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BIS AZO DYE LIQUID CRYSTALLINE MICELLES AS POSSIBLE DRUG CARRIERS IN IMMUNOTARGETING TECHNIQUE

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Lyotropic liquid crystal mesophases, represented in this work by Congo Red bis azo dye solution, were proposed as systems to carry drugs to immuno-selected targets. The possible use of Congo Red for this aim arises from its liquid crystalline features, enabling it to attach to immune complexes as polymolecular, ordered conglomerates and simultaneously to incorporate many organic compounds into its mesophase, largely independent of the water with a specific but adaptive molecular organization. Molecules with planar rigid structure, and/or large hydrophobic fragments, especially those with a positively charged group in the molecule, are found to be incorporated best. Rhodamine B, Rhodamine 6G and adriamycine, which have the assumed binding features, were tested as model compounds and were found to be readily engaged into Congo Red mesophase. The effect of hydrophobicity on ligand binding was evaluated following the incorporation of homologic 10, 12 14, 16 carbon chain organic acids and the effect of charge using small mobile tandem molecules of pI differing by a few pH units (lysine-norleucine, tyrosine-tyramine). Positive charge seems to affect binding especially by influencing the organization of molecules and the shape of the micelle simultaneously.
Congo Red immobilized to heat-aggregated immunoglobulins and antibodies in the immune complex was found to retain its binding ability, confirming its possible usefulness for drug transport.

Key words: *immunotargeting, liquid crystals, Congo Red*

INTRODUCTION

The search for target-directed carriers of drugs is an important pharmacological and medical task at present (1).

Recently there have been many efforts to develop and improve the technique which uses liposomes as drug carriers. However, their instability,

especially in contact with phagocytes, still poses an obstacle to effective application (2, 3, 4). Thus the search to improve this system and/or to device other techniques.

The finding that some dyes displaying lyotropic liquid crystal properties bind selectively to immunoglobulin molecules engaged in the immune complex promises a new way to carry immunotargeted drugs (5—7). Bis azo dyes (Congo Red, Evans Blue) — rigid polarizable polyaromatic compounds with long, rodlike, planar molecules that form an ordered supramolecular organisation in water — fit this group of mesophases well (6). Liquid crystalline micelles, which anchor to immune complexes, as the result may be used to carry chemicals solubilized in the lyotropic mesophase, and hence are proposed here as carriers. Planar rigid probe molecules are generally expected to dissolve when inserted into liquid crystals consisting of rodlike molecules, but organic compounds containing hydrophobic and/or positively charged groups may also be predicted to form mixed phases with bis azo dyes.

The advantage of using bis azo dyes for immunotargeting is their well-known low toxicity (8), arising mostly from their micellar liquid crystalline character, which prevents binding and easy penetration to the cell interior.

This work is a preliminary investigation of Congo Red bis azo dye's ability to form mixed micellar systems, in order to evaluate the possible drug-carrying usefulness of this system in immunotargeting.

MATERIALS AND METHODS

Reagents

All reagents were of analytical grade. Human IgG was purchased from Biomed, Poland. Rabbit anti-TNP IgG was isolated as described earlier (5).

Evaluation of the capture ability of Congo Red dye solution for different compounds

1.8 ml samples containing a mixture of the given compound and Congo Red in five fold molar excess were dialyzed at room temperature against 100 ml of 0.05 M Tris-HCl pH 7.0, 0.15 M NaCl buffer. Fifty-microliter aliquots were taken from the sample at different times, and the amount of the retained compound was estimated spectrophotometrically after Congo Red was separated out by adsorption to cellulose. This same procedure was applied to estimate the amount of compound in the control sample in the absence of Congo Red. Because of adsorption of Congo Red to cellulose, dialysis membranes used for samples containing Congo Red were previously saturated with this dye.

Equilibrium dialysis

0.5 ml samples of Congo Red (1.7×10^{-7} M) in 0.15 M NaCl were dialyzed for 22 hours at room temperature against 250 ml aliquots of 0.15 M NaCl containing Rhodamine B. The range of Rhodamine B concentration used was 5.2×10^{-6} M—2×10^{-4} M. The amount of Rhodamine B bound to Congo Red was estimated spectrophotometrically after Congo Red was separated out.

