EtOAc was washed with brine and dried over anhydrous Na_2SO_4. The solvent was evaporated at 32 °C (15 mm) to an oily residue, which was dissolved in 30 mL of acetone and 30 mL of ether. The solution was treated with solid potassium 2-ethylhexanoate until the solution was pH 6. The precipitate was collected and dried to yield 3.9 g (76%): IR (KBr) 1790 (s), 1700 (m), 1620 (s), 1460 (m), 1370 (s), 1310 (s), 1200 (s), 1140 (s), 955 (m), 740 (m) cm^{-1}; ^1H NMR (D_2O) δ 1.68 (s, 3, CH_3), 3.2–3.9 (m, 2, C_6H_2), 4.0–4.4 (m, 2, CH_2Cl), 4.3 (s, 1, C_3 H), 5.02 (d, 1, C_5 H). Anal. ($C_8H_7ClKNO_5S\cdot 2H_2O$) C, N; H: calcd, 3.24; found, 3.69.

Pivaloyloxymethyl 2β-(Chloromethyl)-2α-methyl-3α-carboxylate 1,1-Dioxide (8). To a solution of 1 g (0.0031 mol) of 7 dissolved in 15 mL of DMAC was added 470 mg (0.0031 mol) of pivaloyloxymethyl chloride. The solution was stirred for 24 h. The mixture was filtered, poured into 120 mL of H_2O, and extracted with EtOAc. The EtOAc was washed 9 times with H_2O and dried over anhydrous $MgSO_4$. The EtOAc was evaporated at 35 °C (15 mm) to an oil, which was chromatographed in Sili*CAR* CC-7 (80 $MeCl_2$/20 EtOAc). The amorphous solid obtained was dissolved in 50 mL of Skellysolve B and stored at 15 °C for 10 h. The crystals were collected by filtration, affording a total of 239 mg: mp 94–95 °C; IR (KBr) 1800 (s), 1770 (s), 1320, 1125 (w) cm^{-1}; ^1H NMR ($CDCl_3$) δ 1.25 [s, 1, $(CH_3)_3C$], 1.65 (s, 1, CH_3), 3.3–3.75 (m, 2, C_6H_2), 3.75–4.2 (m, 2, CH_2Cl), 4.6 (s, 1, C_3 H), 4.6–4.8 (m, 1, C_5 H), 5.7–6.0 (m, 2, OCH_2O). Anal. ($C_{13}H_{20}ClNO_5S$) C, H, N.

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Interaction of Aromatic Dyes with the Coenzyme A Binding Site of Choline Acetyltransferase

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The interaction of a series of aromatic dyes with the coenzyme A binding site of choline acetyltransferase was studied. Several of the dyes were very potent inhibitors of the enzyme. With few exceptions, inhibition was competitive with respect to acetylcoenzyme A and noncompetitive with respect to choline. It appears likely that inhibition by dyes such as Reactive Blue 2 (Cibacron Blue F3GA) or Congo Red, as in the case of coenzyme A interactions, involves hydrophobic bonding, as well as a coulombic interaction with an arginine residue.

Considering that chemotherapy was based on Ehrlich's studies of the specificity of dye interactions, it seems surprising that, even though studies of the structure–action relationships of aromatic dyes led to the development of many medicinally useful agents, the molecular basis of the specificity of dye interactions was studied little until recent years. Glazer[1] noted that aromatic dyes with no obvious structural relationships to normally attached ligands can bind very tightly to globular proteins, predominantly to areas overlapping binding sites for coenzymes or substrates. Crystallographic studies of dehydrogenase–dye complexes have confirmed this.[2-4] The specificity of the interaction of aromatic dyes with the coenzyme-binding sites of certain enzymes is confirmed by the utility of solid supports to which aromatic dyes are attached in the chromatographic purification of such biopolymers. While numerous dyes have been utilized for this purpose, columns carrying Cibacron Blue F3GA have found particularly wide use.[5]

The present study is concerned with the specificity of the interaction of aromatic dyes with choline acetyltransferase (ChA), the enzyme catalyzing the reaction of choline with acetyl-CoA to form acetylcholine, a molecule essential for the functions of the nervous system.

