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Relationship Between Azo Dye Structure and Rat Hepatic Azoreductase Activity

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Abstract □ The rate of reduction was determined for a variety of azo dyes using the rat hepatic azoreductase enzyme system. In decreasing order, the rates of reduction for the azo dyes expressed as nmol of arylamine product formed/min/0.25 g of liver were amaranth (33.2), azosulfamide (32.5), orange G (12.4), 1,2-dimethyl-4-*p*-(carboxyphenylazo)-5-hydroxybenzene (CPA) (9.27), brilliant crystal scarlet (8.00), sulfachrysoidine (7.27), and Sudan I (1.03). A comparison of the partition coefficient with its rate of reduction indicated that the water-soluble azo dyes were reduced more rapidly than the lipid-soluble ones. Furthermore, higher rates of reduction were observed for those dyes containing electron-withdrawing groups on the aromatic rings.

Keyphrases □ Azoreductase—rat hepatic activity, relationship to azo dye structure, amaranth, azosulfamide, orange G, 1,2-dimethyl-4-*p*-(carboxyphenylazo)-5-hydroxybenzene, brilliant crystal scarlet, sulfachrysoidine, Sudan I □ Azo dyes—relationship between structure and rat hepatic azoreductase activity, amaranth, azosulfamide, orange G, 1,2-dimethyl-4-*p*-(carboxyphenylazo)-5-hydroxybenzene, brilliant crystal scarlet, sulfachrysoidine, Sudan I

The azo dyes and pigments form a large group of synthetic colorants used in many dye applications including foods, cosmetics, and pharmaceuticals. The chromophoric system consists of the azo group (N=N—) in association with one or more aromatic system. Monoazo, diazo, triazo, and polyazo dyes contain one, two, three, or more azo groups, respectively, yielding a large range of colors including reds, oranges, and purples (1).

In mammals, several enzyme systems are capable of reducing the azo bond including the liver (2–7) and the GI flora (4, 8–10). These azoreductase enzyme systems reduce a variety of azo dye substrates and do not appear to have a strict structural requirement other than the azo bond (2, 4, 10). A number of azo dyes, such as dimethylaminoazobenzene (butter yellow), amaranth, and others have carcinogenic or suspected carcinogenic activity (4). This toxic activity may be due to the parent compound, which contains the intact azo bond, or to the resulting arylamine. Although the mechanism of the reduction of the azo bond has been studied (2, 3, 5, 11, 12) only a few papers have compared chemical structure with the rates of reduction of the azo bond (2, 13). The objective of this study was to investigate the relationship of the structure of selected azo dyes to the rate of reduction to the corresponding amine by the rat hepatic azoreductase enzyme system. The rat hepatic azoreductase enzyme system obtained from the 10,000 × *g* supernatant fraction of liver homogenates was chosen since the mechanism of azo bond reduction by this enzyme preparation has been characterized by a number of investigators (2, 3, 5–7, 11, 12).

EXPERIMENTAL

Reagents and Chemicals—Azo dyes including brilliant crystal scarlet[1], orange G[1], Sudan I[1], amaranth[1], sulfachrysoidine[2], azosulfamide[2], and 1,2-dimethyl-4-(carboxyphenylazo)-5-hydroxybenzene[3] (CPA) were checked for chemical purity by either TLC on silica gel plates (*n*-butyl alcohol–acetic acid–water, 6:1:3) or ascending paper chromatography (*n*-butyl alcohol–acetic acid–water 12:3:5). Cation-exchange[4] and anion-exchange[4] resins were washed with several volumes of distilled water prior to use. NADP[5], glucose-6-phosphate[5], and nicotinamide[5], as well as other reagents and solvents, were used without further purification.

Partition Coefficient—A partition coefficient, K, in *n*-octanol–0.05 M phosphate buffer (pH 7.4) was determined for each azo dye in duplicate at room temperature. For water-soluble dyes, 1 mL of an aqueous dye solution (~10 μmol) was placed in an extraction tube containing 4 mL of 0.05 M phosphate buffer (pH 7.4). To this sample was added 5 mL of *n*-octanol previously saturated with distilled water. Lipid-soluble dyes were initially dissolved in the water-saturated 1-octanol and added to 5 mL of 0.05 M phosphate buffer (pH 7.4). Each sample was shaken, allowed to stand for a few minutes, and centrifuged. The distribution of the dye in each phase at equilibrium was obtained from absorbance of the dye in a spectrophotometer[6] at an appropriate wavelength. The partition coefficient for each dye was calculated with respect to a standard curve or from the absorbance of the aqueous or lipid phase before and after equilibration.

