Inactivation of Glyceraldehyde-3-phosphate Dehydrogenase and Yeast Alcohol Dehydrogenase by Arene Oxides†

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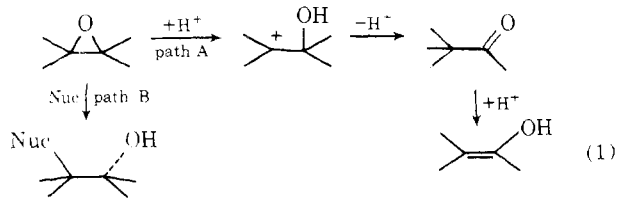
ABSTRACT: Yeast alcohol dehydrogenase (YADH) and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (GPDH), enzymes that contain sulfhydryl groups essential to their catalytic activity, have been found to be rapidly inactivated by small concentrations of several arene oxides with K_Is of $\sim 10^{-4}$ M. The effectiveness of the arene oxides as inhibitors decreases in the order: 4-carbo-*tert*-butoxybenzene oxide > 4-carboxybenzene oxide > phenanthrene 9,10-oxide. The half-life of enzymatic activity decreases with increasing arene oxide concentration and increasing pH of the incubation mixture in the pH range 7.0–9.5. Prior incubation of the enzymes with a saturating concentration of substrate decreases the rate of inactivation by the arene oxides, and incubation of GPDH with arene oxide results in increased absorption of the Racker band at 360 nm for ~5 h followed by a decrease in absorption. These observations suggest that alkylation of the active-site sulfhydryl groups has occurred. Titration with 5,5′-dithiobis(2-nitrobenzoic acid) shows that the arene oxides show specificity toward the active-site cysteine residues. The greater effectiveness of 4-carbo-*tert*-butoxybenzene oxide as an inhibitor of YADH and GPDH activity is as would be predicted from its comparatively large nucleophilic susceptibility index. The β values obtained from Brønsted plots for the reaction of thiolate anions with the arene oxides suggest that the site of nucleophilic attack on 4-carbo-*tert*-butoxybenzene oxide is different from that of other investigated arene oxides. Although lysozyme is inhibited by certain diphenyl ethers and epoxides structurally related to the substrate, phenanthrene 9,10-oxide has no effect on the activity of the enzyme.

Membrane-bound cytochrome P-450 monoxygenases convert aromatic hydrocarbons to arene oxides (Tomaszewski et al., 1974) which serve as intermediates in aromatic compound detoxification (Oesch, 1973; Jeffrey and Jerina, 1975) as well as in the biosynthesis of metabolically important phenols (Daly et al., 1972). Certain arene oxides have been shown to be causative agents of carcinogenesis (Grover et al., 1971; Levin et al., 1976), mutagenesis (Ames et al., 1972), and necrosis (Brodie et al., 1971) as a result of their being bound covalently to cellular macromolecules. At present, it is not known whether the critical site of attachment is protein or nucleic acid. Several investigators (Grover and Sims, 1970 and 1972; Jeffrey et al., 1976a,b; Blobstein et al., 1975, 1976) have established that K-region arene oxides bind covalently to DNA and RNA with the purine bases, in particular guanine, exhibiting the greatest reactivity toward the arene oxides. To date, however, there is essentially no data available on the interaction of arene oxides with proteins.

It has been shown (Bruice et al., 1976a,b) that in aqueous solution arene oxides rearrange to phenols (path A) and are attacked by nucleophiles to give primarily trans-addition products (path B). Thiolate anions exhibit considerably greater



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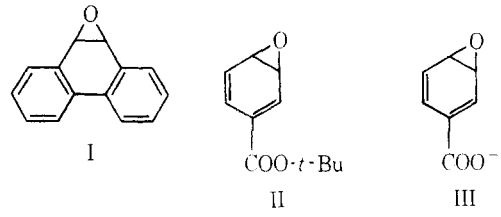
nucleophilic reactivity toward arene oxides than do amines, which in turn are more effective than oxygen nucleophiles. That arene oxides are particularly susceptible to nucleophilic attack by thiols suggests the possibility of their being effective alkylating agents for protein cysteine residues. Consequently, we have examined the effect of three arene oxides [phenanthrene 9,10-oxide (I), 4-carbo-*tert*-butoxybenzene oxide (II), and 4-carboxybenzene oxide (III)] on the activity of yeast



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alcohol dehydrogenase (YADH)[1] and glyceraldehyde-3-phosphate dehydrogenase (GPDH), enzymes that contain sulfhydryl groups essential to their catalytic activity. The results of this study are reported herein.

Experimental Section

Materials. Phenanthrene 9,10-oxide (Newman and Blum, 1964), 4-carbo-*tert*-butoxybenzene oxide (DeMarinis et al., 1974), and 4-carboxybenzene oxide (Richardson et al., 1974) were prepared by previously published procedures. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) was obtained from Sigma as a crystalline suspension in ammonium sulfate (14 mg of protein/mL, 105 units/mg of protein). Yeast alcohol dehydrogenase (EC 2.2.2.2) was purchased from Worthington (two times crystallized; 300 units/mg of protein). Egg-white lysozyme (EC 3.2.1.17) was obtained from Sigma (three times crystallized; 23 000 units/mg

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[1] Abbreviations used: YADH, yeast alcohol dehydrogenase; GPDH, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

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TABLE I: Half-Lives and Inhibitor Constants for the Inactivation of Yeast Alcohol Dehydrogenase and Glyceraldehyde-3-phosphate Dehydrogenase by Arene Oxides.

