach (*10*), spleen (*10*), muscle (*10*), and fat (S. M. Abernathy and G. A. Hamilton, unpublished results). Since the enzyme is located in the peroxisomes and microperoxisomes of animal cells (*4, 11, 12*), and since these organelles have been identified histochemically in almost all cell types (*13*),

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[2] Abbreviations used: D-AA oxidase, D-amino acid oxidase; CG, cysteamine–glyoxylate adduct, i.e., thiazolidine-2-carboxylate; TC, thiazoline-2-carboxylate.

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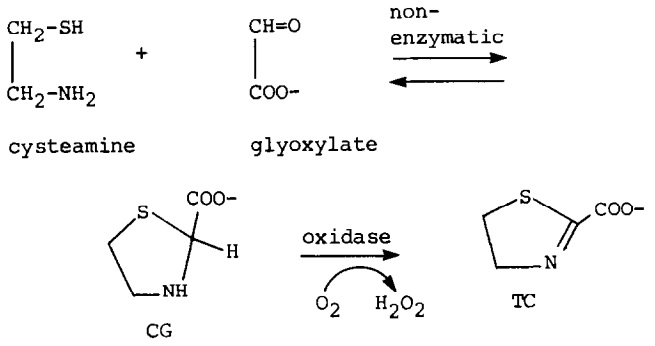
one suspects that D-AA oxidase may be present in virtually all animal cells. Despite this wide distribution, which implies that the enzyme has some important metabolic role, its physiological function has remained unknown. Because D-amino acids are not present in animal cells to any significant extent, we recently suggested (*14*) that the true physiological substrates may be adducts of amines with glyoxylate. Following up on this suggestion, we found that the adduct CG, formed from the reaction of cysteamine with glyoxylate, is a particularly effective substrate for the enzyme (Eq. [1]). Considerable circumstantial evidence (*14*) suggests that this reaction is occurring physiologically.



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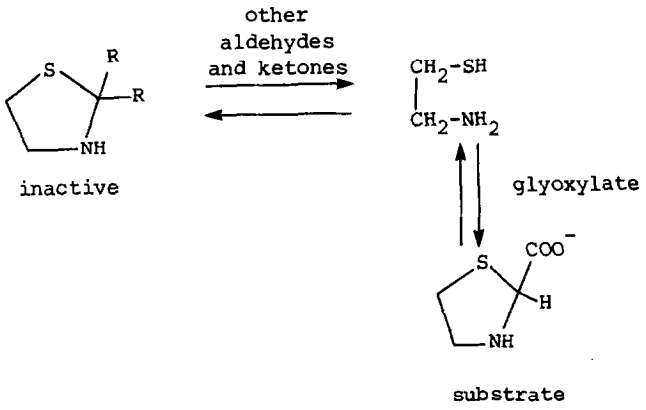
If the conversion of CG to TC is a reaction catalyzed by D-AA oxidase *in vivo*, then several questions still remain unanswered. For example, what is the metabolic function of such a reaction, and in what physiological processes is it involved? In connection with the proposal that TC may be a metabolic effector (*14*), what does it affect? One approach to answering these questions is to consider what types of compounds inhibit D-AA oxidase, and then to correlate the results with the known physiological effects of such compounds. Therefore, in this article we report results from a survey of a large number of compounds (especially metabolites and drugs) as potential inhibitors of D-AA oxidase with CG as substrate, and then under Discussion we use this information to make some suggestions concerning processes in which this enzymic reaction may be involved.

Using the classical D-α-amino acids as substrates, previous investigators have shown that D-AA oxidase can be inhibited in several different ways (*2, 15–17*), including (a) competition with substrate (especially by aromatic and α,β-unsaturated carboxylic acids), (b) competition with FAD (e.g., by FAD analogs, various anions, and some drugs), and (c) reagents which attack a sensitive thiol group. With the cysteamine–glyoxylate adduct (CG) as substrate, the enzymic reaction will be susceptible to two additional types of inhibition, namely, (d) competition with glyoxylate for reaction with cysteamine (Eq. [2]), and (e) competition with cysteamine for reaction with glyoxylate (Eq. [3]). To the extent that these reactions occur they will effectively lower the concentration of the enzymatic substrate and thus slow down the reaction. Since the D-AA oxidase reaction involving classical substrates has a K_m for oxygen which is in the millimolar range

(*15, 18, 19*), another condition which will decrease the rate of the enzyme catalysis is (f) low oxygen pressure. Examples of all six methods for slowing the D-AA oxidase reaction involving CG as substrate are reported here.



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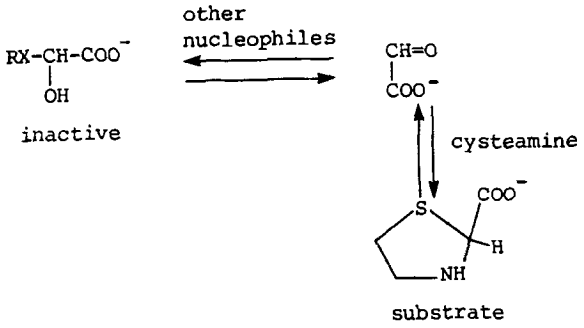

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EXPERIMENTAL

Materials. Crystalline hog kidney D-AA oxidase, obtained from Sigma Chemical Company, was used for all the experiments reported here. By gel electrophoresis this material was estimated to be 80–95% pure, and its specific activity was comparable to that previously reported for the homogeneous enzyme (*19, 20*). The enzyme employed in most of the experiments was dialyzed versus either 100 m*M* sodium pyrophosphate buffer, pH 7.4, or 20 m*M* potassium phosphate buffer, pH 7.4, to remove the ammonium sulfate from the commercial preparation. In some experiments the ammonium sulfate was not removed but no differences in the results obtained with the dialyzed or undialyzed preparations were detectable. In those cases where the deflavo enzyme was used, the FAD was removed as previously described (*21*), with the final dialysis performed using 100 m*M* sodium pyrophosphate buffer, pH 7.4.