Preparation of Liver Homogenates—Male albino Charles River CD rats (180–220 g) were used for all studies. Rats were weighed and then sacrificed by decapitation. Each liver was excised and immediately rinsed with ice-cold 1.15% KCl (isotonic). The livers were trimmed, weighed, and homogenized in three volumes of 1.15% KCl using a polytef pestle and glass mortar. All subsequent procedures were performed at 4°C. The tissue homogenates were centrifuged at 10,000×*g* for 15 min in a refrigerated centrifuge. The 10,000×*g* liver supernatant containing the microsomal enzymes and soluble fraction was filtered through gauze. Approximately 15 mL of the 10,000×*g* liver supernatant was placed in 50-mL glass ampules and shell-frozen in dry ice–acetone. This enzyme preparation is stable at dry ice temperature for a week or more. Previous studies have shown that lyophilization of this preparation stabilized the enzymes for as long as 6 months with no apparent loss of activity (14).

[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] Sterling-Winthrop Research Institute, Rensselaer, N.Y.
[3] ICN Nutritional Biochemics, Cleveland, Ohio.
[4] Cation exchange resin (AG50W-X8, 200-400 mesh) and anion exchange resin (AGL-X2, 200-400 mesh); Bio-Rad Labs, Richmond, Calif.
[5] Sigma Chemical Co., St. Louis, Mo.
[6] UV/Vis; Beckman Instruments, Inc., Irvine, Calif.

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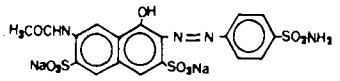
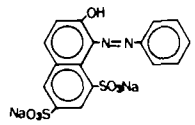
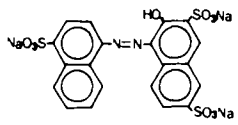
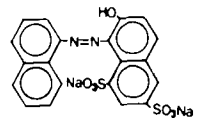
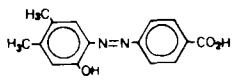
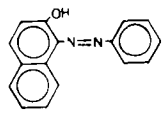
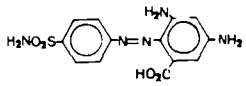
Table I—Structure and Partition Coefficients of Various Azo Dyes

Dye	Structure	Partition Coefficient[a]
Azosulfamide		0.149
Orange G		0.163
Amaranth		0.176
Brilliant crystal scarlet		0.212
CPA[b]		0.950
Sudan I		4.33
Sulfachrysoidine		5.67

[a] Partition coefficient for *n*-octanol–0.05 M phosphate buffer. [b] 2-Dimethyl-4-*p*-(carboxyphenylazo)-5-hydroxybenzene

Enzyme Assays—The shell-frozen 10,000×*g* liver supernatant was used within 24–48 h and was slowly thawed on ice just prior to the azoreductase assay. Each azo dye (10 μmol) was incubated anaerobically in 5 mL of a reaction mixture containing 0.5 μmol of NADP, 40 μmol of glucose-6-phosphate, 20 μmol of nicotinamide, 22 μmol of magnesium chloride, 50 μmol of phosphate buffer, pH 7.4, and enzyme (10,000 × *g* liver supernatant, equivalent to 250 mg of liver). The reaction mixtures were incubated for 0, 5, 15, 30, 45, and 60 min at 37°C using a continuous nitrogen flush to obtain anaerobic conditions, with gentle shaking. The reaction was stopped by the addition of 5 mL of 10% trichloroacetic acid solution. The mixture was centrifuged, and the supernatant was assayed for the arylamine. The *in vitro* azoreductase activity of each azo dye was measured in parallel with the azoreductase activity of CPA, which acted as a positive control.

