Human Liver Aldehyde Oxidase: Differential Inhibition of Oxidation of Charged and Uncharged Substrates *

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Summary. Human liver aldehyde oxidase (aldehyde: O_2 oxidoreductase, EC 1.2.3.1) has been purified 60-fold and some of its properties studied. Like aldehyde oxidase from other mammalian species, human liver aldehyde oxidase is an enzyme with dual substrate specificity, possessing the ability to catalyze not only the oxidation of aldehydes to the corresponding carboxylic acids, but also the hydroxylation of a number of nonaldehydic heterocyclic compounds; its relative activity towards the latter group of substrates is low, however, when compared with that of liver aldehyde oxidase from rabbit and guinea pig. When the aromatic aldehyde benzaldehyde is used as substrate, human liver aldehyde oxidase, like the rabbit enzyme, is strongly inhibited by menadione, estradiol-17β, antimycin A, Triton X-100, and N-alkylphenothiazines; the human enzyme differs from the rabbit enzyme, however, in being relatively insensitive to oligomycin and Amytal. Like the rabbit enzyme, the human enzyme can catalyze the 3-hydroxylation of phenazine methosulfate (PMS) and the 6-hydroxylation of N-methylnicotinamide (NMN). With the rabbit enzyme, however, the aerobic hydroxylation of these substrates proceeds by a conventional mechanism, while with the human enzyme, the aerobic hydroxylation of PMS and NMN is anomalous in that the reaction is inhibited only by agents with affinity for the substrate-binding site, such as cyanide and N-alkylphenothiazines, and not by agents which inhibit the "internal electron transport chain" of the enzyme, such as menadione and diethylstilbestrol. This mode of oxidation appears to be unique to substrates with a positively charged quaternary nitrogen; the hydroxylation of other nonaldehydic heterocyclic substrates for the human enzyme is sensitive to conventional aldehyde oxidase inhibitors.

Introduction

The enzyme hepatic aldehyde oxidase (aldehyde: O_2 oxidoreductase, EC 1.2.3.1) was first described by Gordon, Green, and Subrahmanyan (1). They demonstrated that the enzyme was a flavoprotein distinct from the rather similar enzyme xanthine oxidase (xanthine: O_2 oxidoreduc-

tase, EC 1.2.3.2); like xanthine oxidase, however, it could catalyze the oxidation of a variety of aliphatic and aromatic aldehydes. The source of enzyme for these early studies was hog liver. In 1946, Knox (2) demonstrated that aldehyde oxidase from rabbit liver possessed the ability to catalyze not only the oxidation of aldehydes, but also the hydroxylation of a number of nonaldehydic heterocyclic compounds, e.g., N-methylnicotinamide and quinine. He noted also that the ability of the rabbit liver enzyme to catalyze the oxidation of the latter group of substrates was much greater than that of aldehyde oxidase from other mammalian species. Mahler, Mackler,

Green, and Bock (3) further purified the hog liver enzyme, and demonstrated the presence of molybdenum and iron. Hurwitz (4) demonstrated the ability of rabbit liver aldehyde oxidase to catalyze the oxidation of pyridoxal, and described a number of properties of the enzyme, in particular, its inhibition by cyanide and p-hydroxymercuribenzoate. Rajagopalan, Fridovich, and Handler (5), and Rajagopalan and Handler (6, 7) have published a detailed and comprehensive study of the rabbit liver enzyme; they prepared rabbit liver aldehyde oxidase of greater than 90% purity and demonstrated the presence of coenzyme Q_{10} in the purified enzyme.

Our attention was directed to this enzyme by our finding that, in several species, it was the previously unidentified catalytic agent responsible for the 7-hydroxylation (8) and consequent biological inactivation (9) of the anti-tumor agent 3',5'-dichloromethotrexate, a 6-substituted 2,4-diaminopteridine (10). We subsequently noted that the rabbit liver enzyme could catalyze also the oxidation of several pyrimidines (11) and purines (12) of pharmacologic interest. Since the rabbit liver enzyme has much greater catalytic activity towards nonaldehydic heterocyclic compounds than does aldehyde oxidase from other species, it appeared that information on the properties of human liver aldehyde oxidase was necessary before these findings could be extended to man. It was found, however, that the human enzyme had not been described, despite the possible significance of this enzyme both in the oxidation of aldehydes arising in the course of intermediary metabolism (particularly in the metabolism of ethanol and its congeners) and in the hydroxylation of pharmacologically active heterocyclic compounds. Studies concerned particularly with the sensitivity to inhibitors and with the substrate specificity of human liver aldehyde oxidase were therefore carried out, and are described below.

Methods

Gifts of the following compounds are gratefully acknowledged: trifluoperazine, trimeprazine, chlorpromazine (CPZ), and N-methylphenothiazine from Dr. E. J. Van Loon, Smith, Kline & French Laboratories, Philadelphia, Pa.; promethazine and promazine from Dr. F. E. Hamerslag, Wyeth Laboratories, Philadelphia, Pa.; 3',5'-dichloromethotrexate (DCM) from Dr. J. M. Rueg-segger, Lederle Laboratories Div., American Cyanamid Co., Pearl River, N. Y.; 2-hydroxy-5-fluoropyrimidine from Dr. Z. Buděšínský of the Research Institute of Pharmacy and Biochemistry, Prague; 4-hydroxypyrazolo [3,4-d]pyrimidine from Dr. G. B. Elion, Burroughs Wellcome & Co., Tuckahoe, N. Y.; and disulfiram from Ayerst Laboratories, New York, N. Y. 2,6-Dichlorophenolindophenol (DCIP), α,α-dipyridyl, and DEAE-cellulose were obtained from the Eastman Kodak Co., Rochester, N. Y.; phenazine methosulfate (PMS), N-methylnicotinamide (NMN), diethylstilbestrol, coenzyme Q_{10}, progesterone, and p-chloromercuribenzoate from Calbiochem, Los Angeles, Calif.; oligomycin, flavin adenine dinucleotide (FAD), flavin adenine mononucleotide (FMN), quinacrine, and ammonium sulfate (enzyme grade) from Mann Research Laboratories, Inc., New York, N. Y.; estradiol-17β from California Foundation for Biochemical Research, Los Angeles, Calif.; benzaldehyde, acetaldehyde, propionaldehyde, methanol, potassium cyanide, ammonium chloride, and Versene Fe-3 from Fisher Scientific Company, Pittsburgh, Pa.; Amytal and diethyldithiocarbamic acid from K & K Laboratories, Inc., Plainview, N. Y.; o-chlorobenzaldehyde from Paragon Testing Laboratories, Orange, N. J.; o-nitrobenzaldehyde from Matheson, Coleman, & Bell, E. Rutherford, N. J.; menadione and pyridoxal hydrochloride from Nutritional Biochemicals Corporation, Cleveland, Ohio; antimycin A, nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide reduced (NADH), nicotinamide adenine dinucleotide phosphate (NADP), nicotinamide adenine dinucleotide phosphate reduced (NADPH), from Sigma Chemical Co., St. Louis, Mo.; Triton X-100 from Rohm & Haas Co., Philadelphia, Pa.; and frozen mature rabbit liver from Pel-Freez Biologicals, Inc., Rogers, Arkansas.

Enzyme assays. Spectrophotometric assay methods were employed throughout. For assays at a single wavelength, a Gilford multiple sample absorbance recorder with a Beckman DU monochromator was used; a Cary 15 recording spectrophotometer equipped with a 0–0.1 OD and 0–1.0 OD slide wire was used for serial assays requiring complete ultraviolet absorption spectra.