Roskoski et al.[6] noted that Blue Dextran columns could be used for the purification of ChA. This procedure was improved by Hersh et al.,[7] who used acetyl-CoA as eluant.

Thompson and his co-workers[8] had claimed that the inhibitory action of Reactive Blue 2 (Cibacron Blue F3GA), the chromophore of Blue Dextran, was based on the structural resemblance of this dye to the adenosyl diphosphoryl portion of coenzymes; however, several workers[9-11] questioned the assumption that dye-binding sites and coenzyme-binding sites were very similar. During a study of the abilities of a series of derivatives of CoA to serve as inhibitors of ChA, we noted that not only Reactive Blue 2 but the much simpler molecule 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) appeared to be a competitive inhibitor of acetyl-CoA.[12,13] These findings

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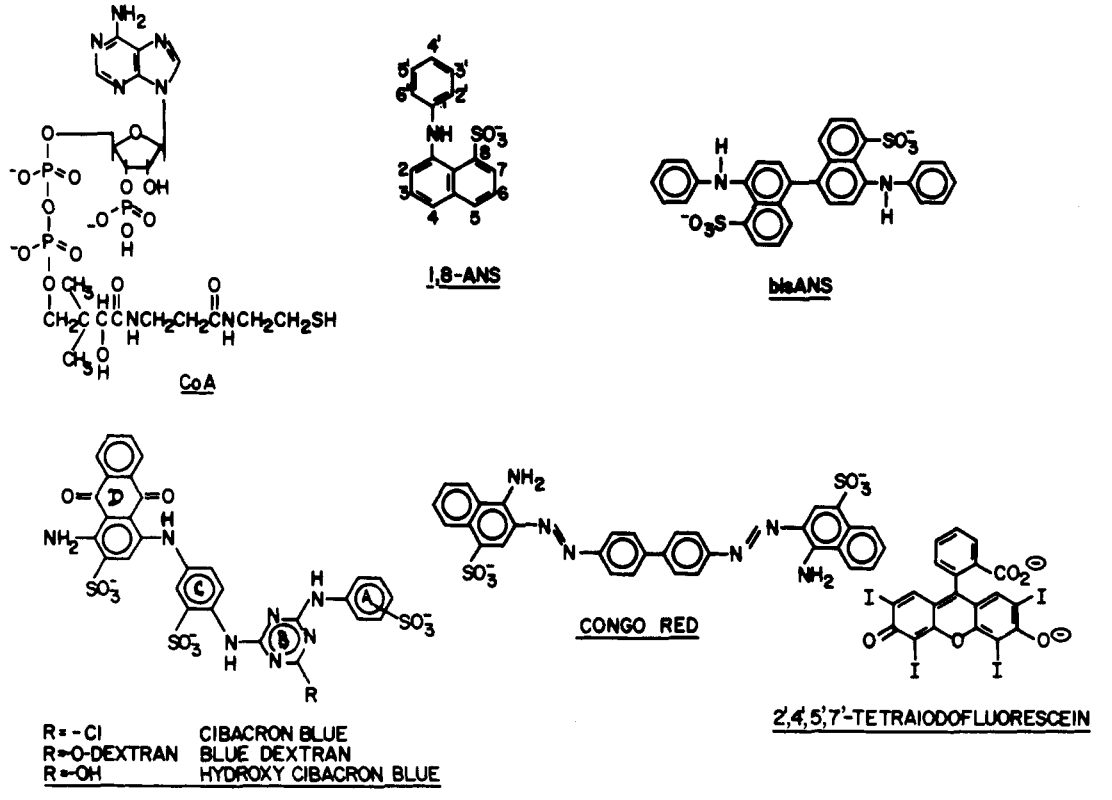
Chart I. Structures of CoA and Several Dyes Capable of Inhibiting ChA



