Here p_i is the probability for the rubidium ion to be located in a site i on the humate ion and ΔB_i is the ^{85}Rb line width characterizing this site. p_0 is the mole fraction of unbound rubidium ions and ΔB_0 the corresponding line width. At moderate humate concentrations p_0 is close to unity whereas the p_i's are proportional to the humate concentration. Thus if the number of rubidium ions bound to the humic acid is much smaller than the total number of rubidium ions the line width should vary linearly with the humic acid concentration.

The studies are continued along the lines presented here.

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Visible Absorption and Circular Dichroism Spectra of 5(4′-Sulfamylphenylazo)-8-hydroxyquinoline Bound to Carbonic Anhydrase and Alcohol Dehydrogenase

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Spectral studies of the binding of inhibitors and other ligands containing chromophoric groups (*i.e.* groups absorbing some kind of electromagnetic radiation) to biological macromolecules have given valuable information about the state of such macromolecular complexes, because the spectral properties of the bound ligand are changed in a characteristic way. The ligands may exhibit changes in fluorescence, microwave absorption (due to paramagnetic groups), or light absorption in the ultraviolet and visible parts of the spectrum.[1–3]

A recent example is given by the studies of azosulfonamide-carbonic anhydrase complexes by Coleman.[4] He found that the induced circular dichroism spectra of the bound chromophore disodium 2-(4 sulfamylphenylazo)-7-acetamido-1-hydroxyphthalene-3,6-disulfonate are sufficiently specific as to distinguish between the various isoenzymes from erythrocytes of human and *Macaca mulatta*. As the three-dimensional structure of human carbonic anhydrase form C (HCAC) is known to a resolution of 5.5 Å [5] and is being worked out at high resolution,[6] and as X-ray studies of the form B are in progress,[7] the carbonic anhydrase is a valuable reference system for comparative spectrochemical studies of bifunctional reversible inhibitors. This means, that many specific, reversible enzyme inhibitors containing a reporter group and an additional 4-sulfamyl-phenyl group could also be bound to carbonic anhydrase, and the spectral properties of the various enzyme inhibitor adducts could then be compared with the adduct of the inhibitor and carbonic anhydrase. In a search for bifunctional protein ligands, we have prepared and studied 5-(4′-sulfamylphenylazo)-8-hydroxyquinoline (SAPAO) and describe here visible absorption and circular dichroism spectra of complexes of human carbonic anhydrase, form B and C, and horse liver alcoholdehydrogenase with the chromophoric ligand.

Experimental. HCAB* was a gift from Dr. Per-Olof Nyman, Gothenburg, HCAC a gift from Dr. Kerstin Fridborg, Uppsala, and LADH a gift from Dr. Åke Åkeson, Stockholm. SAPAO was prepared by azocoupling of diazotized sulfanilamide with 8-hydroxyquinoline according to general procedures [8] and

* *Abbreviations.* HCAB and HCAC, human carbonic anhydrase form B and C, respectively. LADH, liver alcohol dehydrogenase. SAPAO, 5-(4′-sulfamylphenylazo)-8-hydroxyquinoline. NAD^+, NADH, nicotinamide adenine dinucleotide, oxidized and reduced, respectively.

recrystallized several times from ethanol-water.

The enzyme-inhibitor complexes were prepared in metal-free phosphate buffer, $\mu = 0.05$ and pH = 7.5. The absorption spectra of the 1:1 adducts were measured in a Zeiss PMQ II spectrophotometer equipped with the MM12 double monochromator with fluorite prisms. The circular dichroism spectra were recorded with a JOUAN-Dichrographe at the instrument station of the chemistry center, Lund, by the courtesy of Dr. Bengt Nordén.

Results. The visible spectrum of the dye SAPAO is very sensitive to solvent effects (Fig. 1.): The absorption band at the highest wavelength appears at 503 nm in ethanol ($\varepsilon = 3.1 \times 10^4$ l M^{-1} cm^{-1}) at 483 nm in water (pH 8—10), at 460 nm in water (pH 4—7, $\varepsilon = 2.1 \times 10^4$ l M^{-1} cm^{-1}) and at 400 nm in dioxane ($\varepsilon = 2.6 \times 10^4$ l M^{-1} cm^{-1}). In dimethylsulfoxide, *two* high intensity bands appear at 550 nm ($\varepsilon = 1.53 \times 10^4$ l M^{-1} cm^{-1}) and at 405 nm ($\varepsilon = 1.87 \times 10^4$ l M^{-1} cm^{-1}).