(*a*) *Benzaldehyde as substrate.* Examination of the ultraviolet absorption spectra of benzaldehyde and of benzoate at equimolar concentrations at pH 7.8 revealed that the latter compound lacked the strong absorption maximum of benzaldehyde at 249 mμ (Σ max $= 17,540$) (Fig. 1). It therefore appeared possible that aldehyde oxidase activity could be assayed by monitoring the decrease in absorption at 249 mμ consequent upon the oxidation of benzaldehyde to benzoate. Serial spectra of the enzymic conversion of benzaldehyde as catalyzed by the rabbit enzyme are shown in Fig. 2; details of the assay are given in the legend for the figure. In adapting the assay to the Gilford multiple sample absorbance recorder, the 0–0.250 OD scale was used, and a cuvette with all constituents of the reaction except benzaldehyde used as a blank. Sample cuvettes contained, in addition, 5.6×10^{-5} M benzaldehyde. Because of the high protein levels required in the blank and sample cuvettes for
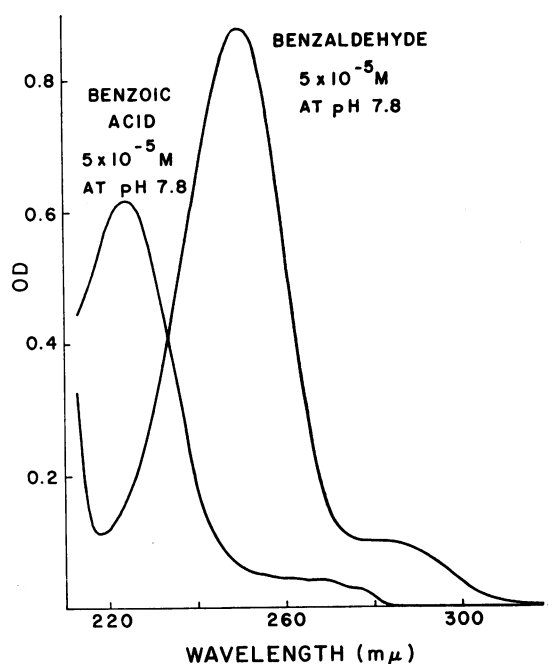
FIG. 1. ULTRAVIOLET ABSORPTION SPECTRA OF BENZAL-
DEHYDE AND BENZOIC ACID IN SODIUM PHOSPHATE BUFFER
AT pH 7.8.

measurable activity at early stages of the purification of
the human enzyme, proper adjustment of the deuterium
lamp mirror for maximum light intensity at 249 mμ
was found to be essential. The assay could be performed
successfully with two randomly selected monochromators
requiring slit widths of 0.35 and 0.42 mm, respectively,
when blanked against distilled water at 249 mμ; the
assay was not practicable with a third monochromator
requiring a slit width of 1.02 mm when blanked under
the same conditions. Rates were measured over a period
of 2 min; detailed assay conditions are described in later
sections.

(b) *PMS as substrate.* The spectra of PMS and its
oxidation product, methylphenazine-3-one, have been
published by Rajagopalan and Handler (13). In the
routine assay used here, the sample cuvettes contained
PMS, 0.5 μmole, sodium phosphate buffer, pH 7.8, 100
μmoles, Versene Fe-3, 15 μg, ammonium chloride, 100
μmoles, and enzyme solution, containing varying amounts
of protein, 0.1 ml; total volume was 1 ml. The increase
in absorption at 520 mμ consequent on the oxidation of
PMS was monitored at 23°C over a period of 4 min
with the Gilford multiple sample absorbance recorder;
controls without substrate and without enzyme were in-
cluded in all assays. A molar extinction coefficient of
11,700 for methylphenazine-3-one at 520 mμ was as-
sumed (13). Fresh PMS solutions were prepared
hourly, as accumulation of enzyme-inhibitory PMS oxida-
tion products was noted in PMS stock solutions exposed
to air.

(c) *Dichlorophenolindophenol (DCIP) as electron ac-
ceptor.* The enzyme assays described above were per-
formed aerobically, with molecular oxygen as electron
acceptor; reaction rates were determined from disap-
pearance of substrate, in the case of benzaldehyde, or
appearance of product, in the case of PMS. With ali-
phatic aldehyde substrates, however, conversion of sub-
strate to product was not accompanied by measurable
spectral changes. In these instances, DCIP, rather than
oxygen, was used as electron acceptor, and the rate of
reaction followed by measuring the rate of reduction of
this compound at 600 mμ. In these assays, 0.25 ml of a
DCIP solution of 10 mg/100 ml was added to the cuvette;
sodium phosphate, Versene Fe-3, and ammonium chloride
concentrations were the same as in the PMS assay.
Rates were measured over a period of 4 min at 23°C,
and a molar extinction coefficient of 19,100 was assumed
for DCIP (14).

(d) *Slowly oxidized substrates.* The enzymic conver-
sions of 2-hydroxy-5-fluoropyrimidine to 5-fluorouracil
(11), DCM to 7-hydroxyDCM, and NMN to NMN-6-
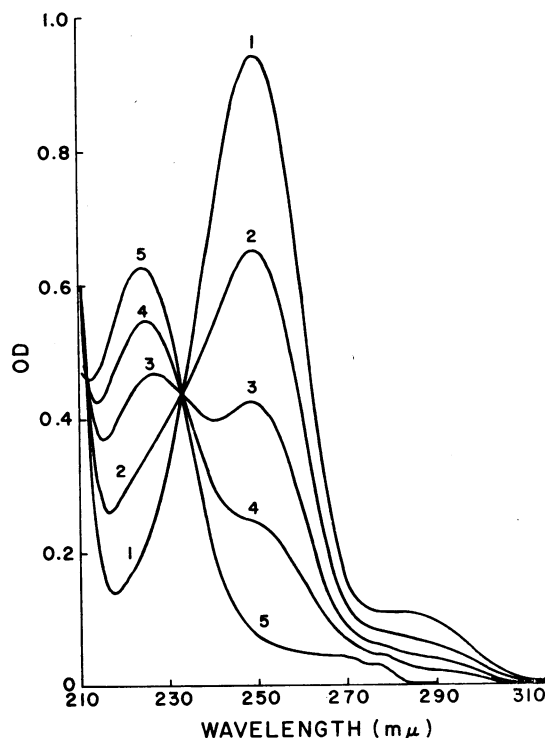pyridone were followed by serial ultraviolet absorption

FIG. 2. ENZYMIC CONVERSION OF BENZALDEHYDE TO
BENZOATE BY RABBIT LIVER ALDEHYDE OXIDASE. The refer-
ence cuvette contained sodium phosphate buffer, pH 7.8,
500 μmoles, Versene Fe-3, 20 μg, and rabbit liver alde-
hyde oxidase, 45 μg, in a total volume of 3 ml. The
sample cuvette contained the same constituents, plus benz-
aldehyde, 0.17 μmole. Spectra were recorded at 4-min
intervals.

spectra, with the Cary 15 recording spectrophotometer. Reference cuvettes contained sodium phosphate buffer, pH 7.8, 500 μmoles (for DCM, buffer of pH 6.9 was used), Versene Fe-3, 20 μg, ammonium chloride, 600 μmoles, and enzyme, in a total volume of 3 ml. The sample cuvette contained the same constituents plus substrate. Since the rate of hydroxylation of these three substrates was slow when the human enzyme was used, maximum possible amounts of enzyme protein were added to reference and sample cuvettes, compatible with accurate recording of spectra of substrate and product.