	YADH[a]		GPDH[b]	
Arene oxide	$t_{1/2}$ (min)	K_I (M)	$t_{1/2}$ (min)	K_I (M)
None	740		90	
4-Carbo-*tert*-butoxybenzene oxide	0.8	11×10^{-4}	2.6	5.1×10^{-4}
4-Carboxybenzene oxide	1.2	13×10^{-4}	13	4.0×10^{-4}
Phenanthrene 9,10-oxide	27	6.8×10^{-4}	16	4.4×10^{-4}

[a] Enzyme was incubated with 1.7×10^{-4} M arene oxide at pH 7.5 at 30 °C; assays were determined at pH 8.8 at 30 °C. [b] Enzyme was incubated at pH 8.5 (30 °C) with 4.4×10^{-3} M arene oxide; assays were determined at pH 8.5 at 30 °C.

of protein). DL-Glyceraldehyde 3-phosphate was prepared from the barium salt of DL-glyceraldehyde 3-phosphate diethyl acetal (Sigma). 5,5′-Dithiobis(2-nitrobenzoic acid) (Nbs_2), NAD^+ (grade III), dithiothreitol, thioglycolic acid, the hydrochlorides of 2-mercaptoethanol and 2-mercaptoethylamine (all from Sigma), guanidine hydrochloride (ultrapure, Schwarz/Mann), and glutathione (Calbiochem) were used without further purification. Ethyl alcohol was from Commercial Solvents. Doubly glass-distilled water was used throughout, and all inorganic reagents were of analytical grade.

Enzyme Assays. A solution of YADH was prepared by adding 1.0 mg of YADH to 10.0 mL of sodium phosphate buffer (0.01 M, pH 7.5); 0.5 mL of this solution was added to 4.5 mL of sodium phosphate buffer (0.01 M, pH 7.5). YADH activity was determined by adding 0.1 mL of the enzyme solution to a solution containing 1.5 mL of 0.032 M sodium pyrophosphate buffer (pH 8.8), 0.5 mL of 2 M ethanol, and 1.0 mL of 2.5×10^{-2} M NAD^+. A GPDH solution was prepared by adding 0.1 mL of the ammonium sulfate suspension to 5.0 mL of a pyrophosphate–arsenate buffer (0.015 M sodium pyrophosphate, 0.03 M sodium arsenate, pH 8.5). Activity was measured by adding 0.4 mL of DL-glyceraldehyde-3-phosphate (4×10^{-3} M) to a solution containing 0.1 mL of the enzyme solution, 0.1 mL of NAD^+ (7.5×10^{-3} M), 0.1 mL of dithiothreitol (0.1 M), and 2.4 mL of the pyrophosphate–arsenate (pH 8.5) buffer. For both enzymes, NADH formation was monitored by the initial increase in absorption of 340 nm (Vallee and Hoch, 1955). For determinations of the pH dependence of inactivation (Tables II and III), the pHs of the YADH and GPDH solutions were varied; the pH of the assay solutions was held constant at 8.8 and 8.5, respectively. The activity of lysozyme was measured by adding 0.1 mL of enzyme solution (0.05 mg of enzyme/mL of phosphate buffer) to 2.9 mL of a solution containing 0.3 mg of dried *Micrococcus luteus* cells/mL in 0.1 M phosphate buffer (pH 7.0). The rate of decrease in absorption was monitored at 450 nm. All enzymatic assays were carried out at 30.0 ± 0.2 °C.

Inactivation of Enzymes. In a typical inactivation experiment, an aliquot (0.1 mL for GPDH and lysozyme, 0.05 mL for YADH) of the arene oxide in an organic solvent was added to the enzyme solution (1.5 mL for GPDH, 2.0 mL for lysozyme, 5.0 mL for YADH) at 30 °C, argon was passed over the solution, and the mixture was incubated at 30 °C. At measured time intervals, the enzyme–arene oxide solution was assayed for loss of activity. Control experiments were run to determine the rate of loss of activity of the enzyme under the same conditions in the absence of arene oxide; an aliquot of organic solvent was added to the enzyme, the mixture was incubated at 30 °C, and assays were determined at appropriate time intervals. Acetonitrile was the organic solvent employed for assays of GPDH and lysozyme; tetrahydrofuran was used with YADH.

Substrate Protection. Substrate sufficient for saturation was added to the enzyme before the addition of arene oxide. Assays were carried out as described above.

Kinetic Measurements. Assays of GPDH, YADH, and lysozyme were run on either a Cary 16, Cary 118, or Gilford Model 2000 spectrophotometer. All spectrophotometers were thermostated at 30 °C. Reactions of thiolate anions with arene oxides were followed at 285 nm. Thiolate buffer solutions (μ = 1.0 with KCl) were prepared immediately prior to use as described previously (Bruice et al., 1976b). Readings of pH were determined on a Radiometer Type PMH 26 pH meter. Calculations of rate constants and least-squares slopes and intercepts were done using a Hewlett-Packard Model 9820A computer.

Titration of Sulfhydryl Groups. To 2.0 mL of a pH 8.2 solution that is 8.0 M in guanidine hydrochloride, 0.2 M in Tris, and 0.2 M in EDTA was added 0.5 mL of a solution of YADH incubated with arene oxide at pH 7.5 at 30 °C (Vanaman and Stark, 1970). The mixture was allowed to sit for 15 min at 30 °C at which time 0.3 mL of 0.01 M 5,5′-dithiobis(2-nitrobenzoic acid) was added. Absorption was monitored for ~20 min at 412 nm. The pH of the final mixture was 8.2. A control with no arene oxide and two blanks, one with arene oxide and no enzyme and one with no arene oxide and no enzyme, were run simultaneously. A similar procedure was used for the titration of GPDH, except that the enzyme was incubated with arene oxide at pH 8.5 and the pH of the titration mixture was maintained at 8.0. The extinction coefficient at 412 nm of the 5-thio-2-nitrobenzoate anion obtained by titration of cysteine was found to be 14.4×10^3 M^{-1} cm^{-1} at pH 8.0 and 18.6 M^{-1} cm^{-1} at pH 8.2.