The azoreductase activity of CPA was measured by the rate of formation of *p*-aminobenzoic acid (13, 15), using the Bratton and Marshall procedure (16). Sulfanilamide was separated from azosulfamide or sulfachrysoidine using a cation-exchange resin. The resin was prepared according to the method of Hernandez *et al.* (3), and the amount of sulfanilamide was quantitated by the Bratton and Marshall procedure (16). Aniline and naphthylamine were separated similarly from their corresponding amines by column chromatography using an anion-exchange resin. For these assays, a 5-mL aliquot of the trichoroacetic acid supernatant was placed on the column along with an additional 5 mL of distilled water. The combined eluates were collected, mixed, and a 5-mL aliquot was used for the determination of either aniline or naphthylamine by the method of Bratton and Marshall (16). For each azo dye the quantitation of the arylamine product (either *p*-aminobenzoic acid, sulfanilamide, aniline, or naphthylamine) from each incubation flask was calculated by a suitable processed standard curve.

The enzymatic azoreduction of amaranth was measured by following the rate of formation of its metabolite, 4-amino-1-naphthalenesulfonic acid. Samples (15 μL) of the trichloroacetic acid supernatant collected after incubation with amaranth were subjected to HPLC[7] at room temperature on a microparticulate reverse-phase column[8] (4 mm × 30 cm). Amaranth and its metabolites were eluted with a mobile phase containing 2.5% acetonitrile in 0.01 M acetate buffer, pH 4 at a flow rate of 4 mL/min chart speed 1.27 cm/min. The compounds were detected by UV absorbance at 254 nm. The metabolite, 4-amino-1-naphthalenesulfonic acid, was quantitated from a processed standard curve.

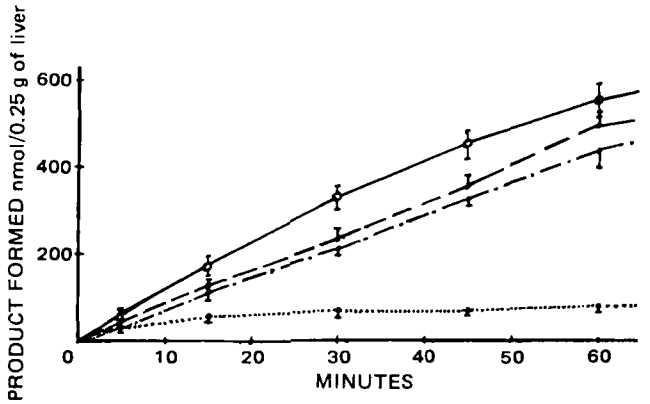


Figure 1—*Average hepatic azoreductase activity. Each azo dye (10 μmol) was incubated anaerobically with the 10,000×g rat liver supernatant and a NADPH-generating system (see text). The amount of arylamine product formed by the reduction of each dye was measured at the indicated time intervals. Key: (—) CPA, results include the* SEM; *(- - -) sulfachrysoidine; (- - -) brilliant crystal scarlet (·····) Sudan I. See Table I for individual values.*

RESULTS

The structures of various azo dyes and their partition coefficients (1-octanol–0.05 M phosphate buffer, pH 7.4) are shown in Table I. Dyes were chosen for their availability and chemical purity. The very lipid-soluble dyes sulfachrysoidine and Sudan I have partition coefficients of 5.67 and 4.33, respectively; the highly water-soluble dyes azosulfamide and amaranth have partition coefficients of 0.149 and 0.187, respectively.