Partial purification of rabbit liver aldehyde oxidase. Aldehyde oxidase was purified approximately 90-fold from rabbit liver by ammonium sulfate fractionation and chromatography on Sephadex G-75 and DEAE-cellulose, as previously described (10). Enzyme activity was assayed with DCM as substrate, and protein was determined by the method of Waddell (15).

Partial purification of human liver aldehyde oxidase. It early became apparent that fresh human liver biopsy material would yield insufficient enzyme for comprehensive studies. Previous studies with the rabbit, mouse, guinea pig, and monkey enzymes indicated, however, that aldehyde oxidase was recoverable from the livers of these species with no detectable alteration in yield or in properties, as long as 24 hr after death, provided that the livers were stored cold (but not frozen) during this interval. For partial purification of the human enzyme, therefore, postmortem human livers were obtained at time of autopsy, within 4 hr after death, with the cooperation of the Department of Pathology, Yale University School of Medicine. Livers from patients with a history of hepatic disease, and livers with gross pathological changes, were excluded; otherwise no selection was made. A total of nine livers was used in this study, from three female and six male subjects ranging in age from 4 to 71 yr. No marked variation in enzyme activity was noted among livers from which enzyme was prepared on the day of collection; two livers which were frozen after collection and used for enzyme preparation some weeks later were found to have much lower enzyme activity. A similar observation of low yield of aldehyde oxidase from horse liver after freezing and thawing was reported by Carpenter (16).

Some studies were repeated with enzyme from fresh wedge biopsy material obtained at laparotomy; no differences were noted from enzyme obtained from necropsy liver.

Livers were homogenized for 1 min in 2.5 volumes of 0.05 M sodium phosphate buffer, pH 7.8, containing Versene Fe-3, 0.005%, the homogenate contrifuged at 14,600 g at 4°C for 30 min, and an aliquot of the supernatant fraction removed for determination of enzyme activity. The supernatant was then heated with continuous stirring for 10 min at 60°C in a water bath. Heat-precipitated protein was removed by centrifuging at 14,600 g for 30 min, the supernatant fraction collected, and sufficient saturated ammoniacal ammonium sulfate solution added to give 45% saturation; saturated ammoniacal ammonium

sulfate solution was prepared by adding 60 ml of concentrated (29%) ammonium hydroxide solution to 940 ml of saturated ammonium sulfate solution. The supernatant solution was allowed to stir slowly at room temperature for 30 min, and the precipitate recovered by centrifuging as above. The precipitate was extracted with sufficient ammoniacal ammonium sulfate solution of 30% saturation to give a protein concentration in the supernatant of approximately 25 mg/ml (in a typical preparation, this required 120 ml of ammonium sulfate solution when liver of starting weight 1300 g was used). The supernatant was recovered by centrifugation, and the precipitate subjected to successive reextractions with ammoniacal ammonium sulfate of 15% saturation, and with distilled water. Typically, most of the aldehyde oxidase activity was present in the 15–30% ammonium sulfate fraction, although activity was usually detectable in the 0–15% ammonium sulfate fraction; if the specific activity of the latter approached that of the 15–30% fraction, the two fractions were combined before the next step, which otherwise was performed on the 15–30% ammonium sulfate fraction alone.

In earlier runs, salts and diffusible low molecular weight compounds were removed at this stage of the purification by overnight dialysis of the 15–30% ammonium sulfate fraction against distilled water at 4°C; it was later found, however, that an alternate procedure, desalting by gel filtration through Sephadex G-75, resulted in less loss of enzyme activity then did overnight dialysis, possibly because gel filtration could be carried out much more rapidly. In a typical run, enzyme solution containing 320 mg of protein was applied to a Sephadex G-75 column, 2 × 18 cm, previously washed for several hours with distilled water. The protein solution obtained from the Sephadex column was then applied to a column of DEAE-cellulose, which had been preequilibrated with distilled water, and eluted stepwise with Trischloride buffers, pH 7.5, of concentrations 0.05, 0.15, 0.25, and 0.50 mole/liter. In a representative run, 280 mg of protein was applied to a DEAE-cellulose column of 2 × 18 cm; the enzyme activity typically appeared in the 0.25 M Tris effluent, although activity was often detectable in the 0.15 M Tris effluent also.

The 0.25 M Tris effluent from several such columns was combined and concentrated osmotically to 0.1 volume by applying powdered sucrose to the external surface of a dialysis bag containing the protein solution. Protein was precipitated again from the concentrated Tris effluent by adding saturated ammoniacal ammonium sulfate solution until 45% saturation was reached. The precipitated protein was redissolved in distilled water to give a solution of approximately 5 mg/ml. The purification procedure for the human enzyme is summarized in Table I.

For determination of the specific activity of the fractions obtained during purification, the benzaldehyde assay described above was used. Before assay, aliquots from the early fractions were subjected to recentrifugation at 105,000 g for 1 hr in a Spinco model L centrifuge;

TABLE I

Partial purification of human liver aldehyde oxidase

Fraction	Total volume	Total protein	Total activity	Specific activity	Yield	Purification
	ml	*mg*	*units**	*units/mg*	*%*	*-fold*
Supernatant from crude homogenate	4000	260,000	3900	0.015		
Supernatant from crude homogenate after heat treatment (60°C for 10 min)	3550	124,000	3100	0.025	79	1.7
Ammonium sulfate (15–30%)	140	5,379	1824	0.339	47	23
DEAE-cellulose (precipitated with ammonium sulfate and redissolved)	262	1,362	1248	0.915	32	61

* One unit = amount of enzyme producing $\Delta 249$ mμ of 1 optical density unit per 10 min at 23°C with benzaldehyde, 5.6×10^{-5} mole per liter, as substrate. In all assays, activity was determined from initial rates.

assays were carried out on the supernatant.[1] The other assay methods described above, although of value with the partially purified enzyme, were not useful for determining over-all fold purification, since they were not applicable to the determination of aldehyde oxidase activity in supernatants from crude homogenates: the DCIP assay gave a high blank rate in the absence of substrate,[2] while the PMS assay gave a spuriously high rate with crude homogenates, due possibly to the presence of one or more enzymes, in addition to aldehyde oxidase, with PMS oxidase activity. This additional PMS oxidase activity could not be inhibited by cyanide, quinacrine, or methanol, and therefore was not attributable to alde-

[1] Rajagopalan, Fridovich, and Handler (5) have shown rabbit liver aldehyde oxidase to be a supernatant enzyme; the hog liver enzyme appears to be mitochondrial, but is readily released into the supernatant fraction during homogenization (17).

[2] A similar observation was reported by Palmer, with the hog liver enzyme (18).

hyde oxidase (5). The benzaldehyde oxidase activity of the crude fractions, on the other hand, was completely inhibited by cyanide (1×10^{-4} mole per liter), diethylstilbestrol (4×10^{-5} mole per liter), and menadione (2.5×10^{-5} mole per liter), and is therefore tentatively attributable to aldehyde oxidase; the validity of these assumptions is discussed in a later section.

The rapid assay methods used in the purification of the rabbit liver enzyme by Rajagopalan, Fridovich, and Handler (5) (the conversion of NMN to its 6-pyridone) and by the present author (10) (the conversion of DCM to 7-hydroxyDCM) were of no value in determining fold purification of the human enzyme: the rates of oxidation were too low to be measured in crude fractions, and the conversion of these substrates could be demonstrated spectrophotometrically only with partially purified human liver aldehyde oxidase.