Results

Inactivation of Yeast Alcohol Dehydrogenase by Arene Oxides. The half-life of yeast alcohol dehydrogenase is greatly reduced by the presence of small concentrations of arene oxides (Table I). For each of the YADH experiments of Table I, the concentration of enzyme and arene oxide in the incubation mixture was 7×10^{-8} and 1.7×10^{-4} M, respectively. The reported half-lives are the average of several determinations. Inactivation of YADH by arene oxides I, II, and III is a first-order process as indicated in Figure 1A for loss of activity in the presence of I. Incubation of the enzyme with a saturating concentration of substrate before the addition of phenanthrene 9,10-oxide (I) offers almost complete protection to inactivation by the arene oxide; the half-life increases from 27 to 152 min. In the case of inactivation by 4-carbo-*tert*-butoxybenzene oxide (II), pretreatment of the enzyme with substrate gives only slight protection to inactivation; the half-life increases from 0.8 to 2.0 min.

The half-lives of YADH in the presence of I, II, and III (Table I) were determined by incubating the enzyme and arene oxide at pH 7.5 and assaying the enzyme at pH 8.8. Since it is the thiolate anion that is alkylated by arene oxides, it is to be expected that the half-life of the enzyme should exhibit a dependence on the pH of the incubation mixture in the vicinity of the pK_a of the protein sulfhydryl groups. In Table II are given the half-lives of YADH determined in the presence of a constant arene oxide concentration and at a constant assay

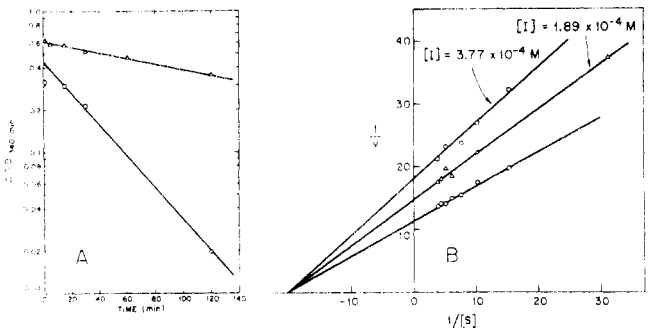


FIGURE 1: (A) (○) Semilog plot of the rate of inactivation of yeast alcohol dehydrogenase by 1.7×10^{-4} M phenanthrene 9,10-oxide (30 °C, incubation pH = 7.5, assay pH = 8.8); (△) the above experiment repeated with the exception that the enzyme is incubated in 0.32 M ethanol before the addition of phenanthrene 9,10-oxide. (B) Lineweaver–Burke plots for inhibition of yeast alcohol dehydrogenase by phenanthrene 9,10-oxide (30 °C, pH 8.8).

pH but at varying incubation mixture hydrogen ion concentrations. It is apparent from the table that a large decrease in the half-life of the enzyme occurs as the pH is increased from 6.5 to 9.5. The inactivation of YADH by styrene oxide, in which 2 mol of reagent is incorporated per mol of subunit, was found to exhibit a dependence on a side chain(s) of pK_a 8.5 (Klinman, 1975). Inactivation of YADH by II is in agreement with an active side-chain residue with a pK_a of approximately the same magnitude. The microscopic pK_as of the cysteine thiol group in aqueous solution at 30 °C have been found to be 8.21 [$HSCH_2CH(NH_3^+)CO_2^-$] and 9.56 [$HSCH_2CH(NH_2)$-CO_2^-] (Reuben and Bruice, 1976).

In addition to the dependence on the pH of the incubation mixture, the half-life of YADH is also markedly affected by the concentration of the arene oxide in the incubation mixture (Table II). The concentration dependence of the rate of inactivation of YADH indicates the much greater effectiveness of the arene oxides as alkylating reagents as compared to styrene oxide. The half-lives of Table I obtained in the presence of 1.7×10^{-4} M arene oxide may be compared with the half-life of 53 min obtained for YADH inactivation in the presence of 9×10^{-3} M styrene oxide (Klinman, 1975). The difference in the effectiveness of the arene oxides and the epoxide as alkylating agents is more striking when the pH of the incubation mixture is considered; the data of Table I were obtained at pH 7.5, while the half-life of the enzyme in the presence of styrene oxide was determined by incubation at pH 8.5.

Titration of YADH with Nbs_2 after incubation for 30 min with 4-carbo-*tert*-butoxybenzene oxide (II) indicates that 3.4 sulfhydryl groups have been alkylated by the arene oxide. Yeast alcohol dehydrogenase is a tetramer; thus, 0.85 mol of arene oxide is incorporated per subunit. Reaction of YADH with iodoacetate or butyl isocyanate has also been shown to result in the alkylation of one sulfhydryl residue per subunit (Belke et al., 1974), while iodoacetamide (Twu et al., 1973) and styrene oxide (Klinman, 1975) are reported to alkylate two thiol residues per subunit.

In Figure 1B are shown Lineweaver–Burke plots for the inhibition of yeast alcohol dehydrogenase by phenanthrene 9,10-oxide (I). The noncompetitive inhibition evidenced in the figure is also shown by 4-carbo-*tert*-butoxybenzene oxide (II) and 4-carboxybenzene oxide (III) with YADH. As found for GPDH (see below), replots of intercept vs. inhibitor concentration and plots of λ_{max} vs. $[E]_T$ indicate that the inhibition of YADH by arene oxides is reversible. Inhibition constants determined for each of the arene oxides are given in Table I. These may be compared to the value of $K_I = 3.7 \times 10^{-2}$ M

TABLE II: The pH Dependence and Arene Oxide Concentration Dependence of the Half-Life of YADH in the Presence of 4-Carbo-*tert*-butoxybenzene Oxide.

pH	[II] (M)	$t_{1/2}$ (min)
6.5	1.5×10^{-4}	135
7.0	1.5×10^{-4}	45
7.5	1.5×10^{-4}	34
8.0	1.5×10^{-4}	28
8.5	1.5×10^{-4}	22
9.5	1.5×10^{-4}	2
7.5	1.3×10^{-5}	502
7.5	3.9×10^{-5}	237
7.5	1.5×10^{-4}	34
7.5	1.7×10^{-4}	0.8