Unless otherwise specified, reagent-grade commercial chemicals were used

without further purification. The following compounds were kindly supplied by the companies indicated: mefenamic and flufenamic acids, Parke, Davis and Company; cromolyn sodium, Fisons Corporation; haloperidol and droperidol, McNeil Laboratories; molindone, Endo Labs.; 5-methylpyrazole-3-carboxylic acid, The Upjohn Company; benzquinamide, Pfizer; mersalyl, Sterling–Winthrop; furosemide, Hoechst–Roussel Pharmaceuticals; chlorothiazide and hydrochlorothiazide, Merck, Sharp and Dohme; 3-aminopicolinic and 3-mercaptopicolinic acids, Smith, Kline and French.

Methods. The enzymic rates and protein concentrations were determined as previously described (*14*). The units used for expressing enzyme activity have also been given (*14*). Unless otherwise noted, all reactions were carried out at 25°C with 0.25 m*M* O_2 (air saturated solutions) present. Also, except where indicated, the substrate was added as an aliquot of a stock solution containing 200 m*M* cysteamine hydrochloride and 300 m*M* glyoxylic acid which had been neutralized to pH 7.4 with NaOH. Prior to use, this stock solution remained at room temperature for at least 1 hr to ensure complete formation of CG. With glyoxylate in 50% excess, all the cysteamine is converted to CG as evidenced by the observation that no O_2 uptake is seen in the absence of the enzyme (because free cysteamine has a thiol group it undergoes nonenzymatic autooxidation (*14*) whereas CG does not). The concentration of the substrate in the reaction mixtures was calculated assuming all the cysteamine used in preparing the solutions is present as CG. In a few cases, crystalline CG, prepared as described by Johnson *et al.* (*22*), was used. The enzymic rate, observed using solutions prepared from this crystalline material, is identical within experimental error to that obtained with the same concentration of CG prepared by incubating cysteamine and 50% excess glyoxylate as described above. At high concentrations glyoxylate is an inhibitor of D-amino acid oxidase (*14*), but at the concentrations of substrate used in the present research the 50% excess glyoxylate has a negligible effect. It is obvious that the CG prepared by the above procedures will be racemic.

Cysteamine and glyoxylate were not added separately to reaction mixtures because the formation of CG under the reaction conditions is not instantaneous, especially at the lower concentrations. Once formed, however, CG does not readily dissociate to cysteamine and glyoxylate even when present in dilute solution. For example, after diluting the concentrated stock solution into a reaction mixture to give 0.4 m*M* CG, the same rate is observed when the reaction is initiated by adding enzyme 30 min later as is observed when it is initiated immediately on dilution.

RESULTS

Inhibition by Some Drugs

A general survey of the effects of various types of drugs on the D-AA oxidase catalyzed oxidation of CG was carried out under conditions (relatively low substrate and FAD concentrations) where inhibition by competition with either sub-

strate or FAD would be observed. Some of these results are summarized in Table 1. Most of the experiments were performed using buffer and salt conditions (footnote *a* of the table) which were designed to approximate those present intracellularly. In addition to the compounds listed in Table 1, several other drugs, which were shown to inhibit specifically by competition with substrate, were studied more thoroughly and are considered later. Under conditions similar to those used for experiments of Table 1, the following compounds at 1.0 m*M* concentrations caused negligible inhibition (less than 10%) of the D-AA oxidase reaction: daunomycin, actinomycin D, antimycin A, chromomycin A_3, antipyrine, *N*-acetyl-*p*-aminophenol, haloperidol, droperidol, chlorpromazine, molindone, benzquinamide, chromolyn sodium, caffeine, theophylline, indole-3-acetic acid, and abscissic acid.

Although a detailed kinetic investigation with each of the inhibitory drugs has not yet been carried out, most of the compounds listed in Table 1 appear to inhibit by competition with FAD, or by mixed competition with substrate and FAD. Since the concentration of FAD used in these survey experiments is 3–6 times its reported K_m of 0.2 to 0.45 μM at pH 7.3 to 7.5 (*23, 24*) any inhibition observed represents a fairly potent inhibition. Two of the listed compounds, namely ethacrynic acid and mersalyl, almost certainly inhibit by reacting with a sensitive sulfhydryl group (*25–29*). Consistent with this interpretation is the observation that the inhibition by ethacrynic acid is time dependent, and thus the amount of inhibition reported in Table 1 is not the maximum obtainable but merely the amount observed following a 15-min incubation with the enzyme under the given conditions. For example, when incubated under the same conditions for 30 min, 1.0 m*M* ethacrynic acid causes 84% inhibition. Because the inhibition by ethacrynic acid presumably involves the formation of a covalent bond by Michael addition of the enzymic thiol to the inhibitor, one expects that, at physiological temperatures (37°C), the rate and extent of inhibition would be still greater. Mersalyl is a particularly effective inhibitor; even at a concentration of 1 μM over 50% inhibition is observed. Since FAD is known to protect the enzyme from inactivation by sulfhydryl reagents (*25–29*), greater inhibition by ethacrynic acid and the diuretic mercurials, such as mersalyl, would also be expected at lower FAD concentrations.