Most of the studies described below were carried out both with human liver aldehyde oxidase purified 20- to 30-fold (the "15–30% ammonium sulfate enzyme") and

TABLE II

*Effect of inhibitors on the conversion of benzaldehyde to benzoate by human liver aldehyde oxidase**

	Inhibitor concentration	Inhibition
		%
α,α-Dipyridyl	1×10^{-4} mole per liter	53
Chlorpromazine	5×10^{-6} mole per liter	51
Diethylstilbestrol	4×10^{-7} mole per liter	43‡
Progesterone	6×10^{-5} mole per liter	71§
p-Hydroxymercuribenzoate	2×10^{-5} mole per liter	88
Oligomycin	60 μg/ml	28
Quinacrine	5×10^{-5} mole per liter	51
Estradiol-17β	8×10^{-8} mole per liter	50‡
Methanol	0.3 mole per liter	67
Potassium cyanide	1×10^{-4} mole per liter	66
Antimycin A	8 μg/ml	46‡
Triton X-100	8×10^{-4} %	65
Menadione	1.5×10^{-5} mole per liter	55
Testosterone	6×10^{-5} mole per liter	44

* Sample cuvettes contained benzaldehyde, 0.057 μmole, sodium phosphate buffer, pH 7.8, 100 μmoles, Versene Fe-3, 15 μg, ammonium chloride, 100 μmoles, and enzyme solution, 0.03 ml, in a total volume of 1 ml. Initial rates were measured, except where otherwise noted.
‡ At the end of 5 min.
§ At the end of 3 min.

40- to 60-fold (the "DEAE-cellulose enzyme"). No significant differences were noted in the human enzyme at these two stages of purification.

Results

Inhibitors of benzaldehyde oxidation

Benzaldehyde (5.6×10^{-5} mole per liter) was used as substrate, and enzyme activity monitored at 23°C by measuring the rate of conversion to benzoate at 249 mμ (see above).

(a) *Steroids as inhibitors.* As shown in Table II, the oxidation of benzaldehyde to benzoate by human liver aldehyde oxidase was inhibited by a variety of agents: the inhibition by the steroid estradiol-17β and the nonsteroidal estrogen diethylstilbestrol was particularly marked (Fig. 3). Estradiol-17β is the most potent inhibitor of human liver aldehyde oxidase found in the present studies; 50% inhibition of the rate of oxidation was noted at an estradiol concentration of 8×10^{-8} mole per liter, when control and inhibited rates were measured 5 min after the start of the reaction. The slow development of maximal inhibition at low estradiol concentrations is apparently due to a slow rate of binding of the steroid to the enzyme: when estradiol (8×10^{-8} mole per liter)

FIG. 4. STRUCTURAL FORMULAS OF N-METHYLPHENAZINE AND CHLORPROMAZINE.

and enzyme were preincubated for 5 min before the reaction was started by adding substrate, maximal inhibition was noted at zero time. The situation thus differs from that seen with antimycin A, where preincubation did not shorten the period required for maximal inhibition to develop (see below).

The nonphenolic steroids progesterone and testosterone were much weaker inhibitors of the enzyme.

(b) *N-alkylphenothiazines as inhibitors.* The possible activity of N-alkylphenothiazines as inhibitors of human liver aldehyde oxidase was suggested by the resemblance of the phenothiazine structure to that of the known aldehyde oxidase substrate PMS (N-methylphenazine) (Fig. 4). The N-alkylphenothiazines studied here are inactive as substrates for the enzyme; this inactivity appears to be due, not to the substitution of sulfur for one of the ring nitrogens, but to the bulky N-alkyl side-chains of the phenothiazines tested: N-methylphenothiazine, unlike the other members of this group, but like N-methylphenazine (PMS), possesses substrate activity for the rabbit liver enzyme.[3]

The N-alkylphenothiazines are more potent inhibitors of the human enzyme than of the rabbit enzyme, irrespective of whether benzaldehyde or PMS is used as a substrate (Tables III and IV): with benzaldehyde (5.7×10^{-5} mole per liter) as
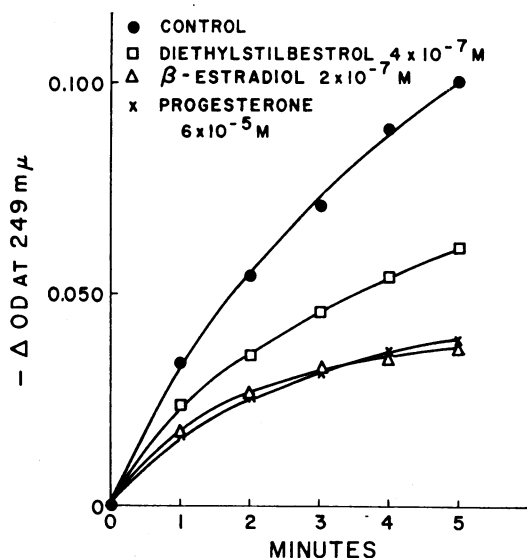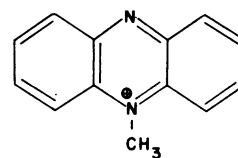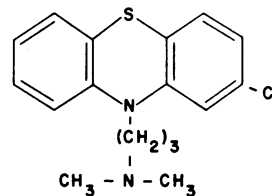

FIG. 3. INHIBITION OF HUMAN LIVER ALDEHYDE OXIDASE BY ESTROGENS AND PROGESTERONE. The rate of oxidation of benzaldehyde (5.6×10^{-5} mole per liter) was followed by the change in optical density at 249 mμ; reaction conditions were identical to those given in Table VII.

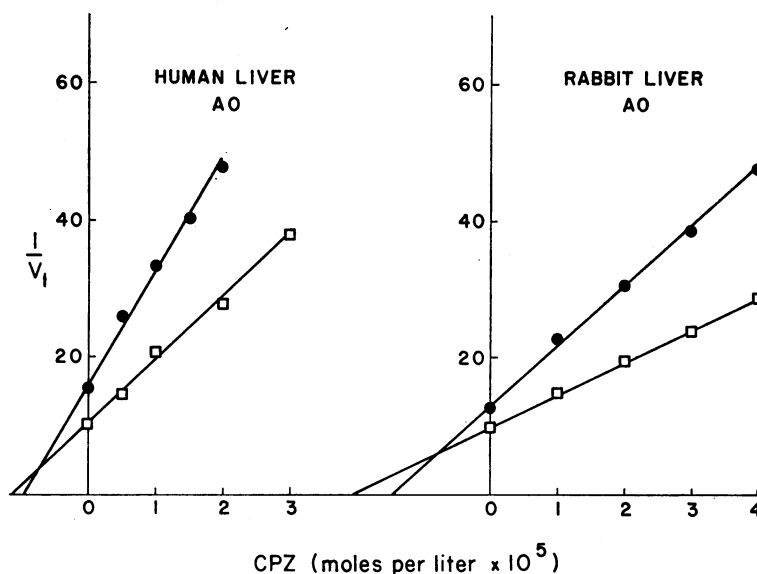[3] G. S. Farnham and D. G. Johns. Unpublished data.

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FIG. 5. COMPETITIVE INHIBITION BY CHLORPROMAZINE OF OXIDATION OF
PMS (5×10^{-4} MOLE PER LITER) BY ALDEHYDE OXIDASE FROM HUMAN LIVER
AND RABBIT LIVER (DIXON PLOT). Reaction conditions were identical to those
given in Table IV. *AO*, aldehyde oxidase; *CPZ*, chlorpromazine.

substrate, the level of chlorpromazine (CPZ) re-
quired for 50% inhibition was 2×10^{-5} mole per
liter in the case of the rabbit enzyme, but 5×10^{-6}
mole per liter in the case of the human enzyme.
Both with the rabbit and with the human enzyme,
CPZ is formally competitive with substrate (Fig.
5).