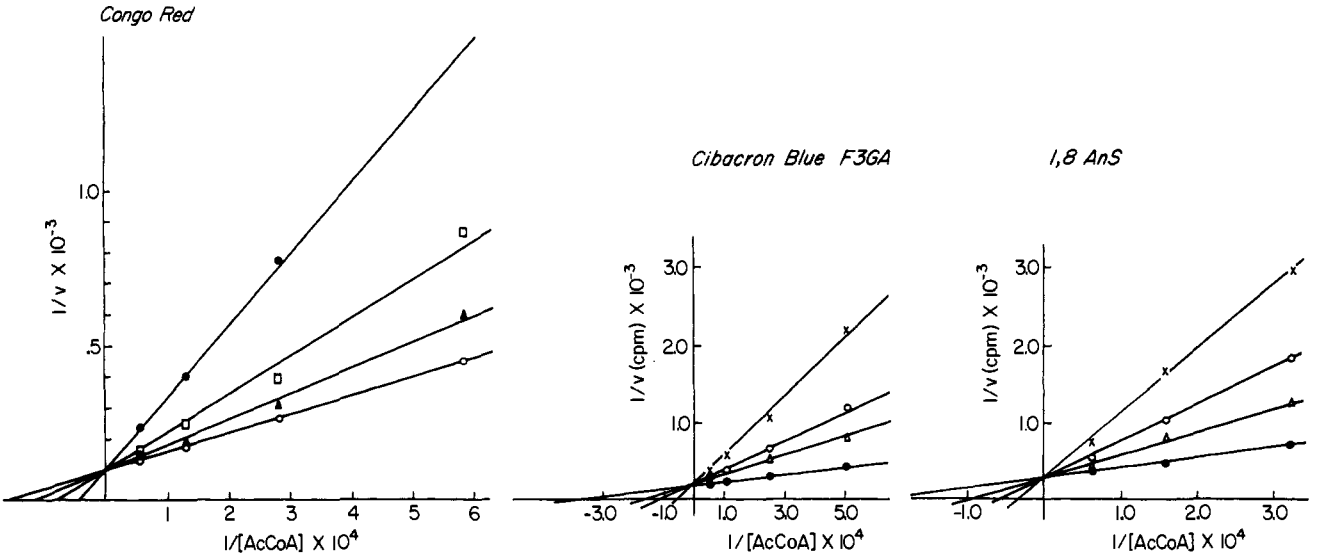


Figure 1. Double-reciprocal plots for inhibition of ChA by Congo Red, Reactive Blue 2, and 1,8-ANS. Congo Red concentrations: (○) no inhibitor, (▲) 1.56×10^{-7} M, (□) 2.6×10^{-7} M, (●) 3.6×10^{-7} M. Reactive Blue 2 concentrations: (●) no inhibitor (△) 1.86×10^{-6} M, (○) 2.48×10^{-6} M, (X) 3.72×10^{-6} M. 1,8-ANS concentrations: (●) no inhibitor, (△) 3.48×10^{-4} M, (○) 4.18×10^{-4} M, (X) 5.22×10^{-4} M.

suggested that a systematic study of the specificity of the dye interactions of ChA might provide useful information about the topography of this enzyme's binding sites. The present study describes the interaction of ChA with 1,8-ANS, its dimer, and several of its positional isomers, with the meta and para isomers of Reactive Blue 2, and with the hydroxy derivative of this dye. Also studied were the inhibitory potencies of Blue Dextran, Reactive Red 120 (Procion Red HE3B), tetraiodofluorescein, and Congo Red.