The lack of inhibition by chlorpromazine, under the conditions given in Table 1, was unexpected, because Gabay and Harris (*23, 30, 31*) reported that this, and closely related phenothiazines, are potent inhibitors which compete with FAD. To more closely approximate the conditions used by Gabay and Harris, D-AA oxidase was prepared FAD free (*21*), and the inhibition experiments repeated at 37°C. Under these conditions however, (buffer as described in footnote *b* of Table 1), only about 35% inhibition was obtained using 200 μM chlorpromazine (with 0.5 μM FAD present). With D-alanine as substrate rather than CG, again only a low level of inhibition was observed. In earlier work Lasslo and Meyer (*32*) had also detected very little inhibition by chlorpromazine. A possible explanation for the much higher levels of inhibition observed by Gabay and Harris is that a significant amount of the chlorpromazine radical was formed under their experimental conditions; with other enzymes (*33*) it has been shown that the radical is a much better

TABLE 1

INHIBITION OF D-AA OXIDASE BY SOME DRUGS[a]

Inhibitor	Inhibition (%) at the following inhibitor concentrations (m*M*)	
	0.1	1.0
Diuretics		
Furosemide	56	89
Ethacrynic acid	31	64
Mersalyl	90	100
Chlorothiazide	18	32
Hydrochlorothiazide	29	36
Anti-inflammatory agents		
Indomethacin		81
Phenylbutazone[b]		64
Acetylsalicylic acid (aspirin)[b]		59
Mefenamic acid		47
Flufenamic acid	18	59
Central nervous system depressants		
Barbital[b]		18
Phenobarbital[b]		20
Hydroxyzine		37
Miscellaneous		
Papaverine		68
Lidocaine		35
Streptonigrin[b]		35
Menadione		50
Acriflavine	41	92
2-(*p*-chlorophenoxy)-2-methyl-propionic acid		30
Warfarin[b]		60

[a] Unless otherwise specified, the reactions were carried out as follows. Solutions containing 10 m*M* potassium phosphate buffer, pH 7.4, 10 m*M* KCl, 10 m*M* $NaHCO_3$, 10 m*M* $MgSO_4$, 10–15 μg/ml D-AA oxidase (dialyzed vs phosphate buffer), 1.3 μM FAD, and inhibitor at the given concentration, were incubated at 25°C for 15 min prior to initiation by adding racemic CG to give a final concentration of 0.4 m*M* (with 0.2 m*M* excess glyoxylate). With no inhibitor present the specific activity observed under these conditions is about 50 mkat/kg.

[b] Solutions containing 20 m*M* pyrophosphate buffer, pH 7.4, 8 μg/ml undialyzed D-AA oxidase, 0.7 μM FAD, and inhibitor at the indicated concentration were incubated 15 min at 25°C prior to initiation by adding racemic CG to give a final concentration of 0.4 m*M* (with 0.2 m*M* excess glyoxylate). With no inhibitor present the specific activity observed under these conditions is about 90 mkat/kg.

inhibitor than chlorpromazine itself. Indeed, when the incubations were performed in the presence of a strong light, considerably more inhibition is seen, and when they are carried out in the presence of 60 μM H_2O_2 and 0.7 μg/ml horseradish peroxidase, the D-AA oxidase catalyzed reaction is completely inhibited even when only 10 μM chlorpromazine is present. Since it is not expected that the free radical would be formed *in vivo* (*33*), it is thus unlikely that inhibition of D-AA oxidase is responsible for the antipsychotic response to chlorpromazine and related compounds.

Inhibition by Some Nucleotide Metabolites

It is known from earlier work (*24, 34–36*) that the adenosine diphosphate part of FAD is mainly responsible for the binding of the coenzyme to D-AA oxidase. Since this structural feature, either with the same base (adenine), or with a different base, is present in a large number of physiologically important nucleotides, a general survey of the effects of such nucleotides on the enzymic activity was carried out in order to clarify which ones might be controlling the activity of the enzyme *in vivo*. Some of these results are summarized in Table 2. Under similar conditions, it was found that the following compounds, even when present at 5 mM concentrations, inhibit the D-AA oxidase reaction by less than 20%: UTP, CTP, GTP, CDP ethanolamine, CDP choline, adenosine 3′,5′-diphosphate, thiamine pyrophosphate, nicotinic acid mononucleotide, and uric acid.

The effects of some of the compounds listed in Table 2, especially the marked inhibition by AMP, ADP, and ADP ribose, have been noted by others (*34, 36*), but the extent of the inhibition caused by most of the other compounds had not been reported heretofore. The results given in Table 2, taken in conjunction with the suspected physiological concentrations of the various nucleotides (*37*), make it unlikely that any guanosine, cytidine, or uridine nucleotide is involved to any significant extent in the control of D-AA oxidase *in vivo*. Similar considerations, however, strongly imply that the levels of several adenosine containing nucleotides, particularly NADH, NADPH, dephospho CoA, ADP ribose, ADP, and AMP (but not cAMP), will be controlling the enzymic activity physiologically, unless the local concentrations of these nucleotides in peroxisomes are considerably different from the general level in the cytosol. Since the reduced forms of the nicotinamide coenzymes are good inhibitors while the oxidized forms are not, the amount of inhibition caused by these coenzymes will depend on the oxidation–reduction state of the biological system, a condition which is known to vary considerably, depending on the availability of substrates, O_2, etc., and on the presence or absence of various hormones.

It is somewhat surprising that NADPH, which has a 2′-phosphate on the adenosine moiety, is a reasonably good inhibitor, while CoA and acetyl CoA, which have a 3′-phosphate group, are poor inhibitors. When the 3′-phosphate is removed from CoA, then a very effective inhibitor, namely dephospho-CoA, is obtained. The inhibition by dephospho-CoA is of special interest because it is an intermediate in the formation of cysteamine from CoA (*38*). For this reason the FAD competitive inhibition constant (K_i) for dephospho-CoA was determined (at 25°C

TABLE 2

INHIBITION OF D-AA OXIDASE BY SOME NUCLEOTIDES[a]