The LADH complex (Fig 2.) shows two Cotton effects, one strongly negative at 495 nm and one weaker, positive at 415 nm.

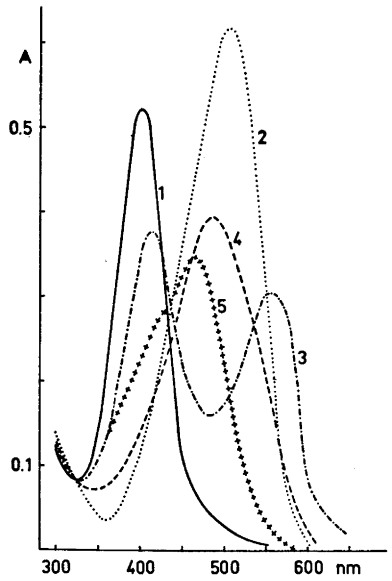

Fig. 1. Absorption spectra of 5-(4′-sulfamylphenylazo)-8-hydroxyquinoline, 2×10^{-5} M. Solvent: (1) dioxane; (2) ethanol; (3) dimethylsulfoxide; (4) phosphate buffer, pH 7.5; (5) biphthalate buffer, pH 4.0.

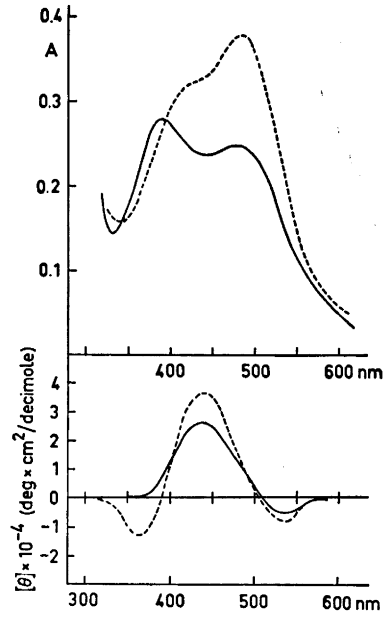

Fig. 2. Absorption and circular dichroism spectra of the adduct of SAPAO with human carbonic anhydrase form B (solid line) and form C (dashed line). 1.2×10^{-5} M in phosphate buffer. $\mu = 0.05$, pH = 7.5.

The strong, negative C. D. band corresponds to the visible absorption maximum of the ligand at the highest wave length (see above). The position of this absorption maximum is very little changed upon binding of the ligand to LADH, *i.e.* from 483 to 480 nm. The second Cotton effect at 415 nm corresponds to a weak shoulder in the absorption spectrum of the dye-enzyme adduct.

The complex of SAPAO with the human carbonic anhydrases B and C is characterized by three Cotton effects (Fig. 3.): One weak, negative band at 540 nm, one strong, positive at 440 nm and one negative at 365 nm, the last one being much more pronounced in HCAC than in HCAB. The absorption spectra of the carbonic anhydrase complexes lack the qualitative correspondance of absorption and C.D. bands as shown by the LADH complex.

Discussion. A comparison of the spectra of both pairs of dye-enzyme complexes shows: The ligand's absorption spectrum is not changed by binding to LADH in such

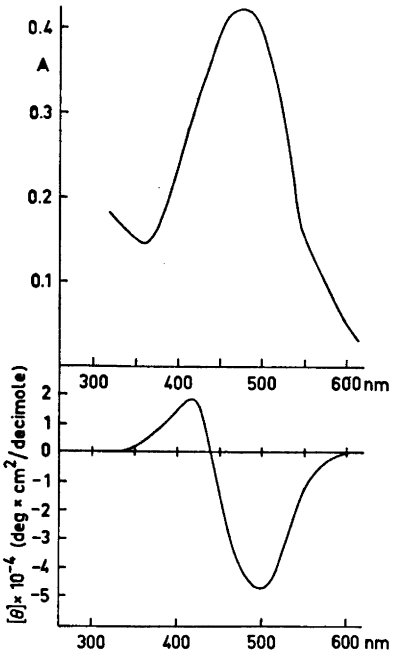

Fig. 3. Absorption and C. D. spectra of the adduct of SAPAO with horse liver alcohol dehydrogenase. 1.2×10^{-5} M in phosphate buffer. $\mu = 0.05$, pH = 7.5.

a way, that it gives rise to any solvent-analogue perturbation of the chromophore. Such an effect is, on the other hand, to be considered in the case of the carbonic anhydrase-dye complexes. Here, a broad region of strong absorption and optical activity appears at lower wavelengths, another one at higher wavelengths as compared to the free ligand. This resembles qualitatively the influence of the dipolar solvent dimethylsulfoxide, where high intensity bands appear below and above the absorption band of the dye in water (Fig. 1).