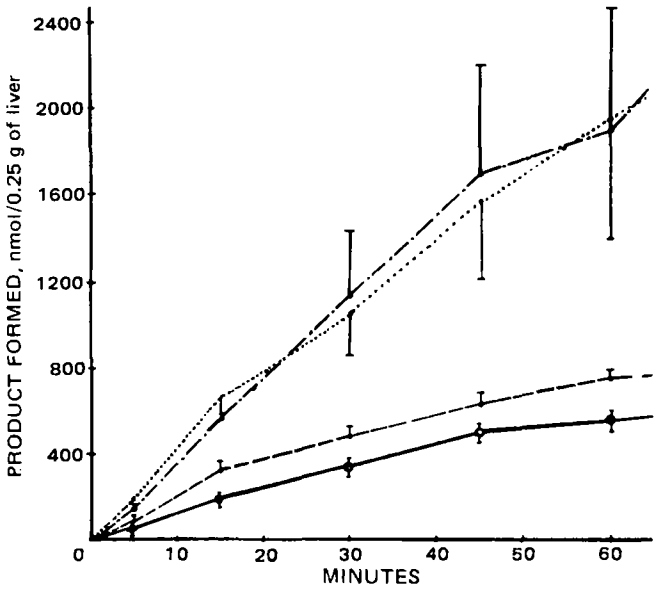


Figure 2—*Average hepatic azoreductase activity. Each azo dye (10 μmol) was incubated with the 10,000×g rat liver supernatant and a NADPH-generating system (see text). The amount of arylamine product formed by the reduction of each dye was measured at the indicated time intervals. Key: (—) CPA, results include the* SEM; *(- - -) amaranth; (·····) azasulfamide (- - -) orange G. See Table I for individual values.*

[7] Model ALC/GPC 204; Water Associates, Milford, Mass.
[8] μBondapak C_{18}; Water Associates, Milford, Mass.

Table II—Hepatic Azoreductase Activity [a]

Dye	n	Amount of Product Formed, nmol/0.25 g of liver					Azoreductase Activity[b]	r
		5 min	15 min	30 min	45 min	60 min		
CPA[c]	15	49.6	187	331	450	548	9.27	0.993
		(10.9)	(15.3)	(29.7)	(36.5)	(43.5)	(0.74)	
Amaranth	2	163	579	1130	1700	1890	33.2	0.990
		(50.1)	(19.1)	(304)	(577)	(577)	(11.9)	
Azosulfamide	3	198	669	1050	1576	1958	32.5	0.996
		(36.4)	(191)	(209)	(350)	(534)	(8.58)	
BCS	3	53	127	230	357	493	8.00	0.968
		(3.34)	(6.67)	(25.2)	(23.4)	(63.7)	(0.93)	
Sulfachrysoidine	3	36	124	226	329	438	7.27	0.996
		(6.94)	(18.3)	(10.9)	(6.67)	(36.3)	(0.40)	
Orange G	3	85	327	485	632	745	12.4	0.976
		(45.1)	(37.2)	(32.9)	(36.1)	(32.6)	(0.59)	
Sudan I	2	38	58	64	70	78	1.03	0.849
		(7.5)	(7.5)	(6.0)	(5.0)	(2.5)	(0.07)	

[a] Standard error in parentheses. [b] Azoreductase activity is equal to the slope of the linear regression line calculated from the nmoles of azo dye formed with respect to time (0–60 min). The azoreductase activity is expressed as nmoles of product formed/min/0.25 g liver. Each azoreductase activity was measured concurrently with CPA activity. [c] 1,2-Dimethyl-4-*p*-(carboxyphenylazo)-5-hydroxybenzene.

The rate of reduction of each azo dye by the 10,000×*g* supernatant fraction obtained from rat liver homogenates was measured by observing the formation of the arylamine with respect to time. The rate of reduction of CPA was measured concurrently with the azoreductase activity of each of the other azo dyes. Therefore, the integrity and consistency of the azoreductase enzyme preparation was verified. The rates of formation of the arylamine from each substrate are shown on Figs. 1 and 2. Azoreductase activity appeared to be linear for the reduction of each azo dye from 0 to 60 min. A regression line was calculated by the least-squares method comparing the product formed with respect to time (0 to 60 min). The slope of this computed regression line is equivalent to the azoreductase activity for each dye and is expressed as nmol of product formed/min/0.25 g of liver (Table II). With the exception of Sudan I, the coefficients of correlation for each azoreductase activity were excellent, implying that the azoreductase activities were linear for 60 min. The correlation coefficient for the azoreductase activity of Sudan I was 0.85.