Nucleotide	Inhibition (%) at the following inhibitor concentrations (m*M*)		
	0.2	1.0	5.0
NAD^+	6	13	50
NADH	35	68	93
$NADP^+$		3	4
NADPH	21	56	87
$NaAD^{+b}$		18	
CoA		4	16
Acetyl CoA	6	21	34
Dephospho CoA	84	94	95
ADP ribose	40	80	
AMP	25	68	91
ADP	60	86	92
ATP		18	52
cAMP			37
GMP	21	44	73
GDP		24	36
cGMP		36	68
CMP		18	46
CDP		22	44
UMP			34
UDP		9	45
UDP glucose		11	44
UDP glucuronate		37	72

[a] Reaction conditions: solutions containing 20 m*M* potassium phosphate buffer, pH 7.4, 10 m*M* KCl, 17 μg/ml D-AA oxidase (dialyzed vs phosphate buffer), 1.4 μM FAD, and inhibitor at the given concentration were incubated at 25°C for 15 min prior to initiation by adding racemic CG to give a final concentration of 0.4 m*M* (with 0.2 m*M* excess glyoxylate). With no inhibitor present the specific activity observed under these conditions is about 50 mkat/kg.

[b] Nicotinic acid adenine dinucleotide.

in 20 m*M* sodium pyrophosphate buffer, pH 7.4, with 5 m*M* CG and 0.25 m*M* O_2 present), and found to be 10 μM. Since the concentration of dephospho-CoA, at least in liver, is believed to be 10–50 μM (*39*), it thus seems very likely that this precursor of cysteamine also controls its conversion to TC catalyzed by D-AA oxidase.

Substrate Competitive Inhibition

That aromatic carboxylic acids are potent substrate competitive inhibitors of D-AA oxidase has been known for some time (*40–44*). Such inhibition is characteristic of this enzyme and is a relatively unique phenomenon. Listed in Table 3 are some inhibition constants obtained at pH 7.4 using racemic CG and 0.25 m*M* O_2 as

substrates. Also, given in the table is the percent inhibition of the 0.4 m*M* CG reaction that results when 0.1 m*M* inhibitor is present; these data are listed so that the extent of inhibition caused by these compounds can be more easily compared to that of the compounds given in the earlier tables. Such results emphasize the fact that several of these substrate competitive inhibitors are indeed very effective inhibitors. Under conditions similar to those used for the experiments in Table 3, it was found that 1 m*M* concentrations of 3,4,5-trimethoxybenzoate, 3,4,5-trimethoxycinnamate, 3-mercaptopicolinate, and *N*-methylnicotinate cause less than 10% inhibition when CG is present at 0.4 m*M*. Also relatively ineffective as inhibitors are the tryptophan metabolites, quinolinic, kynurenic, and xanthurenic acids, which inhibit to less than 25% under similar conditions. Compounds that are reasonable inhibitors, but which were not studied sufficiently extensively to obtain K_I's, include (percent inhibition with 1 m*M* inhibitor and 0.4 m*M* CG is

TABLE 3

SUBSTRATE COMPETITIVE INHIBITORS OF D-AA OXIDASE

Inhibitor	Inhibition constant[a] (K_I, μM)	Inhibition[b] (%) with 0.1 m*M* inhibitor and 0.4 m*M* CG
Indole-2-carboxylate	0.6	98
5-Methylpyrazole-3-carboxylate	1.1	97
Pyrrole-2-carboxylate	2.0	95
5-Methylthiophene-2-carboxylate	3.7	91
Thiophene-2-carboxylate	4.5	89
Benzoate	6.0	86
Salicylate	13	74
Tropolone	40	48
Anthranilate	43	46
Furan-2-carboxylate	58	39
Nicotinate	65	36
5-Methoxyindole-2-carboxylate	67	35
Cinnamate	70	34
Furosemide	100	27
Orotate	200	15

[a] Reaction conditions: 25°C, 20 m*M* sodium pyrophosphate buffer, pH 7.4, 0.25 m*M* O_2 (air saturated), 10 μM FAD, 8 μg/ml undialyzed D-AA oxidase, 0.13–1.0 m*M* racemic CG (with glyoxylate in 50% excess), and the inhibitor at various concentrations from about $\frac{1}{2}$ to 3 times its K_I value. The reactions were initiated by adding an aliquot of the enzyme. Each K_I was determined by standard methods from kinetic data obtained using at least 4 different inhibitor concentrations and 6 different substrate concentrations. Under these reaction conditions with no inhibitor present, the apparent K_m for racemic CG was found to be 0.23 ± 0.02 m*M*, and the maximum velocity varied from 210 to 250 mkat/kg with different enzyme samples.

[b] Calculated using the standard equation for substrate competitive inhibition, the experimentally determined K_I's and K_m, and the given concentrations of the inhibitor and CG.

given following each compound) picolinate, 78; 3-aminopicolinate, 70; α-cyanocinnamate, 67; 3-hydroxyanthranilate, 56; and sorbate, 50.

Inhibition constants for many of the compounds listed in Table 3 have been obtained by others (*41–44*) at higher pH's using other substrates. However, no data for several of the physiologically active compounds, including the pyrazole and thiophene carboxylates, tropolone, and furosemide have been reported previously. Although the trends in the observed K_i's as a function of structure are in the same directions as others have noted, the absolute values obtained here are considerably lower than those kinetically determined with other substrates at the nonphysiological higher pH's.

The inhibition by pyrrole-2-carboxylate is of some interest in relation to the proposal that a cysteamine-glyoxylate adduct is the physiological substrate for D-AA oxidase. Both glyoxylate and pyrrole-2-carboxylate are known products of hydroxyproline metabolism in animals (*45, 46*). It seems possible that the minor pathway to pyrrole-2-carboxylate may have evolved to control the flow through D-amino acid oxidase when the animal is faced with a hydroxyproline load that would lead to large amounts of glyoxylate being formed.