X-Ray work demonstrated a rather narrow cleft at the active site of human carbonic anhydrase, form C. It is tempting to interpret the spectral properties of the HCAC-complexes as a consequence of the substitution of part of the water around the chromophore for several other groups of different polarity which build up the cleft at the active center. Apparently, the protolysis of the sulfonamide from the acid to the basic form

$$RSO_2NH_2 \longrightarrow RSO_2NH^- + H^+$$

changes the spectrum of the free ligand in water very little (Fig. 1) and cannot explain the absorption characteristics of the binary complex. On the other hand, the spectra given here do not help to decide the question still under discussion, whether arylsulfonamides are attached to carbonic anhydrase in their acid or basic forms.

The spectra of the LADH complex do not indicate any general similarity with those of the HCA-complexes. The appearance of a distinct "hydrated" form of the ligand in the LADH adduct makes it unlikely, that the chromophore should be bound inside a narrow, pre-formed cleft. This is somewhat unexpected, since it is believed, that chelating ligands, like *o*-phenanthroline and 8-hydroxyquinoline bind to a protein-bound zinc ion in the active site, competing thereby with NAD^+ and NADH.[13] However, there is evidence from X-ray work,[9,10] ^{35}Cl-nuclear magnetic resonance studies,[12] and other spectroscopic and kinetic measurements,[11] that the binding of coenzyme, but neither of chelating ligands nor nicotinamide-free dinucleotides, leads to a significant conformational change, which prepares the protein for its catalytic function. Thus, the hydroxyquinoline dye introduced here is an optical probe for the coenzyme-free enzyme protein; it is probably not able to give structural information about the catalytically active state of LADH.

We are now studying in greater detail the binding of SAPAO to LADH under varying conditions, and also the properties of other dyes which may form ternary complexes with the enzyme in the presence of coenzyme.

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On the Localization of Aminopeptidase B and Separation of its two Molecular Forms by Automated Recycling Chromatography

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Earlier studies [1,2] have shown that rat liver contains two molecular forms of aminopeptidase B, one of which is evidently derived from red blood cells present in livers which are used to purify aminopeptidase B. The purpose of this communication is to present a detailed method for preparing the two molecular forms by recycling chromatography on Sephadex® G-200, and to provide evidence that the actual liver enzyme occurs in the soluble phase of the cell.

All reagents and enzyme assay methods have been published earlier.[3,4] The automated recycling chromatography was performed with the LKB ReCyChrom system, which was equipped with the LKB 4938 Programmer (LKB Produkter, Bromma, Sweden).

Wistar albino rats, 10 months old and of both sexes, were used. The livers were removed after the animals were killed by a blow on the neck. A single liver was put for 10 min in 20–30 ml of cold (+4°C) 0.25 M sucrose solution, after which the tissue was cut into fine particles. From this crude homogenate a 10 % suspension (containing appr. 1 g of fresh tissue) was made in cold 0.25 M sucrose. The crude homogenate was treated in 20 ml portions (containing appr. 2 g of fresh tissue) with a Thomas (B) hand homogenizer equipped with a teflon pestle. Two downward strokes were sufficient. The resultant homogenate was filtered through a double-layered lace cloth and the filtrate was centrifuged for 5 min at 600 *g* to remove nuclei, whole cells, larger remnants of tissue, *etc.* The resulting centrifugate was again centrifuged for 20 min at 10 000 *g* to remove lysosomes and mitochondria. The pH of the supernatant fluid was adjusted to pH 5.0 with 0.1 N HCl at room temperature while mixing with a magnetic stirrer. The acid treatment was performed to remove microsomes.[5,6] The suspension was allowed to stand for 60 min at +4°C, after which it was centrifuged for 30 min at 78 000 *g*. The sediment was discarded. The pH of the supernatant fluid was adjusted to pH 7.0 with cold 0.1 N NaOH. No visible precipitate was observed. Ammonium sulphate was added to the supernatant fluid to achieve a final saturation of 80 %. The mixture was centrifuged 60 min later for 15 min at 78 000 *g*. The sediment was dissolved in 0.1 M tris-HCl buffer, pH 7.15, producing a final volume of 2.0 ml (per 2 g fresh tissue).

A typical result from the automated recycling chromatography of the two molecular forms of aminopeptidase B is shown in Fig. 1, and Table 1 shows the time schedule for the separation of the two molecular forms. The third cycle revealed the two molecular forms, but