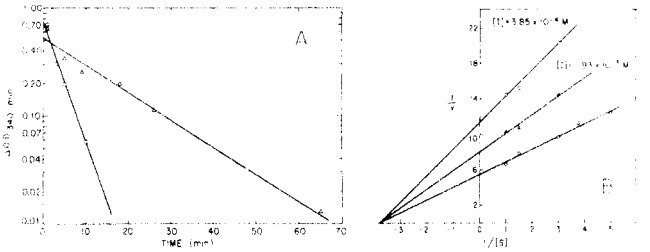


FIGURE 2: (A) (○) Semilog plot of the rate of inactivation of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase by 4.4×10^{-3} M 4-carbo-*tert*-butoxybenzene oxide (30 °C, incubation and assay pH = 8.5). (△) The above experiment repeated with the exception that the enzyme is incubated in 2.5×10^{-3} M DL-glyceraldehyde-3-phosphate before the addition of 4-carbo-*tert*-butoxybenzene oxide. (B) Lineweaver–Burke plots for inhibition of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase by 4-carboxybenzene oxide (30 °C, pH 8.5).

reported for inactivation of YADH by styrene oxide. Thus, when compared to styrene oxide, the arene oxides bind more tightly to the enzyme and inactivate it more rapidly.

In the course of this work, it was discovered that small quantities of acetonitrile evidence an inhibitory effect on YADH. Nonaqueous solutions of arene oxides were employed for the inhibition studies; 0.05 mL of the arene oxide solution was added to 5.0 mL of the buffered enzyme. Thus, in order to determine the half-life of the enzyme in the absence of arene oxide, 0.05 mL of nonaqueous solvent was added to the enzyme solution to be assayed. When the solvent employed was acetonitrile, the enzyme was found to have a half-life of 300 min. The half-life increased to 740 min in the presence of the same volume of tetrahydrofuran. Consequently, THF was employed as solvent for the arene oxides for all determinations done with YADH.

Inactivation of Glyceraldehyde-3-phosphate Dehydrogenase by Arene Oxides. In the absence of arene oxide, rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was found to have a half-life of 90 min at 30 °C and at pH 8.5. Under the same conditions, the half-life of the enzyme was markedly decreased in the presence of arene oxides I, II, and III (Table I). In each case, the concentration of the arene oxide employed in the incubation mixture was 4.4×10^{-3} M and the enzyme concentration was 2×10^{-6} M. With I or II as inhibitor, inactivation of GPDH is a first-order process as indicated in Figure 2A for inactivation by 4-carbo-*tert*-butoxybenzene oxide. In the presence of 4-carboxybenzene oxide (III), the rate of loss of enzymatic activity observed during the first 30–40 min is followed by a somewhat slower rate of inactivation. The half-life of GPDH in the presence of III given in Table I was obtained directly from a plot of activity vs. time of addition of arene oxide. In the presence of I or II, the half-life of GPDH

TABLE III: The pH Dependence of the Half-Life of Glyceraldehyde-3-phosphate Dehydrogenase in the Presence of 4.4 × 10^{-3} M 4-Carboxybenzene Oxide.

pH	$t_{1/2}$ (min)
7.5	25
8.0	15
8.5	13
9.0	9
9.5	6

obtained in this manner is in agreement with the half-life calculated from the observed first-order rate constant.

Assays of GPDH require the addition of a thiol such as dithiothreitol to reduce that portion of the enzyme that exists in the inactive oxidized form. For the experiments reported herein, dithiothreitol was not added to the incubation mixtures since it would compete with the enzyme sulfhydryl groups for the arene oxide; it was, however, added to the assay solution. If dithiothreitol is omitted from the assay as well as from the incubation mixture, the initial activity of the enzyme is decreased by about 30%, the half-life is reduced from 90 to about 30 min, and the kinetic data are not readily reproducible. Thus, the data of Table I were obtained with some of the incubated enzyme present in the inactive form which presumably is not fully susceptible to the inhibitor. This suggests that the potency of the arene oxides as inhibitors of GPDH may be somewhat greater than indicated by the half-lives of the table.

Lineweaver–Burke plots for inhibition of the enzyme by 4-carboxybenzene oxide are given in Figure 2B. Similar plots were obtained with arene oxides I and II. The inhibitor constants (K_I) obtained from these double-reciprocal plots are listed in Table I. From Figure 2B it is apparent that the arene oxides act as noncompetitive inhibitors. The reversible nature of the inhibition is indicated by linear replots of intercept vs. inhibitor concentration (Cleland, personal communication). A plot of V_{max} vs. $[E]_T$, where $[E]_T$ represents total units of enzyme activity added to the assay, for GPDH in the absence and presence of 2.2 × 10^{-3} M 4-carbo-*tert*-butoxybenzene oxide is shown in Figure 3. The difference in slopes of the control and plus inhibitor plots is also indicative of the reversible noncompetitive nature of the inhibition (Segel, 1975).

Figure 2A shows the first-order inactivation of GPDH by 4.4 × 10^{-4} M II. Also in this figure is shown the effect of the same concentration of II on GPDH preincubated for 2–3 min with a saturating concentration of D-glyceraldehyde 3-phosphate. Inactivation remains a first-order process, but the half-life of the enzymatic activity has been increased from 2.6 to 13 min. Similarly, the half-life of the enzyme in the presence of phenanthrene 9,10-oxide (I) increases from 16 to 34 min when the enzyme is preincubated with substrate. Thus, pretreating the enzyme with substrate offers some protection to inhibition by the arene oxide, indicating that at least some of the inhibitor is found at or near the active site of the enzyme.

The half-lives of GPDH given in Table I were determined by incubating the enzyme with the arene oxide at pH 8.5. The data of Table III indicate the dependence of the rate of inactivation of GPDH on the pH of the incubation mixture; in all cases assays were determined at pH 8.5. The pH dependence of the rate of inactivation of GPDH by III is similar to that found for inactivation of YADH by II.