All of the compounds listed in Table 3 are carboxylates except for tropolone. Presumably tropolone is also an effective substrate competitive inhibitor because it exists at pH 7.4 as an anion (pK_a = 6.92) (*47*), and it is a planar pi-bonded molecule like the aromatic carboxylates.

Recently, Marcotte and Walsh (*48*) reported that enols of α-ketoacids are very effective inhibitors of D-AA oxidase. Since the tyrosine metabolite, *p*-hydroxyphenylpyruvate, exists in water solution partially as the enol, its ability to inhibit the enzyme was determined. When present at a concentration of 1 m*M* (with 0.4 m*M* CG as substrate), however, *p*-hydroxyphenylpyruvate inhibited the reaction by only 11%. In another attempt to observe inhibition by an enol of an α-ketoacid which might also be physiologically significant, the D-AA oxidase catalyzed reaction of CG (0.4 m*M*) was carried out (pH 7.4, 10 m*M* Tris–HCl buffer, 37°C) in the presence of calf intestine alkaline phosphatase (6.5 μg/ml) and phosphoenolpyruvate (1.0 m*M*), but again no inhibition of the D-AA oxidase reaction was observed. It was shown that under these conditions the alkaline phosphatase is active and produces 2.5 μ*M* pyruvate (determined using lactate dehydrogenase) per minute. Presumably no inhibition was seen because the enol form of pyruvate, the initial product of the phosphatase reaction, is converted too rapidly to the keto form; recent evidence (*49*) indicates that the half time for enolpyruvate under these reaction conditions is probably considerably less than 0.5 min. Although it is expected that one would observe inhibition at lower temperatures if massive amounts of phosphatase were used, the present results indicate that under physiological conditions it is unlikely that inhibition by enolpyruvate is important in controlling D-AA oxidase.

Inhibition by Glyoxylate and Cysteamine Competitors

As illustrated by the results summarized in Table 4, the rate of O_2 uptake catalyzed by D-AA oxidase is decreased if aldehydes are present during the for-

TABLE 4

INHIBITION OF THE D-AA OXIDASE CATALYZED REACTION INVOLVING CG BY COMPOUNDS WHICH COMPETE WITH GLYOXYLATE FOR REACTION WITH CYSTEAMINE[a]

Competitor	Inhibition (%) with the competitor to glyoxylate ratio equal to: 1:1	5:1
Formaldehyde	29	90
Acetaldehyde	22	54
Succinate semialdehyde	13	65
Glyceraldehyde-3-phosphate	6	8
α-Ketoglutarate	0	0
Acetone	0	0
Glucose	2	8
Glucose 6-phosphate	0	0

[a] Solutions containing 10 m*M* glyoxylate, either 10 or 50 m*M* competitor, 20 m*M* pyrophosphate buffer, pH 7.4, and 10 m*M* cysteamine were incubated at room temperature for 30 min. These solutions were prepared by adding as the final reagent an aliquot of a concentrated cysteamine hydrochloride solution (neutralized to pH 7.4) to a solution of all the other reagents. A sample of the incubated mixture was diluted 25-fold with 20 m*M* sodium pyrophosphate buffer, pH 7.4, containing 10 μM FAD, to give a final total concentration of glyoxylate and glyoxylate derived products of 0.4 m*M*. The enzymic rate of O_2 uptake supported by this solution at 25°C was determined following initiation of the reaction by adding undialyzed D-AA oxidase (to a final concentration of 8 μg/ml). The percent inhibition was calculated taking as the standard reaction (0% inhibition) that involving cysteamine and glyoxylate alone with no competitor present, but all other incubation and assay conditions the same. Under these conditions the specific activity observed is 120 mkat/kg. In controls where no glyoxylate was added, but cysteamine and competitor were present, no enzyme catalyzed O_2 uptake was observed.

mation of CG from cysteamine and glyoxylate. Presumably this is caused by a lowering of the concentration of CG due to the formation of adducts of cysteamine with the competitor aldehydes, as illustrated in Eq. [2]. Because adducts formed from ketones are not as stable as those formed from aldehydes, ketones are ineffective inhibitors by this mechanism. Aldehydes which exist mainly as internal hemiacetals would also not be expected to compete well with glyoxylate for reaction with cysteamine, and this is evidenced by the results obtained with glucose and its phosphate.

In a similar manner, the rate of the enzyme catalyzed O_2 uptake is decreased when various thiols or amines are present during the formation of CG (Table 5). Again, this is presumably caused by a lowering of the concentration of CG, due in most cases to the formation of inactive adducts of glyoxylate with the competitor as illustrated in Eq. [3]. Thiols, especially β-amino thiols, are particularly effec-

TABLE 5

INHIBITION OF THE D-AA OXIDASE CATALYZED REACTION INVOLVING CG BY COMPOUNDS WHICH COMPETE WITH CYSTEAMINE FOR REACTION WITH GLYOXYLATE[a]

Competitor	Inhibition (%) with the competitor to cysteamine ratio equal to: 1:1	5:1
L-Cysteine	49	71
D,L-Penicillamine	42	71
Dithiothreitol	10	53
2,3-Dimercaptopropanol	25	73
β-Mercaptoethanol	9	44
Glutathione	8	13
Dihydrolipoic acid	10	40
H_2O_2	17	100
2,3-Diaminopropionate	14	46
1,3-Diaminopropane	0	0
Putrescine	0	0
Spermidine	0	0
Spermine	0	0

[a] The incubations, assays, and calculations of percent inhibition were done as described in the footnote to Table 4 except that the incubation solutions were prepared by adding glyoxylate as the final reagent, and they contained 3 m*M* EDTA when a thiol was present as a competitor. In controls where no cysteamine was added, but glyoxylate and competitor were present, no enzyme catalyzed O_2 uptake was observed except in the case where 1,3-diaminopropane is the competitor; in this case the specific activity with either 0.4 or 2.0 m*M* 1,3-diaminopropane is 11 mkat/kg. When thiols were used as competitors, some nonenzymatic O_2 uptake is observed due to autooxidation but this is minimized by having EDTA present. The enzymatic rates were calculated in those cases by subtracting the nonenzymatic rate from the observed rate with the enzyme present. In no case was the nonenzymatic autooxidation rate more than 10% of that observed with the enzyme present. In control experiments it was shown that 0.12 m*M* EDTA has no effect on the enzymatic reaction.