(c) *Inhibitors of electron transport.* Antimycin
A, a compound active in mitochondrial systems as
an inhibitor at a site subsequent to the reduction

of coenzyme Q (19), was effective as an inhibitor
of benzaldehyde oxidation by human liver alde-
hyde oxidase (Fig. 6; Table V); the level re-
quired was slightly higher than that required to
give equivalent inhibition of the rabbit liver en-
zyme. At low levels of antimycin A, maximal in-
hibition was not seen until several minutes after
the start of the reaction; the rate of onset of inhibi-
tion was not increased by preincubation. As with
the rabbit enzyme (6), antimycin A may, there-

TABLE III

*N-Alkylphenothiazines as inhibitors of the conversion of
benzaldehyde to benzoate by aldehyde oxidase from human
liver and rabbit liver**

Inhibitor‡	Inhibition	
	Human enzyme	Rabbit enzyme
	%	%
Promethazine	32	53
Promazine	41	63
Trimeprazine	49	85
Chlorpromazine	51	50
Trifluoperazine	72	63

* Control cuvettes contained benzaldehyde, 0.057
μmole, sodium phosphate buffer, pH 7.8, 100 μmoles,
Versene Fe-3, 15 μg, ammonium chloride, 100 μmoles, and
enzyme solution, 0.03 ml, in a total volume of 1 ml.
‡ Inhibitor concentrations were 5×10^{-6} mole per liter
for assays with human liver aldehyde oxidase, and 2×10^{-5}
mole per liter for assays with rabbit liver aldehyde oxidase.

TABLE IV

*N-Alkylphenothiazines as inhibitors of the 3-hydroxylation
of PMS by aldehyde oxidase from human liver
and rabbit liver**

Inhibitor‡	Inhibition	
	Human enzyme	Rabbit enzyme
	%	%
Trifluoperazine	30	70
Promazine	43	79
Promethazine	44	62
Chlorpromazine	45	48
Trimeprazine	60	88

* Control cuvettes contained PMS, 0.5 μmole, sodium
phosphate buffer, pH 7.8, 100 μmoles, Versene Fe-3,
15 μg, ammonium chloride, 100 μmoles, and enzyme solu-
tion, 0.1 ml, in a total volume of 1 ml.
‡ Inhibitor concentrations were 5×10^{-6} mole per liter
for assays with human liver aldehyde oxidase, and 2×10^{-5}
mole per liter for assays with rabbit liver aldehyde oxidase.

TABLE V

Electron transfer inhibitors: relative effectiveness as inhibitors of aldehyde oxidase from human liver and rabbit liver

Enzyme source	Substrate	Concentration of inhibitor required to reduce reaction rate to 50% of control rate		
		Antimycin A	Oligomycin	Amytal
		μg/ml	*μg/ml*	*moles/liter*
Rabbit liver	Benzaldehyde*	2	0.4	3×10^{-4}
Human liver	Benzaldehyde*	8	>60‡	$>3 \times 10^{-4}$§
Rabbit liver	PMS‖	2	0.4	5×10^{-4}
Human liver	PMS‖	>40	>60	$>1 \times 10^{-3}$

* Control cuvettes contained benzaldehyde, 0.057 μmole, sodium phosphate buffer, pH 7.8, 100 μmoles, Versene Fe-3, 15 μg, ammonium chloride, 100 μmoles, and enzyme solution, 0.03 ml, in a total volume of 1 ml.
‡ Slight inhibition was noted at this concentration (see Table II).
§ Higher Amytal concentrations could not be used because of the absorption of this compound at the wavelength used for the assay (249 mμ).
‖ Sample cuvettes contained PMS, 0.5 μmole, sodium phosphate buffer, pH 7.8, 100 μmoles, Versene Fe-3, 15 μg, ammonium chloride, 100 μmoles, and enzyme solution, 0.1 ml, in a total volume of 1 ml.

fore, react with a reduced form of the human enzyme.

By contrast, oligomycin and Amytal, both effective inhibitors of the rabbit liver enzyme (6), were ineffective as inhibitors of human liver aldehyde oxidase (Table V). Slight inhibition of the human enzyme was noted when the oligomycin concentration was raised to 60 μg/ml; this concentration, however, is of a different order of magnitude from that required to inhibit the oxidation of benzaldehyde by the rabbit liver enzyme under identical conditions (0.4 μg of oligomycin per ml) (Table V).

(*d*) *Other inhibitors.* Cyanide, quinacrine, methanol, *p*-hydroxymercuribenzoate, menadione, and Triton X-100 were effective inhibitors of hu-

man liver aldehyde oxidase at levels comparable to those reported for the rabbit liver enzyme (6) (Table II). The iron-chelating agent, α,α-dipyridyl, was an effective inhibitor of the oxidation of benzaldehyde by the human enzyme at a concentration of 1×10^{-4} mole per liter. 4-Hydroxypyrazolo[3,4-*d*]pyrimidine (3×10^{-4} mole per liter), a highly potent inhibitor of human liver xanthine oxidase (20), was ineffective as an inhibitor of human liver aldehyde oxidase.

(*e*) *Coenzymes.* NAD, NADH, NADP, and NADPH had no effect on the rate of oxidation of benzaldehyde by the 40- to 60-fold purified human enzyme. Some oxidation of NADH to NAD was noted in crude fractions; further investigation showed this to be due to the presence of alcohol dehydrogenase, catalyzing the reduction of benzaldehyde to benzyl alcohol. DEAE-cellulose
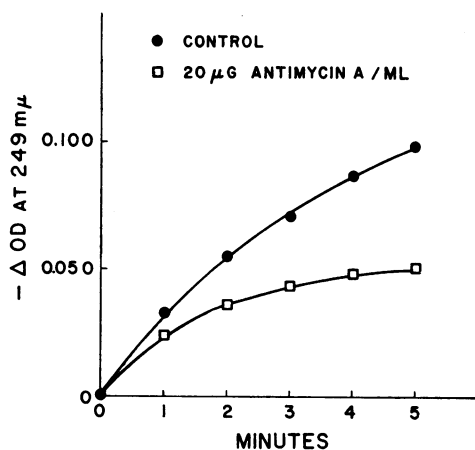

FIG. 6. INHIBITION OF HUMAN LIVER ALDEHYDE OXIDASE BY ANTIMYCIN A. Substrate: benzaldehyde, 5.6×10^{-5} mole per liter.