Yeast glyceraldehyde-3-phosphate dehydrogenase has been reported to be irreversibly inactivated by three aliphatic epoxides structurally similar to the substrate (McCaul and Byers, 1976). Glycidol phosphate is the most effective of the inhibitors followed by glycidol and propylene oxide. Compared to the effectiveness of the arene oxides as inhibitors of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, the aliphatic epoxides are considerably less effective inhibitors of the yeast enzyme. The half-life of the enzyme is linearly related to the concentration of epoxide. To achieve a half-life of 14 min, the enzyme must be incubated with 5 × 10^{-2} M glycidol phosphate.

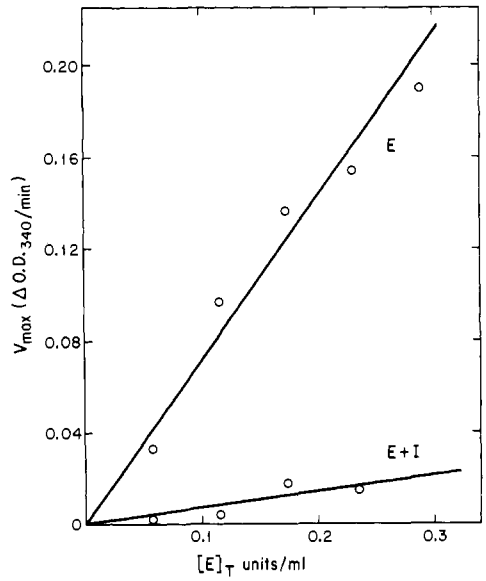


FIGURE 3: Plots of V_{max} vs. total units of rabbit muscle glyceraldehyde-3-phosphate dehydrogenase activity in the absence and presence of 2.2 × 10^{-3} M 4-carbo-*tert*-butoxybenzene oxide (30 °C, pH = 8.5).

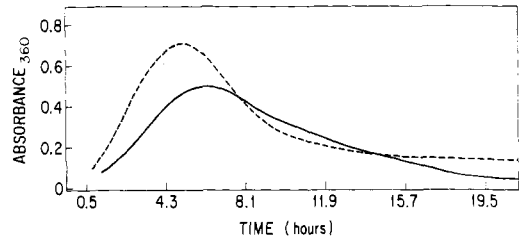


FIGURE 4: Solid line: absorbance at 360 nm of glyceraldehyde-3-phosphate dehydrogenase as a function of time. Dashed line: absorbance at 360 nm of the same concentration of glyceraldehyde-3-phosphate dehydrogenase in the presence of 1 × 10^{-4} M 4-carboxybenzene oxide as a function of time.

Incubation of GPDH with 4-carbo-*tert*-butoxybenzene oxide for 30 min destroys essentially all of the enzymatic activity. Titration of the enzyme at this time with Nbs_2 shows that 2.1 sulfhydryl residues have been alkylated. Four thiol residues of yeast glyceraldehyde-3-phosphate dehydrogenase must be alkylated by glycidol phosphate and other simple epoxides for the enzyme to be completely inactivated, while other alkylating agents such as iodoacetic acid and iodoacetamide are half-of-the-site reagents with this enzyme (McCaul and Byers, 1976).

Plots of absorbance of GPDH in pyrophosphate–arsenate buffer at 360 nm as a function of time are shown in Figure 4 for the enzyme in the absence (solid line) of arene oxide and in the presence (dashed line) of 1.1 × 10^{-4} M 4-carboxybenzene oxide. The experiment was repeated with 4-carbo-*tert*-butoxybenzene oxide as inhibitor, and the relative change in

TABLE IV: Relative Rates of Spontaneous Ring Opening (A) and Thiolate Anion Nucleophilic Attack (B).

Compound	A^a	B^b	B/A^i
Ethylene oxide	1[c]	1[f]	1
Benzene oxide	1 900[d]	4[g]	0.002
Naphthalene 1,2-oxide	5 000[d]	41[h]	0.008
Phenanthrene 1,2-oxide	50 000[d]	39[h]	0.0008
Phenanthrene 3,4-oxide	90 000[d]	50[h]	0.0006
Phenanthrene 9,10-oxide (I)	40	82[h]	2
4-Carbo-*tert*-butoxybenzene oxide (II)	250[e]	7200	30
4-Carboxybenzene oxide (III)	600	11	0.02

[a] Ethylene oxide measured at 25 °C, all others at 30 °C. [b] Attack on ethylene oxide measured at 20 °C, others at 30 °C. [c] Virtanen and Korhonen (1973). [d] Bruice et al. (1976a). [e] Johnson and Bruice (1975). [f] Danehy and Noel (1960). [g] Reuben and Bruice (1976). [h] Bruice et al. (1976b). [i] Nucleophilic susceptibility index.

absorption of the enzyme in the presence and absence of arene oxide was essentially identical to that shown in Figure 4. Neither of the arene oxides individually has an absorption band in the vicinity of 360 nm.

The Effect of Phenanthrene 9,10-Oxide on the Activity of Lysozyme. Lysozyme has been found to be inhibited by thyroxine and triiodothyronine as a result of the formation of an insoluble complex between the enzyme and the diphenyl ethers (Litwack, 1958 and 1963; Litwack and Sears, 1965; Viscidi et al., 1966; Tabachinick and Giorgio, 1966). The enzyme is also inhibited by 2′,3′-epoxypropyl β-glycosides of *N*-acetyl-D-glucosamine, although not by the simple epoxide, propylene oxide (Thomas et al., 1969; Thomas, 1970). The effectiveness of the glycosidic epoxides as inhibitors lies in their ability to alkylate Asp-52 (Eshdat et al., 1974). Because of the ability of certain diphenyl ethers and epoxides to inhibit lysozyme, it was of interest to determine the effect of phenanthrene 9,10-oxide (I) on the activity of the enzyme. However, when lysozyme was incubated for 2 h at 30 °C with I (5.6×10^{-4}–1.3×10^{-3} M), no decrease in catalytic activity was apparent.