tive inhibitors by this mechanism as expected from earlier work on the stabilities of carbonyl adducts (*50*). Although the physiologically important polyamines (putresine, spermidine, and spermine) are ineffective as inhibitors a 1,2-diamine that can form a cyclic 5-membered adduct with glyoxylate is inhibitory if it has a carboxylate group as in 2,3-diaminopropionate; ethylene diamine itself forms a very good enzymic substrate on combination with glyoxylate (*14*). The inhibition by H_2O_2 is probably not caused by the formation of a stable adduct, but rather is most likely due to the removal of glyoxylate by oxidative decarboxylation to formate and CO_2, a reaction that H_2O_2 is known to carry out (the adduct is an intermediate in this process). By whatever mechanism, however, H_2O_2 leads to a

dramatic decrease in the rate of the D-AA oxidase reaction when it is present during the formation of the substrate CG.

If CG is formed from cysteamine and glyoxylate prior to the addition of the competitor, the compounds listed in Tables 4 and 5 do not cause as much inhibition, at least under one specific set of conditions. For example, the rate observed after 0.4 m*M* CG has been incubated with 5 to 6 m*M* acetaldehyde or D,L-penicillamine for 15 min prior to initiation by adding enzyme, is essentially the same as observed in the absence of the competitor. These systems have not been studied extensively enough to be able to conclude which results have a kinetic explanation (i.e., are dependent on the rates of formation and breakdown of the various adducts), and which are due to the differing thermodynamic stabilities of the adducts. One suspects that both are involved, and thus that the percent inhibition will depend on the time of incubation, how the reagents are mixed, etc. Nevertheless, the results given in Tables 4 and 5 do qualitatively illustrate that inhibition by this type of mechanism is possible, and they indicate what types of compounds do lead to such inhibition.

Although relatively high concentrations of the various reagents were incubated in order to obtain the results given in Tables 4 and 5, it is expected that similar results would have been obtained with lower concentrations if the time of incubation was lengthened. The formation of each adduct is presumably a second-order reaction, so the competitors should compete with each other to the same extent as long as the ratio of their concentrations remains the same. Lower concentrations were not used in this research because cysteamine and the other thiols slowly autooxidize, and this would be a greater complication if the incubation periods are longer. It is felt that the present protocol is a reasonable model for the physiological situation where various metabolites, drugs, and other enzymes would be constantly competing for a limited amount of both cysteamine and glyoxylate.

Effects of Oxygen Concentration

The observed K_m for O_2, obtained for various D-amino acid oxidase catalyzed reactions, is very dependent on the substrate employed, and has been found to range from 0.03 to 3 m*M* (*18, 19*). With saturating amounts of FAD (10 μM) and racemic CG (10 m*M*) present at 25°C in 20 m*M* sodium pyrophosphate buffer (pH 7.4), the K_m for O_2 was determined to be 1.1 m*M*. At the more physiological concentration of 0.4 m*M* CG, the apparent K_m for O_2 was found to be about 0.4 m*M*. Since both of these are higher than the concentration of O_2 in solution when it is air saturated (0.25 m*M*), or when it is less than air saturated as expected in most tissues, the rate of the D-AA oxidase reaction involving CG as substrate will be very sensitive to the O_2 concentration present in that tissue.

From the plots of the experimental data used to determine the K_m for O_2, one can obtain the maximum velocity that would be observed when the coenzyme and all of the substrates are present in saturating amounts. This was found to be 1.2 kat/kg, which is the highest maximum velocity ever recorded for a D-AA oxidase substrate at pH 7.4, the physiological pH. At any pH, it is exceeded only by that of its close structural analog, D-proline, which reacts with a maximum velocity of

2.0 kat/kg at 25°C and pH 8.5 (*19*). Such results emphasize the fact that CG is one of the very best substrates known for D-AA oxidase, and it is the best under physiological conditions (*14*).

DISCUSSION

As indicated by the foregoing results, the rate of the D-AA oxidase catalyzed reaction can be decreased by a large number of metabolites and drugs which affect the enzyme by several different mechanisms. In attempting to use this information to pinpoint what physiological processes might involve the enzyme, one must recognize that such evidence is only circumstantial, and the correlations could be due to coincidence. This is especially true if only one type of compound, which inhibits the enzyme by one specific mechanism, has a particular physiological effect. If, however, several unrelated types of compounds inhibit D-AA oxidase by distinctly different mechanisms, but are all known to have the same physiological effect, then it is less likely that the correlation could be due to coincidence. Also, if increasing the concentration of any one of the enzymic substrates (cysteamine, glyoxylate, or O_2) is known to have the opposite physiological effect to the inhibitors, then this adds credence to the correlations. Therefore, in the following discussion special emphasis will be given to cases in which several different effects all point in the same direction.