Results

Table I summarizes the K_i values and standard errors seen when ChA, isolated from squid head ganglia, was inhibited by aromatic dyes in the presence of variable acetyl-CoA concentrations. All experiments were carried out in the presence of a saturating concentration (1×10^{-2} M) of choline. The structures of CoA and of several of the dyes are shown in Chart I. AMP, 3',5'-ADP, NAD^+, NADH, $NADP^+$, and NADPH did not inhibit ChA at a concentration of 10^{-4} M. The dyes were noncompetitive inhibitors with respect to choline. K_i values agreed well when determined with either acetyl-CoA or choline being used as variable substrates. The aromatic dyes yielded Lineweaver–Burk plots suggesting competitive inhibition with respect to acetyl-CoA, except for Procion Red, 1,5-ANS, and 2,6-ANS which were noncompetitive inhibitors.

As can be seen in Table I, a relatively high variability in K_i values was encountered when the inhibition by Re-

Table I

inhibitor	K_i, $\times 10^{-6}$ M (±SE)
CoA	75 (4)
$CoSSCH_3$	2.3 (0.3)
1,8-ANS	200 (50)
2,8-ANS	150 (60)
1,2-ANS	*a*
1,5-ANS	1500 (500)
2,6-ANS	800 (300)
bis(ANS)	3 (0.5)
naphthalene-1-sulfonate	1600 (500)
tetraidofluorescein	8 (3)
Cibacron Blue (mixture, meta and para isomers)	1.2 (0.5)
Cibacron Blue (para isomer)	0.8 (0.5)
Cibacron Blue (meta isomer)	0.8 (0.5)
Hydroxy Cibacron Blue	1.8 (0.5)
Blue Dextran	0.4 (0.1)[b]
Blue Dextran	74 (10)[c]
Congo Red	0.1 (0.06)
Procion Red	0.3 (0.1)
AMP	1800[d]
3',5'-ADP	1800[d]
NAD^+, NADH, $NADP^+$, NADPH	*e*

[a] No inhibition at limit of solubility (4.4×10^{-3} M). [b] K_i based on molecular weight of 2 000 000 for Blue Dextran. [c] K_i based on content of Cibacron Blue F3GA in Blue Dextran as determined by the procedure of Easterday and Easterday.[28] [d] I_{50}. [e] No inhibition at 10^{-4} M.

active Blue 2 and Congo Red was studied. Presumably, the tendency of these dyes to form aggregates at concentrations as low as 10^{-5} to 10^{-6} M, with aggregate formation extremely sensitive to small changes in salt concentration and temperature,[14,15] is responsible for the variability encountered. While slope replots[16] for CoA and CoA methyl disulfide were linear, slope replots for Cibacron Blue, Congo Red, and the other dyes examined were parabolic (concave upwards) at high inhibitor concentrations. Again, the possible role of aggregation in this phenomenon cannot be eliminated.

Discussion

Chromatographic columns carrying Reactive Blue 2 or other aromatic dyes have found wide application in the purification of biopolymers. It has been claimed that Reactive Blue is complementary to the "dinucleotide fold" of enzymes, i.e., the region to which the adenosyl diphosphoryl portion of coenzymes is attached.[8] Indeed, it was postulated that tight binding by Reactive Blue or related dyes was diagnostic for the presence of the "dinucleotide fold" in biopolymers.[17] As already noted, the postulated identity of the dye-binding site and the coenzyme-binding site has been questioned by several workers.[9-11]

Since Reactive Blue 2 contains a chloro-1,3,5-triazino residue, the possibility of this normally very reactive chlorine being involved in enzyme inhibition was examined by comparing the inhibitory potencies of Reactive Blue, its hydroxy derivative, and Blue Dextran. The hydroxy derivative was prepared by the procedure of Moe and Piszkiewicz.[18] It can be seen in Table I that the chloro group of the dye is not involved in the inhibition of ChA.

Reactive Blue 2, even if purified,[19] is a mixture of isomers in which a sulfonate residue is attached either to the meta or the para position of the A ring of the dye. In contrast to recent work with liver alcohol dehydrogenase, which is inhibited more effectively by the para than by the meta isomer,[4] we found the isomers to be equiactive in inhibiting ChA.