Discussion

We have previously shown that phenanthrene 9,10-oxide, the K-region oxide of phenanthrene, undergoes nucleophilic attack by thiolate anions, amines, and oxygen bases. In contrast, the isomeric non-K-region arene oxides (phenanthrene 1,2- and 3,4-oxide) are attacked by thiolate anions but not by amines or oxygen bases. The inherent nucleophilic susceptibilities of the two classes of arene oxides are similar (eq 1, path B) (Bruice et al., 1976b). The rate of the competing aromatization reaction (eq 1, path A), however, is over 1000 times faster for the non-K-region arene oxides than for the K-region oxide. Consequently, only the very reactive thiolate anions can compete with the facile aromatization reaction to give nucleophilic addition adducts with the non-K-region oxides. Nucleophiles of a wide variety of activity react with phenanthrene 9,10-oxide, since this reagent, as a result of its slow rate of aromatization, remains in solution as a candidate for nucleophilic attack. In order to predict whether a nucleophile will form a covalent adduct with a given arene oxide, both the inherent susceptibility of the arene oxide to nucleophilic attack and the rate of the ring opening reaction must be taken into account. To provide a method quantitating these factors, we have employed a "nucleophilic susceptibility index", defined as the relative second-order rate constant for attack by the thiolate anion of 2-mercaptoethanol divided by the relative rate of water-catalyzed epoxide ring opening. The nucleophilic susceptibility indices for a number of non-K-region arene oxides and the K-region oxide I are given in Table IV. From the table it is apparent that the alkylating potential of phenanthrene 9,10-oxide is similar to that of ethylene oxide which is well recognized as an effective alkylating agent (Ross, 1950). All of the non-K-region arene oxides, with the exception of II, have very small nucleophilic susceptibility indices. It was expected that II would prove to be an effective alkylating agent, since the electron-withdrawing ($\sigma^+ = 0.37$) carbo-*tert*-butoxy group should both increase the susceptibility of the ring to nucleophilic attack and decrease the rate of epoxide ring opening to carbonium ion compared to the corresponding rates for benzene oxide. Thus, if the carcinogenicity of arene oxides resides in their potential as alkylating agents, addition of a strongly electron-withdrawing substituent to a biologically harmless non-K-region oxide may result in its conversion to a potent carcinogen.

All experiments with 4-carboxybenzene oxide (III) were carried out at a pH considerably above the pK_a of the carboxy substituent ($pK_a = 3.70$, Richardson et al., 1974). The carboxylate anion has little effect on the π-electron system of the benzene ring, as evidenced by its small substituent constant ($\sigma^+ = -0.02$, Brown and Okamota, 1968) and by the 50–50 mixture of meta- and para-substituted phenols obtained upon aromatization of III under basic conditions (Richardson et al., 1974). Benzene oxides with electron-withdrawing substituents at the 4 position give primarily meta-substituted phenols, while those with electron-donating substituents at the 4 position aromatize primarily to para-substituted phenols (Bruice and Bruice, 1976). Consequently, the nucleophilic susceptibility index of III should be similar to that of benzene oxide. However, the rate of spontaneous ring opening of III is less than and its susceptibility to thiolate anion attack is greater than the corresponding rates for benzene oxide, resulting in a slightly greater nucleophilic susceptibility index (Table IV).

Arene oxides I, II, and III are noncompetitive and, apparently, reversible inhibitors of yeast alcohol dehydrogenase and rabbit muscle glyceraldehyde-3-phosphate dehydrogenase. They bind fairly tightly to the enzymes with K_Is of $\sim 10^{-4}$ M, are effective inhibitors at low concentrations (Table I), and exhibit a pH dependence expected for thiol-group modification (Tables II and III). The arene oxides, particularly 4-carbo-*tert*-butoxybenzene oxide (II), react rapidly with these enzymes; the rate of inactivation of GPDH by II is faster than the rate of inactivation of yeast glyceraldehyde-3-phosphate dehydrogenase by epoxides designed as substrate analogues (McCaul and Byers, 1976), and the rate of inactivation of YADH by II is much greater than that obtained with a 100-fold greater concentration of styrene oxide (Klinman, 1975). Both enzymes are tetramers with each subunit of GPDH containing one and YADH containing two essential cysteine residues at the active site. Alkylation by the arene oxides is apparently specific for the active-site thiol groups. When all enzymatic activity has been destroyed (after incubation with II for 30 min), titration with Nbs_2 indicates that 3.4 thiol groups of YADH and 2.1 thiol groups of GPDH have been alkylated. Alkylation of the cysteine residues at the active sites of YADH and GPDH is evidenced by the decreased effectiveness of the inhibition when the enzymes are saturated with substrate before the addition of the arene oxide. That alkylation of less than four residues is apparent may be due in part to the reversible nature of the inhibition. Thiol blocking reagents such as iodoacetic acid, *p*-nitrophenyl acetate, and acetyl phosphate are generally found to react with less than four cysteine residues of GPDH and to exhibit some variability in the number of sites affected (Park et al., 1961; Mathew et al., 1965; Harris and Perham, 1965; Koeppe et al., 1956). This

has been suggested to be due to the variable degree of oxidation of the thiol groups of the enzyme (Chance and Park, 1967).