TABLE VI

*Aldehydes as substrates for human liver aldehyde oxidase**

	Initial reaction velocity (Benzaldehyde = 100)
Benzaldehyde	100
Propionaldehyde	97
Acetaldehyde	73
Pyridoxal	25
o-Chlorobenzaldehyde	18
o-Nitrobenzaldehyde	<5
Formaldehyde	<5

* Sample cuvettes contained substrate, 2 μmoles, DCIP, 25 μg, sodium phosphate buffer, pH 7.8, 100 μmoles, Versene Fe-3, 15 μg, ammonium chloride, 100 μmoles, and enzyme solution, 0.1 ml, in a total volume of 1 ml. Reaction rates were determined by monitoring the rate of reduction of DCIP, at 600 mμ.
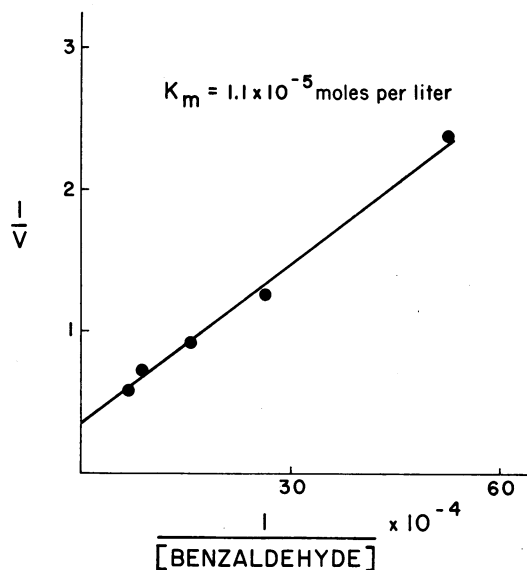
FIG. 7. OXIDATION OF BENZALDEHYDE BY HUMAN LIVER ALDEHYDE OXIDASE (LINEWEAVER-BURK PLOT). Sample cuvettes contained sodium phosphate buffer, pH 7.8, 100 μmoles, Versene Fe-3, 15 μg, ammonium chloride, 100 μmoles, enzyme solution, 0.02 ml, and substrate as indicated, in a total volume of 1 ml. The rate of oxidation of benzaldehyde was followed by the change in optical density at 249 mμ.

chromatography separated all alcohol dehydrogenase activity from the aldehyde oxidase preparation.

Added FAD, FMN, and coenzyme Q_{10} did not affect the rate of oxidation of benzaldehyde by the human enzymye.

Other aldehyde substrates

A number of aliphatic and aromatic aldehydes served as substrates for human liver aldehyde oxidase; in no instance, however, did the maximal reaction velocity exceed that of benzaldehyde (Table VI). The apparent K_m of benzaldehyde for the human enzyme, as determined by the Lineweaver-Burk method, was extremely low for this enzyme (1.1×10^{-5} mole per liter) (Fig. 7); for other substrates, Michaelis constants ranged from 5×10^{-5} to 5×10^{-3} mole per liter. Formaldehyde, unlike the other aliphatic aldehydes tested, was an extremely poor substrate for human liver aldehyde oxidase; a similar observation was reported by Palmer (18) for the hog liver enzyme. Early workers (1), using the hog liver enzyme,

suggested that the low substrate activity with formaldehyde might be due to denaturation of the enzyme by this compound; this view would not appear to be tenable with human liver aldehyde oxidase, since the oxidation of the substrate PMS by the human enzyme was not significantly inhibited by formaldehyde (6×10^{-3} mole per liter), although marked inhibition, as a consequence of competition for the substrate-binding site, was noted in the presence of other aliphatic aldehydes (see below).

Inhibitors of PMS oxidation by human liver aldehyde oxidase

It early became apparent from inhibition studies that the mode of PMS oxidation by human liver aldehyde oxidase differed in some important respects from the mode of oxidation of benzaldehyde. Parallel control studies with the rabbit liver enzyme, on the other hand, showed benzaldehyde and PMS to be handled similarly by the latter enzyme.

(a) *Inhibitors equally effective against PMS and benzaldehyde oxidation by human liver aldehyde oxidase.* Cyanide, quinacrine, N-alkylphenothiazines (Tables III and IV), methanol, and
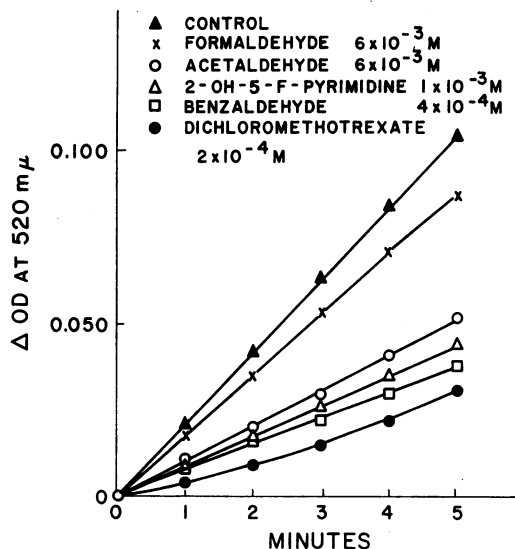

FIG. 8. INHIBITION BY OTHER SUBSTRATES OF OXIDATION OF PMS (5×10^{-4} MOLE PER LITER) BY HUMAN LIVER ALDEHYDE OXIDASE. The rate of conversion of PMS to N-methylphenazine-3-one was followed by the change in optical density at 520 mμ; reaction conditions were identical to those given in Table IV.

TABLE VII

Estrogens and progesterone as inhibitors of human liver aldehyde oxidase

	Concentration of inhibitor required to reduce reaction rate to 50% of control		
Substrate	Estradiol-17β	Progesterone	Diethylstilbestrol
	moles/liter		
Benzaldehyde*	8×10^{-8}	4×10^{-5}	5×10^{-7}
PMS‡	1×10^{-4}	$>4 \times 10^{-5}$	$(8 \times 10^{-6})§$

* Sample cuvettes contained benzaldehyde, 0.056 μmole, sodium phosphate buffer, pH 7.8, 100 μmoles, Versene Fe-3, 15 μg, ammonium chloride, 100 μmoles, and enzyme solution, 0.03 ml, in a total volume of 1 ml. Rates were measured 5 min after the start of the reaction.

‡ Sample cuvettes contained PMS, 0.5 μmole, sodium phosphate buffer, pH 7.8, 100 μmoles, Versene Fe-3, 15 μg, ammonium chloride, 100 μmoles, and enzyme solution, 0.1 ml, in a total volume of 1 ml. Rates were measured 5 min after the start of the reaction.

§ Stimulation, 50% maximal.

p-hydroxymercuribenzoate were equally effective as inhibitors of the oxidation of both PMS and benzaldehyde.

Other substrates for the enzymes, both aldehydes and nonaldehydic heterocyclic substrates, were effective inhibitors of PMS oxidation (Fig. 8), a result compatible with utilization of the same substrate-binding site for both aldehyde and non-aldehydic heterocyclic substrates. Benzaldehyde (4×10^{-4} mole per liter) was particularly effective as an inhibitor of PMS oxidation. Formaldehyde, as noted above, apparently has low affinity for the substrate-binding site of human liver aldehyde oxidase, as indicated by its very slight ability to inhibit PMS oxidation, shown here, and its low substrate activity (Table VI).

(*b*) *Compounds effective as inhibitors of benzaldehyde oxidation, and ineffective as inhibitors of PMS oxidation by human liver aldehyde oxidase.* Menadione, a strikingly effective inhibitor of the oxidation of both benzaldehyde and of PMS by the rabbit liver enzyme, and of benzaldehyde by the human liver enzyme, is ineffective in inhibiting the oxidation of PMS by human liver aldehyde oxidase (Fig. 9). Similarly, the steroid inhibitors (Table VII), Triton X-100 ($5 \times 10^{-4}\%$), and antimycin A (Table V), highly potent inhibitors of benzaldehyde oxidation, are ineffective as inhibitors of PMS oxidation by the human enzyme.