As can be seen in Table I, ChA shows relatively little structural specificity in its ability to bind aromatic dyes. Congo Red, an arylazo dye, is an even better inhibitor than Reactive Blue, an arylamino dye. Indeed, Congo Red appears to be the most potent competitive inhibitor of ChA known. The presence of sulfonate groups is not essential for effective inhibition of the enzyme. Tetraiodofluorescein, a dye lacking sulfonate groups, is a good inhibitor of ChA.

We had noted some time ago that a much smaller molecule than the above aromatic dyes, 1,8-ANS, inhibited ChA competitively with respect to acetyl-CoA.[12] The inhibitory activity of the dimer of 1,8-ANS, 4,4'-bis[1-(phenylamino)naphthalene 8-sulfonate], exceeded that of the monomer. The relatively high inhibitory potency of the dimer had been noted in other enzyme systems.[20] The cyclic analogue of 1,8-ANS, *N*-phenyl-1,8-naphthalene sultam, was synthesized by the procedure of Cory et al.[21] but proved too insoluble for determination of its inhibitory activity. 1-(Phenylamino)naphthalene, the analogue of 1,8-ANS lacking the sulfonate group, also had negligible solubility in water. The inhibitory activity of naphthalene-1-sulfonic acid, the ANS analogue lacking the arylamino group, was very low.

There was some specificity in the inhibitory actions of positional analogues of 1,8-ANS, as can be seen in Table I. The 1,8 and 2,8 isomers were relatively good inhibitors, yielding Lineweaver–Burk plots suggestive of competitive inhibition with respect to acetyl-CoA. The 1,5 and 2,6 isomers were rather poor, noncompetitive inhibitors of acetyl-CoA binding. 1,2-ANS was inactive up to its limit of solubility.

The observation that small aromatic dyes bind relatively tightly to ChA is paralleled by similar observations in the alcohol dehydrogenase system. Bränden et al.[22] showed that NAD^+ attachment in liver alcohol dehydrogenase was blocked competitively by a series of aromatic molecules, including 1,8-ANS. Subsequently, it was demonstrated that these molecules bind to the adenosine binding site of the enzyme.[3,22] On the other hand, the binding of Reactive Blue 2 to horse liver alcohol dehydrogenase involves attachment to the pyrophosphate binding site as well as to the adenosine binding site,[4] while the A ring of the dye and the nicotinamide ribose portion of the coenzyme interact with different portions of the enzyme. It may be assumed that in the case of blockade by Reactive Blue and by Congo Red, the adenosine and the 3'-ribose phosphate binding sites of ChA are involved.

Janin and Chothia[23] had proposed that coenzyme binding can be related directly to the area of protein surface protected from contact with solvent with primarily

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hydrophobic interactions taking place. This conclusion does not appear applicable to CoA–ChA interactions. Elution of ChA from Blue Dextran columns can be accomplished readily with 0.5 M KCl, while 60% ethylene glycol is required for elution to take place in the absence of salt. This observation, coupled with the fact that the 3′-phospho group of CoA and acetyl-CoA is essential for tight binding to ChA,[12,13] suggests the importance of coulombic interactions. We had shown previously that ChA binds increasingly tightly to alkylagarose columns as the length of the alkyl group is extended.[13] These findings have been cited as supporting the importance of hydrophobic interactions in ChA binding. The CoA-binding and dye-binding studies suggest that coulombic interactions are at least as important as hydrophobic interactions when CoA or aromatic dyes are attached to this enzyme.

A recent X-ray diffraction study of the binding of the para isomer of Reactive Blue 2 to horse liver alcohol dehydrogenase[4] showed an interaction of a sulfonate group in the para position of ring A with an arginine guanidino group, while anthraquinone was attached to the adenine binding site.[4] We are assuming that, in the case of ChA, the anthraquinone portion of the dye also interacts with the adenosine binding site, while the sulfonate group interacts with an active-site arginine residue. We have already shown that "specific" arginine reagents, such as phenylglyoxal,[24] inhibit ChA, with CoA being more effective than 3′-dephospho-CoA in protecting against inhibition.[25a,b]

The present studies suggest that aromatic dyes should be useful as probes of the CoA binding site of ChA. Since tetraiodofluorescein is highly fluorescent while Reactive Blue 2 and Congo Red show induced CD spectra when introduced into the asymmetric environment of enzyme binding sites,[26,27] these dyes should be useful not only for studying the topography of the CoA binding site but also for studying conformational changes induced by choline, the other substrate of the enzyme.