Alkylation of the active-site thiol group of GPDH by arene oxides is also evidenced by the change in absorption of the enzyme at 360 nm (Figure 4). This absorption band (Racker band) has been suggested to be due to a covalent bond (Racker and Krimsky, 1952) or to a charge-transfer complex (Kosower, 1956) between bound NAD^+ and an electron donor of the enzyme. Cys-149, the active-site thiol residue, is thought to be the electron donor because of its location with respect to NAD^+ (Moras et al., 1975) and the pH dependence of the light absorption (Cseke and Boross, 1967). Moreover, the Racker band absorption of the GPDH–NAD^+ complex is diminished or abolished by oxidation of the thiol residues, by thiol alkylation with iodoacetic acid, by acylation with 1,3-diphosphoglycerate or acetyl phosphate (Racker and Krimsky, 1952; Trentham, 1968), and on treatment of the enzyme with *p*-chloromercuribenzoate (Velick, 1953). From Figure 4 it is apparent that alkylation of the enzyme sulfhydryl groups with arene oxide results in an actual *increase* in absorption of the Racker band. Increased Racker band absorption has also been observed when the active-site thiol group is alkylated with iodoacetamide or bromacetone (Boross and Cseke, 1967). Addition of heavy metal ions such as Ag^+ or Cd^{2+} results in modification of the charge-transfer complex (Boross, 1965) rather than the abolishment seen upon addition of Hg^{2+}. The above experiments indicate that the active-site thiol group is involved in the enzyme–coenzyme interaction but, apparently the presence of the cysteine residue in the anionic form is not a necessary requirement for this enzyme–coenzyme interaction. The fact that A_{360} may either increase or decrease after the reaction of enzyme with reagent and the fact that with arene oxides the change in absorption takes place over several hours after reaction with reagent is complete suggests that the Racker band absorption change, heretofore associated solely with thiol-group modification, may in fact be the result of changes in the overall tertiary structure of the protein.

As expected from the nucelophilic susceptibility indices of Table IV, 4-carbo-*tert*-butoxybenzene oxide (II) is the most effective of the three arene oxides toward inhibition of YADH activity. The half-life of YADH is decreased from 740 min in the absence of arene oxide to 0.8 min in the presence of 1.7×10^{-4} M II. The half-lives of Table I were determined by incubating YADH with the arene oxide at pH 7.5. If incubation had been carried out at a higher pH, even more dramatic inactivation would have been apparent (Table II). Although phenanthrene 9,10-oxide (I) is more readily attacked by nucleophiles and has a greater nucleophilic susceptibility index than 4-carboxybenzene oxide (III), the latter is more effective as an inhibitor of YADH activity. Presumably, the steric requirements of I decrease its effectiveness as an inhibitor as compared to III.

The relative effectiveness of the three arene oxides as inhibitors is the same toward both YADH and GPDH activity, II > III > I. Only in the case of incubation with 4-carboxybenzene oxide (III) is loss of GPDH activity not a first-order process. Presumably, this is due to loss of some of the inhibitor via aromatization (path A, eq 1) during incubation with enzyme. Inactivation of YADH by III is sufficiently rapid to make the competing aromatization reaction unimportant. The efficiency of II as an alkylating agent toward both enzymes is such that no competition from water-catalyzed decomposition of the inhibitor occurs, and the rate of spontaneous ring opening of I is sufficiently slow that it does not compete with inactivation of the enzymes by alkylation (Table IV).

The data of Table I appear to indicate that the arene oxides

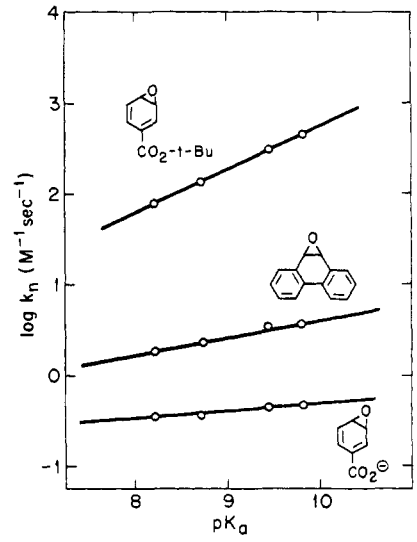
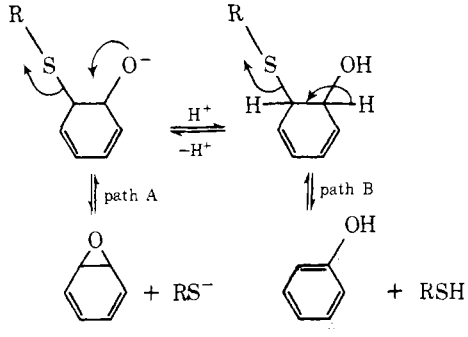


FIGURE 5: Second-order rate constants for attack of thiolate anions on 4-carbo-*tert*-butoxybenzene oxide, phenanthrene 9,10-oxide, and 4-carboxybenzene oxide vs. the pK_a of the thiol: 2-mercaptoethylamine = 8.22, glutathione = 8.72, 2-mercapthoethanol = 9.45, sodium thioglycolate = 9.82. These pK_as were determined in H_2O, 30 °C, μ = 1.0 with KCl (Reuben and Bruice, 1976).

are not as reactive with the active-site thiols of GPDH as with those of YADH. This, however, may be partially due to an artifact of the experimental procedure employed. Most samples of GPDH have some of the enzyme present in the inactive disulfide form which is regenerated by incubation with a thiol such as dithiothreitol. Reduction of the enzyme by thiol before the addition of arene oxide could not be carried out in this study because added thiol would react with the arene oxide. Dithiothreitol, however, was added to the assay solution and, as a result, additional enzyme sulfhydryl groups were released that in their disulfide form had been protected from reaction with arene oxide. In support of this conclusion is a 30% reduction in the initial activity of the enzyme when dithiothreitol is omitted from the assay.

The reversibility of the inactivation of YADH and GPDH by arene oxides may be attributable either to expulsion of the thiol group upon reclosure of the alkylated product back to arene oxide (eq 2, path A) or to expulsion of the thiol group upon aromatization of the alkyl group bound to the thiol (eq 2, path B). Preliminary kinetic evidence would appear to in-

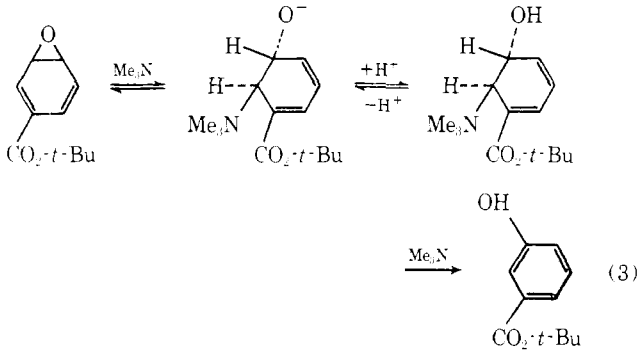


(2)

dicate that the reversible nature of the inhibition is more likely attributable to reclosure of the thiol addition product back to arene oxide. In the reaction of thiols with arene oxides a very slow reaction ($\sim 1 \times 10^{-5}$ s^{-1}) is apparent after the initial nucleophilic attack. This is presumably the aromatization reaction, and its rate of occurrence would appear to be too slow for it to account for the reversibility of the inhibition.