(*c*) *Compounds effective as inhibitors of benzaldehyde oxidation which stimulate the rate of PMS oxidation by human liver aldehyde oxidase.* Diethylstilbestrol, a potent inhibitor of benzaldehyde oxidation by the human enzyme (Table II), stimulates significantly the rate of PMS oxidation

(Fig. 10); the maximal rate reached was 165% that of the control. Half-maximal stimulation was seen at a diethylstilbestrol concentration of 8×10^{-6} mole per liter. Stimulation of PMS oxidation was also noted with the sulfhydryl compound diethyldithiocarbamic acid, a weak inhibitor of benzaldehyde oxidation. The mode of stimulation of PMS oxidation by the two compounds would appear to differ, since the effects were additive when both were added at levels sufficient to give a maximal effect with either compound alone (Fig. 11).

No examples were found of compounds effective as inhibitors of PMS oxidation and ineffective as inhibitors of benzaldehyde oxidation by human liver aldehyde oxidase.
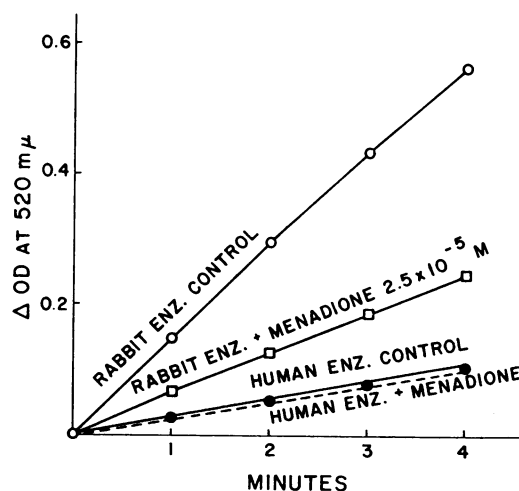

FIG. 9. DIFFERENTIAL INHIBITION BY MENADIONE (2.5 $\times 10^{-5}$ MOLE PER LITER) OF OXIDATION OF PMS (5×10^{-4} MOLE PER LITER) BY ALDEHYDE OXIDASE FROM RABBIT LIVER AND HUMAN LIVER. Experimental conditions were identical to those given in Table IV.
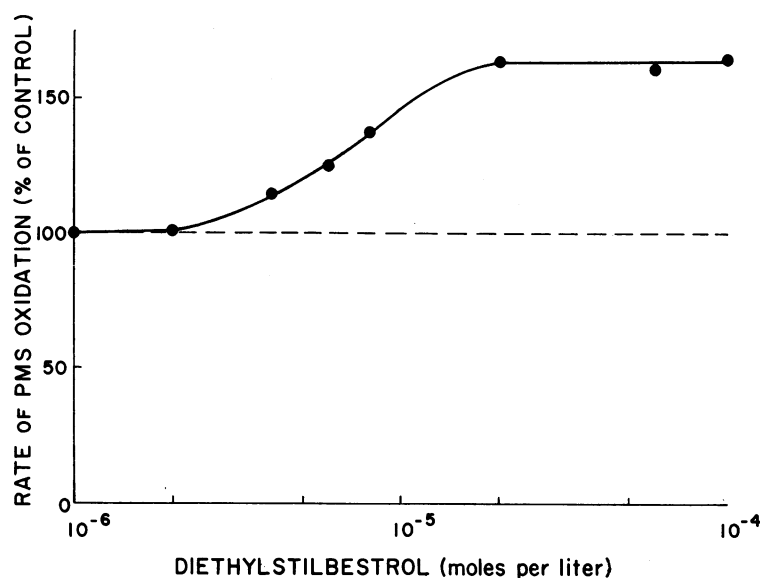
FIG. 10. STIMULATION BY DIETHYLSTILBESTROL OF OXIDATION OF PMS
(5×10^{-4} MOLE PER LITER) BY HUMAN LIVER ALDEHYDE OXIDASE. Experi-
mental conditions were identical to those given in Table IV. Diethylstilbes-
trol was added in ethanol solution (0.01–0.02 ml); corresponding volumes
of ethanol alone were added to control cuvettes.

Other nonaldehydic heterocyclic substrates

The oxidation of N-methylnicotinamide, which,
like PMS, has a positively charged quaternary ni-
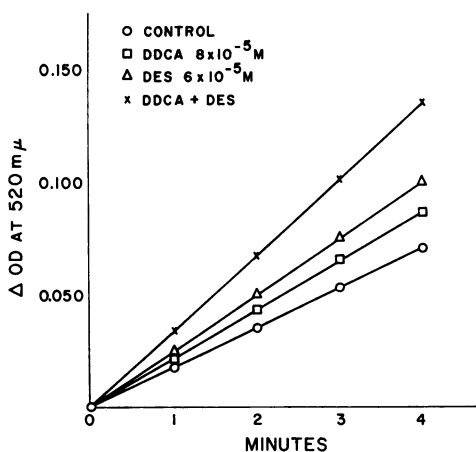trogen, followed the same pattern as that of PMS,

FIG. 11. ADDITIVE STIMULATION BY DIETHYLSTILBES-
TROL, DES, (2×10^{-5} MOLE PER LITER) AND DIETHYLDI-
THIOCARBAMIC ACID, $DDCA$, (8×10^{-5} MOLE PER LITER)
OF OXIDATION OF PMS (5×10^{-4} MOLE PER LITER) BY
HUMAN LIVER ALDEHYDE OXIDASE. Diethylstilbestrol and
diethyldithiocarbamic acid were added in ethanol solu-
tion; corresponding volumes of ethanol alone were added
to control cuvettes.

i.e., the reaction was sensitive to inhibition by cy-
anide, quinacrine, and N-alkylphenothiazines, but
not to inhibition by menadione or diethylstilbestrol.
In contrast, the oxidation of the uncharged non-
aldehydic substrate 2-hydroxy-5-fluoropyrimidine
was sensitive to both the latter agents (Table
VIII); thus, this compound, like benzaldehyde,
apparently utilizes the complete "internal electron
transport chain" of aldehyde oxidase.

TABLE VIII

Effect of inhibitors on the conversion of 2-hydroxy-5-fluoro-
pyrimidine to 5-fluorouracil by human liver
aldehyde oxidase*

	Inhibitor concentration	Inhibition
	moles/liter	%
Potassium cyanide	1×10^{-4}	71
Menadione	2.5×10^{-5}	46
Diethylstilbestrol	3.3×10^{-5}	93

* Reference cuvettes contained sodium phosphate buffer,
pH 7.8, 500 μmoles, Versene Fe-3, 20 μg, ammonium
chloride, 600 μmoles, and enzyme, 0.1 ml, in a total volume
of 3 ml.   Sample cuvettes contained the same constituents,
plus 2-hydroxy-5-fluoropyrimidine, 0.54 μmole. Ultra-
violet absorption spectra were recorded at 20-min intervals,
using a Cary 15 recording spectrophotometer with a
0–1.0 OD slide wire. Reaction rates were determined
from the rate of decrease in the absorption maximum of the
substrate at 310 mμ (11).