Since animal kidneys usually have the highest D-AA oxidase activity of any tissue, the inhibition by diuretics (Table 1) is of considerable interest. The mercurial compound, mersalyl, and the high ceiling diuretics, furosemide and ethacrynic acid, are especially good inhibitors, while the sulfonamides are less effective. Separation of the diuretics into those two particular groups is in itself an indication that the inhibition of D-AA oxidase may be involved in the diuretic response to at least the first group of compounds. There is considerable evidence that furosemide, ethacrynic acid, and the mercurials function in the kidney by a similar mechanism, while the response to the sulfonamides is somewhat different, and is partially due to the inhibition of carbonic anhydrase (*51, 52*). While ethacrynic acid and mersalyl presumably inhibit D-AA oxidase by reacting with a sulfhydryl group, furosemide apparently inhibits by a combination of substrate competitive (Table 3) and FAD competitive mechanisms; evidence for the latter is that considerably greater inhibition is seen with 0.1 m*M* inhibitor and 0.4 m*M* CG when the FAD concentration is 1.3 μM (Table 1) than when it is 10 μM (Table 3). As discussed above, the fact that these diuretics inhibit the enzyme by different mechanisms, but have the same physiological effect, makes it more likely that inhibition of D-AA oxidase could be involved in the diuretic response to these compounds. A further indication that this enzymic reaction may participate in the maintenance of water and ion balance is that cysteamine is a known antidiuretic under some physiological conditions (*53*). Because D-AA oxidase is present in peroxisomes, it is unreasonable to suggest that it is involved directly in the ion pumping mechanisms. Rather, it seems more likely that the product of the D-AA oxidase reaction is acting as a metabolic effector, and thus controlling the activity of the enzymes which actually do the pumping.

The known presence of D-AA oxidase in leukocytes (*5*) makes the inhibition by anti-inflammatory agents worthy of comment. It is generally accepted that inhibition of prostaglandin synthetase is at least partly responsible for the anti-inflammatory response caused by many such reagents (*54*), and several of these compounds, especially those listed in Table 1, certainly inhibit the synthetase at lower concentrations than are required to inhibit D-AA oxidase. Some known anti-inflammatory compounds, however, for example, salicylate and penicillamine, do not inhibit prostaglandin synthetase, so other mechanisms must also be involved. Since these latter compounds are potent inhibitors of the D-AA oxidase reaction involving CG (Tables 3 and 5), it seems possible that this enzymic reaction may also participate in the inflammatory response. This suggestion is substantiated by the observation that antimalarial compounds, which are known (*55*) good inhibitors (FAD competitive) of D-AA oxidase, also exhibit anti-inflammatory activity (*54*). Actually the inhibition of D-AA oxidase by many anti-inflammatory agents *in vivo* is expected to be even greater than the results given in the tables would indicate, because the enzyme will also be affected indirectly. Several anti-inflammatory agents are known to be very effective inhibitors of nicotinate phosphoribosyltransferase (*56*), so the intracellular concentration of nicotinate should increase when such agents are present. As shown in Table 3, nicotinate is a good inhibitor of D-AA oxidase.

Because animal brains and nervous tissue have relatively high concentrations of D-AA oxidase, the effects of various compounds which affect the transmission of nerve impulses is of interest. From the results obtained, it seems possible that at least some of the effects of the sedative hypnotics (*57*), anticonvulsants (*58*), local anesthetics (*59*), ethanol, and tropolone could be due to a modification of D-AA oxidase activity. The barbiturates, being very weak inhibitors (*60*, Table I), could not be operating by inhibiting the enzyme directly. They and other anticonvulsants, however, are known to be effective inhibitors of aldehyde reductase (*61*), and there is some evidence that the sedation caused by barbiturates is due to the increased concentrations of aldehydes which result from such an inhibition (*62*). These aldehydes, which are produced in a monoamine oxidase catalyzed reaction from various biogenic amines, should inhibit the D-AA oxidase reaction involving CG by the mechanism of Eq. [2] as found for several other aldehydes (Table 4). In fact, in the absence of the barbiturates one expects that the flow through D-AA oxidase is normally controlled, at least partially, not only by such aromatic aldehydes, but also by succinate semialdehyde (Table 4) which is a brain metabolite of γ-aminobutyrate, a known inhibitory neurotransmitter. Although not specifically tested in this research, chloral hydrate, which is another sedative hypnotic, should also be an effective inhibitor by the same mechanism. Consistent with the suggestion that anticonvulsants may be functioning by slowing down the D-AA oxidase reaction is the observation that a high concentration of any one of its presumed substrates, namely, cysteamine (*63*), glyoxylate (*64*), or O_2 (*65*), is known to cause convulsions.

Although lidocaine is only a weak inhibitor (Table 1), most other local anesthetics are esters of benzoic acid derivatives (*59*) which are expected to hydrolyze inside cells to produce potent D-AA oxidase inhibitors (Table 3). It has frequently

been proposed that many of the adverse effects of ethanol, on both the central nervous system and on the liver, are due to acetaldehyde (*66*), and that too is a good inhibitor (Table 4). The effects of tropolone on neural behavior have usually been interpreted as being due to the inhibition of catechol-*o*-methyltransferase, but it has been pointed out that other mechanisms must also be involved (*67*). Tropolone is just as effective an inhibitor of D-AA oxidase (Table 3) as it is of the transferase (*47*). In summary, although the evidence is not very conclusive at this stage, there are several hints that the D-AA oxidase reaction may be involved in the transmission of nerve impulses. Again, because of its presence in peroxisomes, D-AA oxidase could not participate directly as a receptor or as an ion pump, but presumably the product of its reaction could act to control the activity of enzymes that do carry out these functions.

Among the best substrate competitive inhibitors (Table 3) are several compounds, especially 5-methylpyrazole-3-carboxylate, salicylate, and nicotinate, which are known to be hypolipidemic and hypocholesterolemic agents in animals (*68, 69*). This suggests that the D-AA oxidase reaction may have a role in controlling lipid and cholesterol metabolism. Another compound which has been used clinically to lower blood lipid levels is clofibrate, whose hydrolysis product, 2(*p*-chlorophenoxy)-2-methylpropionic acid, is only a weak inhibitor (Table 1). Therefore it is unlikely that the effects of this drug on whole animals are due to inhibition of D-AA oxidase. There is, however, considerable evidence that clofibrate functions by a different mechanism from that of the other compounds.