The partition coefficient in 1-octanol–0.05 M phosphate buffer (pH 7.4) was determined for each dye and compared with its azoreductase activity. A direct correlation between partition coefficient and azoreductase activity was not observed. However, if the azoreductase activity is compared with the reciprocal of the partition coefficient, a regression line with a slope of 3.24 and correlation coefficient of 0.74 can be calculated (Fig. 3). However, if the azoreductase activity for orange G is excluded from the regression line calculation, a slope of 4.00 and correlation coefficient of 0.84 are obtained. Moreover, using the same data and comparing the rate of azoreductase activity with the logarithm of the partition coefficient (log K), a regression line calculated with a slope of −0.20 and a correlation coefficient of −0.75. Thus, there appears to be a trend in which azoreductase activity increases with aqueous solubility, but the correlation is not very strong.

DISCUSSION

From observation of each azo dye structure, factors other than lipid solubility may also affect the rate of azoreductase activity. Enzymatic reduction of azo dyes may either be a one- or two-electron transfer (17, 18). Therefore, the rate of reduction should be influenced by the electron density at the azo bond. Structural variations which can modify this electron density include the nature of the substituents around the ring systems and the potential for intramolecular hydrogen bonding of the substituents with the azo bond (19). For example, electron-withdrawing groups will decrease the electron density, thereby enhancing azo bond reduction. Moreover, hydroxyl or amino groups in close proximity to the azo bond can enhance reduction by hydrogen bonding with the azo bond. Furthermore, the nature of the aromatic systems around the azo bond should effect the rate of azo reduction. Thus, naphthalene provides greater steric hindrance, which should decrease the azoreductase activity in comparison with a phenyl ring.

Several different pathways for the reduction of azo dyes exist in the rat hepatic microsomal fraction, including NADPH cytochrome *c* reductase and cytochrome P_{450} (2–4, 10, 12). Apparently azo dyes with low reduction potentials are reduced more rapidly by cytochrome P_{450}, whereas azo dyes with higher reduction potentials are reduced more rapidly by NADPH cytochrome *c* reductase (5). In addition, amaranth azo reduction occurs primarily *via* cytochrome P_{450} and does not stimulate NADPH oxidation nor superoxide formation (5, 17). Furthermore, the cytosol fraction contains an azoreductase enzyme which is more selective in reducing azo dyes compared with the microsomal fraction (6, 7, 20). Many investigators have also noted that the azo dyes may be reduced nonenzymatically using reduced $NADPH_2$ (2, 3, 12) or reduced flavins (2, 12, 21). The nonenzymatic azo dye reduction is related to the reduction potential between $NADP^+$/NADPH and the substrate which acts as a final electron acceptor (20). Therefore, important factors for comparing the rates of reduction of azo dyes by hepatic enzymes include the reduction potential of the substrate and its affinity for the enzyme system.

The results of this investigation demonstrated that Sudan I had the slowest rate of azo dye reduction probably due to both the lack of electron-withdrawing groups on the aromatic ring system and its high lipid solubility. Higher rates of azo reduction are observed for those dyes containing electron-withdrawing substituents such as a sulfonamido group in sulfachrysoidine, a sulfonate group in both brilliant crystal scarlet and orange G, and carboxylic group in CPA. The higher rate of