Discussion

The careful delineation of the "internal electron transport chain" of rabbit liver aldehyde oxidase by Rajagopalan and Handler (6) permits a ready explanation of the results which have been obtained in inhibition studies with the human enzyme. As these authors have demonstrated, cyanide, *p*-hydroxymercuribenzoate, methanol, and quinacrine attack the enzyme at or near the substrate-binding site. The present studies suggest that *N*-alkylphenothiazines, being formally competitive with substrate, also bind at the substrate site, irrespective of whether the rabbit or the human enzyme is used. All these agents are effective in inhibiting the oxidation of either charged or uncharged substrates by the human enzyme. However, a second class of inhibitors, menadione, Triton X-100, antimycin A, and the steroids, apparently influence the reduction and reoxidation of the components of the "internal electron transport chain" of the rabbit enzyme (molybdenum, FAD, coenzyme Q_{10}, and iron) and do not bind at the substrate site [Rajagopalan and Handler (6)]. These agents do not affect the oxidation of charged substrates such as PMS and NMN by human liver aldehyde oxidase, but are highly effective inhibitors of the oxidation of uncharged substrates such as benzaldehyde and 2-hydroxy-5-fluoropyrimidine, when oxygen is used as electron acceptor. It would appear therefore that, under the conditions used in the present studies, the uncharged substrates for the human enzyme utilize the entire "internal electron transport chain" of the enzyme, and are sensitive to inhibitors which act at any site of the chain, while positively charged substrates can utilize only the substrate site, and are sensitive solely to inhibitors binding at the latter. Thus, whereas with the rabbit enzyme, under aerobic conditions, the electronic structure of the substrate appears to be of little importance in determining the route of electron transport (7), with the human enzyme, the electronic structure of the substrate is of much greater significance.

The present studies do not establish whether molecular oxygen acts as electron acceptor in the oxidation of the charged substrates by the human enzyme, or whether the substrate itself serves this function: Rajagopalan and Handler (13) have

shown that, with the rabbit liver enzyme, under anaerobic conditions, PMS can act as an electron acceptor for aldehyde oxidase; and thus, when PMS is used anaerobically as a substrate for the rabbit enzyme, equimolar amounts of *N*-methylphenazine-3-one and leuko PMS are generated. Such a situation may apply with the human enzyme even under aerobic conditions: since the site of electron egress is early in the chain when PMS is used as electron acceptor (6), the insensitivity of PMS oxidation by the human enzyme to electron chain inhibitors such as menadione, which act late in the electron transfer pathway, would thus be accounted for.

The unusual situation of a compound such as diethylstilbestrol acting as either an inhibitor or stimulator of the catalytic activity of an enzyme, depending on which type of substrate is used, is compatible with the two routes of oxidation postulated for human liver aldehyde oxidase. Since diethylstilbestrol and diethyldithiocarbamic acid appear to bind at sites of the internal electron transport chain remote from the substrate-binding site, such compounds could inhibit the oxidation of a compound requiring the complete transport chain (e.g., benzaldehyde) while fortuitously causing a favorable alteration in conformation at the substrate site. Such a favorable conformational change would only be demonstrable when a substrate not requiring the entire electron transport chain, such as PMS, was used.

These studies throw little light on the long standing problem of the metabolic role of aldehyde oxidase. Several other enzymes in addition to aldehyde oxidase are known to possess the ability to catalyze the oxidation of aldehydes, among them xanthine oxidase (EC 1.2.3.2); the steroid-insensitive NAD-linked aldehyde dehydrogenase studied by Racker (21), Deitrich, Hellerman, and Wein (22), and others (EC 1.2.1.3); and the steroid-sensitive NAD-requiring aldehyde dehydrogenase of Maxwell and Topper (23). The studies of Richert and Westerfeld (24) suggest that NAD-linked aldehyde dehydrogenases, rather than metalloflavoproteins such as aldehyde oxidase and xanthine oxidase, are of primary importance in the metabolism of acetaldehyde arising from the oxidation of ethanol. It would appear likely, therefore, that the primary physiologic role of hepatic aldehyde oxidase may

not be the oxidation of aliphatic aldehydes. From the present studies with the human enzyme, and from earlier studies by other authors with liver aldehyde oxidase from other mammalian species, three other possible functions for this enzyme may be postulated. The first of these is that aldehyde oxidase may be serving a protective function as a nonspecific drug-metabolizing enzyme. It has been shown, for example, that the 7-hydroxylation (and consequent biologic inactivation) of the anti-neoplastic agent methotrexate by this enzyme proceeds so rapidly in the rabbit that, for all practical purposes, a toxic level of this compound for this species cannot be obtained (25, 26). Appropriately substituted nonphysiologic purines (12) and pyrimidines (11) can likewise serve as excellent substrates for rabbit liver aldehyde oxidase. The present studies with the human enzyme would tend to suggest, however, that the drug-metabolizing function of aldehyde oxidase is not of importance in all species. In man, the range of nonphysiologic heterocyclic substrates that can be hydroxylated by the enzyme is much narrower than in the rabbit or guinea pig, and the rate of hydroxylation is very much slower.

A second possibility is that these nonphysiologic heterocyclic compounds have the capacity to act as substrates as a consequence of their resemblance to an as yet unidentified physiologic nonaldehydic heterocyclic substrate for the enzyme. The use of aldehyde oxidase inhibitors such as those described in the present paper should permit the testing of this hypothesis: the administration of such an inhibitor at an adequate dosage level should result in an increased urinary excretion of the hypothetical substrate, in the same way that the xanthine oxidase inhibitor 4-hydroxy-pyrazolo[3,4-d]pyrimidine results in an increased excretion of the xanthine oxidase substrates xanthine and hypoxanthine (27).

The third, and perhaps most likely possibility, is that the physiologic role of human liver aldehyde oxidase is the oxidation of aromatic aldehydes arising from dietary sources, or occurring in the course of intermediary metabolism. As noted above, the aromatic aldehyde benzaldehyde has the highest affinity for the human enzyme of all the substrates examined to date; in addition, the maximal reaction velocity for this substrate exceeded that for all other substrates tested. Similar results were obtained with the mouse and rabbit liver

enzymes. Whatever the physiologic role of the enzyme may be, however, the effectiveness as aldehyde oxidase inhibitors of relatively nontoxic pharmacologic agents such as quinacrine and the N-alkylphenothiazines, and physiologic compounds such as estradiol-17β, would suggest either that aldehyde oxidase, like xanthine oxidase, is not essential to life, or that it is present in great excess over the amount required in intermediary metabolism.

The partial structural analogy between the phenothiazine nucleus and the isoalloxazine component of flavin adenine dinucleotide (FAD) has permitted the hypothesis that N-alkylphenothiazines may inhibit flavoproteins in vivo by virtue of an affinity for FAD-binding sites; this suggestion has received experimental support from the observation that N-alkylphenothiazines compete with FAD for the apoenzyme of D-amino acid oxidase (28, 29). This interpretation, however, cannot be extended to the inhibition by phenothiazines of aldehyde oxidase; in this instance, as the present studies show, the inhibitors are formally competitive with substrate. Similarly, Rajagopalan and Handler (7) have shown that quinacrine inhibits rabbit liver aldehyde oxidase by formal competition with substrate. These results can most easily be interpreted by assuming an affinity of the phenothiazine nucleus (or, in the case of quinacrine, of the rather similar acridine nucleus) for the substrate-binding site of aldehyde oxidase. It would appear, in fact, that only steric hindrance from the long N-alkyl side-chains of the phenothiazines examined here prevents these compounds from acting as substrates; as noted above, N-methylphenothiazine, like N-methyl-phenazine (PMS), is rapidly oxidized by the rabbit liver enzyme.[3] Thus, while these studies reveal nothing about the mode of action of the N-alkylphenothiazines at the molecular level as psychopharmacologic agents, they permit the alternative suggestion that, assuming these compounds act in vivo as enzyme inhibitors, the site of action at the hypothetical target enzyme or enzymes of the central nervous system may be a substrate-binding site, as with aldehyde oxidase, rather than an FAD-binding site, as with D-amino acid oxidase. It would follow that the hypothetical target enzyme need not be a protein with a readily dissociable flavin cofactor, and that the resemblance between the phenothiazine nucleus

and the isoalloxazine component of FAD may be fortuitous, and not of pharmacologic significance.

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