An even more extensive role in controlling metabolism is indicated by correlations suggesting that the D-AA oxidase reaction may participate in the intracellular response to insulin, and possibly some other hormones. Numerous experiments have indicated that several of the characteristic effects of insulin are to (1) lower blood glucose (cause hypoglycemia) in animals, (ii) increase the rate of glycolysis in whole cell preparations, (iii) increase the rate of glucose transport through insulin sensitive membranes, and (iv) decrease the rate of lipolysis in adipose tissue. One or more of these same effects has been observed with each type of D-AA oxidase inhibitor considered in this research, and exactly the opposite effects have been found when the concentration of each of the three substrates has been increased.

(a) *Substrate competitive inhibitors.* Most of the compounds listed in Table 3 are reported to be hypoglycemic agents, especially in diabetic animals (*69–75*), several (*69, 76–79*) increase the rate of glycolysis in various tissues, salicylate and nicotinate accelerate the rate of glucose uptake in rat diaphragm muscle (*80*) and adipose tissues (*78, 79*), respectively, and many inhibit lipolysis (*69, 78, 79*).

(b) *FAD competitive inhibitors.* Adenosine nucleotides (*36*, Table 2), antimalarials (*55*), and 2-(*p*-chlorophenoxy)-2-methylpropionic acid (Table 1) inhibit by this mechanism, and are known to cause an insulinlike response in several types of cells (*78, 79, 81, 82*). Also, the intracellular concentration of ADP increases when muscles are contracted, or when cells are treated with uncoupling agents; under such conditions the rate of glycolysis is increased, the rate of glucose uptake is stimulated (*83, 84*), and lipolysis is inhibited (*79*).

(c) *Thiol specific reagents.* Although the effects of such reagents on whole cell

systems are complex, and are markedly dependent on concentrations, under some conditions they cause a stimulation of both glycolysis and glucose uptake, and an inhibition of lipolysis (*79, 83, 85–87*).

(d) *Competitors for reaction with cysteamine* (Eq. [2] and Table 4). Aldehydes formed from amines in a monoamine oxidase catalyzed reaction stimulate glucose oxidation (*88, 89*).

(e) *Competitors for reaction with glyoxylate.* (Eq. [3] and Table 5). Various thiols and H_2O_2 are known to cause insulinlike effects in several different systems (*83, 87, 90–92*).

(f) *O_2 concentration.* There is abundant evidence in the literature that anoxia or anaerobic conditions stimulates both glycolysis (*93*) and glucose uptake (*83, 84*), and inhibits lipolysis (*94*) in a manner very similar to that caused by insulin. The inhibition of glycolysis by high concentrations of O_2 has been known for a long time as the Pasteur effect (*95*).

(g) *Cysteamine concentration.* The results are complicated by the fact that the oxidation–reduction state of the system may change depending on whether the free thiol or disulfide is used; however, in several different systems cysteamine has been shown to have an antiinsulin effect (*96–99*).

(h) *Glyoxylate concentration.* Glyoxylate is a potent inhibitor of a number of cell processes, including glycolysis and glucose uptake (*100, 101*).

These results imply that the D-AA oxidase reaction is involved in an intracellular messenger system for insulin. It seems unlikely that the correlations could be due to coincidence when eight different methods for modifying the rate of this reaction all point in the same direction; they all suggest that insulin is somehow slowing down the D-AA oxidase reaction. Several mechanisms by which it might do this can be envisaged, but such a discussion is outside the scope of this article. Suffice it to say that insulin could not be reacting directly with the enzyme because it interacts with the plasma membrane while D-AA oxidase is present in peroxisomes.

If D-AA oxidase is participating in an intracellular messenger system for insulin, one suspects that it may also be involved in that capacity for other hormones and cell communicants (e.g., neurotransmitters) as well. For most hormones and neurotransmitters, insufficient data are available to make the types of correlations possible with insulin. The previous discussion, however, implied that the D-AA oxidase reaction may be involved in hormonally controlled ion and water balance in the kidney, and in the transmission of nerve impulses. Calcitonin is another hormone which may function by a mechanism involving D-AA oxidase because several substrate competitive inhibitors (Table 3) have hypocalcemic activity very similar to that of calcitonin (*102–104*).

Insulin is a growth factor, and seems closely related to a number of other growth factors, both structurally and in the metabolic responses that each elicits (*105–107*). If insulin functions by slowing down the D-AA oxidase reaction, then this implies that the product of the oxidase reaction is a general metabolic inhibitor which controls the growth of cells. Several observations which point in this direction include: all of cysteamine (*108–110*), glyoxylate (*101*), and O_2 (*65*) are toxic to cells; treatment of experimental animals with cysteamine leads to tumor

rejection (*111*); the activity of D-AA oxidase is markedly lower in neoplastic tissue than in normal tissue (*4, 112*); its activity is also lower in newborn animals than in older fully grown animals (*4*); both fetal and tumor tissue exhibit (*113*) high aerobic glycolysis (Warburg phenomenon), which is a characteristic also observed, as discussed above, when cells are treated with D-AA oxidase inhibitors. The indication that the D-AA oxidase reaction may be partially responsible for the inhibition of glycolysis by O_2 (Pasteur effect) implies that this enzyme is a particularly important "oxygen sensor" (*114*) in animals.

No attempt has been made here to consider how the D-AA oxidase product could function to cause the various effects implied by the above discussion; that will be the subject of future publications. Rather, in this article we have attempted to use the inhibition information to pinpoint what particular processes might be modified by the enzymic product, so that future efforts aimed at determining how it operates would be better focussed. It should be emphasized that all of the suggestions discussed here are based solely on circumstantial evidence, and thus they should be taken only as hypotheses until more direct evidence is obtained. Despite this caveat, the intriguing possibility remains that the D-AA oxidase reaction may play a major role in the control of metabolism in animals.

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