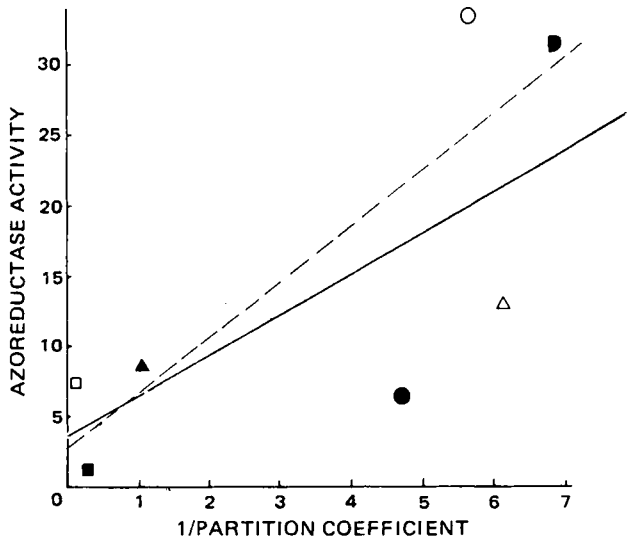


Figure 3—*Correlation of azoreductase activity and partion coefficient for each dye. The azoreductase activity (nmol of product formed/min 0.25 g of liver) for each dye is compared with the reciprocal of the corresponding partition coefficient (n-octanol–0.05 M phosphate buffer, pH 7.4). The calculated regression line (——) including the orange G azoreductase activity gives a slope of 3.24 and correlation coefficient of 0.74. The calculated regression excluding the orange G azoreductase activity (- - -) gives a slope of 4.00 and a correlation coefficient of 0.84. Key: (■) Sudan I; (□) sulfachrysoidine; (▲) CPA; (●) brilliant crystal scarlet; (△) orange G; (◑) azosulfamide; (○) amaranth.*

azo bond reduction observed with orange G compared with brilliant crystal scarlet may be due to steric hindrance in the latter. Amaranth and azosulfamide had the highest rates of azoreductase activity due to the presence of more electron-withdrawing groups (sulfonates) in the ring system. Sulfachrysoidine, a lipid-soluble azo dye, as indicated by its large partition coefficient has a greater than anticipated rate of azo reduction due to the presence of the sulfonamido group. The potential for the formation of an intramolecular hydrogen bond between the hydroxyl or amino group at the *ortho* position of the aromatic rings and the azo nitrogen is common to all the dyes investigated in this study and, therefore, should not be a major contributing factor in the observed differences in the rates of azo bond reduction.

Because of the wide variety of structures of azo dyes, it is difficult to determine which physicochemical factor influences the rate of azo reduction by the hepatic azoreductase enzymes the most. This investigation used the 10,000×*g* supernatant fraction from liver homogenates as the enzyme source to determine if an overall generalization could be determined for the rates of reduction of azo dyes. The results of this investigation show that both the partition coefficient and the presence of electron-withdrawing substituents will influence the rate of reduction of the azo bond. Apparently, the larger the number of electron-withdrawing groups, the more rapid the rate of reduction by the hepatic azoreductase system.

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High-Performance Liquid Chromatographic Analysis of the Semisynthetic Epipodophyllotoxins Teniposide and Etoposide Using Electrochemical Detection

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Received June 28, 1982, from the *Pharmacokinetics and Pharmacodynamics Section, St. Jude Children's Research Hospital, Memphis, TN 38101.* Accepted for publication December 13, 1982.

Abstract □ A high-performance liquid chromatographic (HPLC) assay was developed for the quantitation of two structurally similar and highly active anticancer drugs, etoposide (I) and teniposide (II), and their potential metabolites (hydroxy acid, picrolactone, and aglycone). The assay utilizes electrochemical detection, which imparts specificity and sensitivity sufficient to detect ≥20 ng/mL in plasma, urine, and CSF. The mean assay coefficients of variation were 5.1 and 8.1% for teniposide (10 μg/mL) and etoposide (5 μg/mL), respectively. The extraction efficiencies were 86% for etoposide, 70% for its hydroxy acid metabolite, 66% for teniposide, and 54% for the hydroxy acid of teniposide. The correlation coefficient of the multilevel standard curve was ≥0.995 over the concentration range of 0.05–50 μg/mL for the parent drugs and metabolites extracted from plasma. This method has been used to determine the concentrations of the parent drugs and their metabolites in the plasma, urine, and CSF of patients with cancer.

Keyphrases □ Etoposide—analysis with metabolites, high-performance liquid chromatography with electrochemical detection, human urine, plasma, and CSF □ Teniposide—analysis with metabolites, high-performance liquid chromatography with electrochemical detection, human urine, plasma, and CSF

Two relatively new and highly active antineoplastic drugs, etoposide {4′-demethylepipodophyllotoxin 9-[4,6-*O*-(*R*)-ethylidene-β-D-glucopyranoside], (I)} and teniposide {4′-demethylepipodophyllotoxin 9-[4,6-*O*-(*R*)-2-thenylidene-β-D-glucopyranoside], (II)} have clinical activity in childhood leukemias, lymphomas, neuroblastomas, brain tumors, and germ cell tumors and adult lung, brain, bladder, and testicular cancers as well as adult leukemias and lymphomas (1–5). The proposed metabolic scheme was derived from the known molecular transfor-

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