Experimental Section

Aromatic Dyes. Generous samples of Reactive Blue 2 (isomer mixture), as well as of the meta and para isomers, were obtained from Dr. H. Bosshard of Ciba-Geigy, Basel, Switzerland. Purification was carried out by the procedure of Beissner and Rudolph.[19] The hydroxy derivative of Cibacron Blue was prepared from the chlorotriazine by the procedure of Moe and Piszkiewics.[18] Completion of the reaction was ascertained by showing that the C–Cl stretching band in the infrared spectrum had disappeared.[18] Thin-layer chromatography was used to establish lack of contaminants in the purified compounds. 1,8-ANS and its 1,2, 1,5, 2,6, and 2,8 isomers were purchased from Molecular Probes, Roseville, MN, as was the dimer of 1,8-ANS 4,4′-bis[1-(phenylamino)naphthalene 8-sulfonate]. All compounds were recrystallized. Procion Red HE3B (Reactive Red 120) was a gift from the Amicon Corp., Lexington, MA. The cyclic analogue of 1,8-ANS was synthesized by the procedure of Cory et al.[21] The content of Reactive Blue 2 in Blue Dextran (Pharmacia) was determined by the procedure of Easterday and Easterday.[28]

Choline Acetyltransferase (ChA). ChA derived from squid head ganglia was partially purified by the procedure of Husain and Mautner[29] using the following modifications: The 40–30% $(NH_4)_2SO_4$ extraction step and the chromatography on mercurial Sepharose were omitted. Chromatography on phosphocellulose resin was followed by chromatography on hydroxylapatite, yielding activity ranging from 3 to 7 $\mu M\ min^{-1}$ (mg of protein)$^{-1}$. The enzyme was stabilized by the addition of ethylene glycol to a concentration of 10% (v/v) to all buffers. Recovery was ~40% of starting enzyme activity. The enzyme assay procedure of Fonnum[30] was used; inhibition assays, in the presence of 10 mM choline, were carried out as described previously,[12] except that lysozyme, previously used by us to stabilize ChA, was omitted because of its ability to bind aromatic dyes. [1-^{14}C]Acetyl-CoA (or [2-^{3}H]acetyl-CoA) for enzyme assays was purchased from New England Nuclear Corp. Inhibition data were analyzed by the procedure of Cleland,[31] utilizing the computer programs described to obtain K_i and standard error values. A DEC System 10 computer was used.

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Interaction of 5-Ethynyl-2′-deoxyuridylate with Thymidylate Synthetase

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The interaction of 5-ethynyl-2′-deoxyuridylate (5-ethynyl-dUMP; 1) with thymidylate (dTMP) synthetase has been investigated. The compound was an inhibitor of the enzyme, competitive with 2′-deoxyuridylate (dUMP) when the reaction was initiated by addition of enzyme ($K_i = 2.7 \times 10^{-6}$ M). However, upon preincubation of 1 with dTMP synthetase, the inhibition pattern became noncompetitive. The time course of the enzyme reaction in the presence of 1 was nonlinear, indicating an increase in binding with time. Irreversible inactivation of the enzyme did not occur. The compound did not appear to become altered structurally as a result of interaction with the enzyme. A ternary complex was formed among dTMP synthetase, compound 1, and 5,10-methylenetetrahydrofolate, which was stable enough to survive Sephadex G-25 filtration but dissociated upon denaturation of the enzyme.

A number of compounds containing an acetylenic moiety have been shown to be mechanism-based inhibitors of various enzymes.[1] For example, the mechanism postulated for the irreversible inactivation of lactate dehydrogenase

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