Brønsted plots for the reaction of thiolate anions with arene oxides I, II, and III are given in Figure 5. The second-order rate

constants for nucleophilic attack of thiolate anions on 4-carboxybenzene oxide (III) exhibit only a slight dependence on the pK_a of the thiol ($\beta = 0.1$). Small β values have also been reported for the reaction of thiols with benzene oxide (Reuben and Bruice, 1976) and phenanthrene 1,2-, 3,4-, and 9,10-oxides (Bruice et al., 1976b). A somewhat greater dependence on thiol pK_a ($\beta = 0.4$) is exhibited by the second-order rate constants for attack on 4-carbo-*tert*-butoxybenzene oxide. It may be that the greater β value is the result of a difference in the site of nucleophilic attack on this arene oxide. It has been established that in the case of benzene oxide and phenanthrene 9,10-oxide the primary site of nucleophilic attack is the carbon bound to the epoxide oxygen, resulting in trans 1,2-addition products. Recently, it has been shown that trimethylamine catalyzes the aromatization of 4-carbo-*tert*-butoxybenzene oxide via the addition-elimination mechanism given in eq 3 (Johnson and Bruice, 1975). This mechanism has been substantiated by



(3)

isolation and spectral identification of the addition intermediate. Evidently, the strongly electron-withdrawing carbo-*tert*-butoxy group changes the predominant site of nucleophilic attack. This suggests that in the case of II thiolate anion nucleophilic attack may also take place at the 3 position and give rise to a greater β value than exhibited by those arene oxides whose predominant site of attachment for the nucleophile is the 2 position.

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Two Human Trypsinogens. Purification, Molecular Properties, and N-Terminal Sequences†

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ABSTRACT: The two human trypsinogens have been isolated from human pancreatic juice in a sufficient amount to study molecular and structural properties. The purification procedure included filtration on Sephadex G-100 followed by ion-exchange chromatography on DEAE-cellulose. The two trypsinogens represent 19% of total proteins of pancreatic juice. Trypsinogen 1, the major form, is present in a quantity twice that of trypsinogen 2, which is the most anionic protein in human pancreatic juice. The two proteins have partial immunological identity, close molecular weights (23 438 and 25 006 for trypsinogens 1 and 2, respectively) and similar amino acid compositions. The N-terminal sequences are the same for the first 9 residues: Ala-Pro-Phe-Asp_4-Lys-Ile. The two proteins differ in the activation peptides released during the transformation to trypsins. Trypsinogen 2 liberates one octapeptide Ala-Pro-Phe-Asp_4-Lys while trypsinogen 1 liberates two peptides, the same octapeptide and the pentapeptide $(Asp)_4$-Lys.

The molecular properties of some human pancreatic proteolytic enzymes—chymotrypsins (Coan et al., 1971; Coan & Travis, 1972), trypsins (Travis & Roberts, 1969; Mallory & Travis, 1973; Feinstein et al., 1974), protease E (Mallory & Travis, 1975), elastases (Largman et al., 1976), and carboxypeptidases A (Peterson et al., 1976) and B (Geokas et al., 1975)—have recently been studied. However, very few zymogens of these enzymes have been isolated because of the difficulty in obtaining nonactivated starting material. We have previously reported the purification of the two human chymotrypsinogens (De Caro et al., 1975) and demonstrated that chymotrypsinogen A, the major form, is the only precursor of the three chymotrypsins isolated from autolyzed pancreas. Human pancreatic juice contains two anionic trypsinogens (Figarella et al., 1969; Clemente et al., 1972). The human trypsins have been extensively studied for their molecular properties and their interactions with proteinase inhibitors (Travis & Roberts, 1969; Mallory & Travis, 1973; Feinstein et al., 1974; Feeney et al., 1969). However, our studies of the inhibition spectra of these enzymes derived from the purified trypsinogens (Figarella et al., 1975) have pointed out some differences in the properties of trypsins obtained by controlled activation and trypsins prepared from activated material. These results demonstrate the importance of the isolation of zymogens. In addition, since trypsinogen activation seems to play an important part in pancreatitis (Ohlsson & Tegner, 1973), the study of human trypsinogens may be of clinical significance.

We have previously characterized the activation peptide of trypsinogen 2, the most anionic trypsinogen (Guy et al., 1976). In this paper, we report the purification and some molecular properties of the two human trypsinogens and we discuss the activation peptides of trypsinogen 1.

Materials and Methods

Materials

Human pancreatic juice was collected by catheterization of the pancreatic duct from patients with normal pancreatic function. Samples devoid of free proteolytic activity were lyophilized and stored at −20 °C.

Porcine enterokinase was kindly supplied by Dr. S. Maroux and pancreatic trypsin inhibitor (Kunitz) was a gift of Dr. E. Sache (Laboratoire Choay).

Trypsin inhibitor–Sepharose was prepared by activating Sepharose 4B (Pharmacia) with cyanogen bromide and coupling activated Sepharose with Kunitz pancreatic trypsin inhibitor according to the procedure of Cuatrecasas et al. (1968).

Methods

Column Chromatography. All chromatographic procedures of enzyme purification were carried out at 4 °C and performed in buffers containing 1 mM benzamidine to prevent the activation.

Protein concentration was determined by measuring the absorbance at 280 nm, using a mean extinction coefficient $E_{1cm}^{1\%} = 20.0$.

Trypsin Activation and Trypsin Activity. (a) Routine Activation in Chromatographic Fractions. Fractions containing trypsinogen were activated with porcine enterokinase (1% by

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