Binding of organic aliphatic acids to Congo Red mesophase

Increasing amounts of aliphatic acid solution (1.7×10^{-3} M) in methanol were added to 1.5 ml samples of Congo Red (5.4×10^{-6} M) in 0.15 M NaCl, stabilized with 0.1% gelatine. The mixtures were heated for 10 minutes at 100°C and then slowly cooled. The amount of unbound acids in the sample was determined by standard turbidity measurements at 546 nm.
The same method was used for determination of binding of cholesterol to Congo Red.

Convective filtration

Binding of Rhodamins and other ligands to Congo Red micelles was also estimated by filtration through YMT ultrafiltration membrane (MPS-1 Micropartition system, Amicon). After standard centrifugation (30′, 2000 g) the content of ligand in solutes was measured.

Assessment of Adriamycine binding

The binding to Congo Red was studied following the removal of this drug from the solution of the given concentration by adsorption to the cellulose membrane pieces loaded with Congo Red. This was performed versus control test with Congo Red free cellulose pieces used for incubation and compared to corresponding test with Rhodamine B.

Binding of Rhodamine 6G to aggregated IgG-Congo Red complex

Congo Red attached to heat-aggregated IgG immobilized by disulphide crossbridging (9) was used to capture Rhodamine 6G from the solution. For this aim, glass beads covered by n-octadecylamine anchored in a thin polystyrene layer were thiolated according to standard procedure and crossbridged to thiolated human IgG.

The immobilized IgG was then heated at 63°C for 20 min in the presence of Congo Red (2.14×10^{-3} M). The binding bed thus obtained was washed and incubated for several hours with ligand solutions.

The capture of Rhodamine 6G by aggregated IgG-Congo Red complex was measured spectrophotometrically by estimating the decrease of Rhodamine concentration in the supernatants. The amount of bound Congo Red was estimated after its elution from the glass beads with methanol.

Binding of the Congo Red-Rhodamine 6G comicelle to the antigen-antibody complex

TNP-cysteamin-CM Sephadex was prepared according to (5). The Sephadex grains were incubated for 1 hour at 37°C with increasing amounts of polyclonal anti-TNP IgG in 0.15 M NaCl Tris-HCl buffer, pH 8.0. After unbound IgG was removed, the samples were incubated for 1 hour

at 37°C with a mixture of Congo Red (0.1 mg/ml) and Rhodamine 6G (0.1 mg/ml). The grains were extensively washed and the bound dyes were extracted with methanol and estimated spectrophotometrically (Congo Red) or by fluorescence measurement (Rhodamine 6G). In independent experiments, fluoresceinated anti-TNP IgG (10) was used for binding. The antibodies were released from the complex by sulphitolysis of cysteamine spacer with 0.1 M sodium sulphite, and were estimated spectrofluorimetrically.

Electron microscopy

Images of the IgG-dye complexes were obtained with a TESLA BS 500 electron microscope. The contrast derives exclusively from silver derivative of thiolated Congo Red (6).

Computational methods

The optimal 3-D structure, with the partial charge distribution all over the particular molecule, was calculated using MOPAC (QCPE) ver. 6.0 (11) with the AM1 procedure (12). The calculation was performed on a CONVEX 3220 computer of the Cyfronet-Academic Computer Center in Cracow. Graphic representation of the structures was obtained using PC DISPLAY Serena Software.

RESULTS

Lyotropic mesophases formed in water by associated rigid polarizable polyaromatic compounds, known as chromonic mesophases (13), may create distinct from the water solvent dissolving milieu for numerous chemicals and drugs. The stacking and interaction of amphiphilic liquid crystal monomeric compounds through their hydrophobic regions is the main driving force for the association of molecules and the formation of a micelle-like supramolecular organization (14). The site-to-site arrangement of elongated, bipolar molecules, represented here by bis azo dyes, form rodlike or ribbonlike ordered arrays whose organization is determined mainly by the dyes' internal symmetry. Self-assembly of monomers provides an avenue for the growth of single chain samples, or tangled ones resembling clouds at higher concentrations (6). Dense packing in spontaneously obtained geometries is disturbed by long-range repulsive forces introduced by readily dissociable anionic groups distributed symmetricaly at the ends of the molecule.

Fig. 1 presents the Congo Red dye molecule, its two-molecular-unit arrangement, and a simplified drawing of the predicted image of the liquid crystal, micellar organization.

Planar rigid molecules may be readily incorporated into the liquid crystal ordered arrays formed by Congo Red dye molecules, by slipping in between two neighboring dye molecules. Polyaromatic ring ligands are especially favored for sandwiching into the Congo Red polymolecular entity in a water

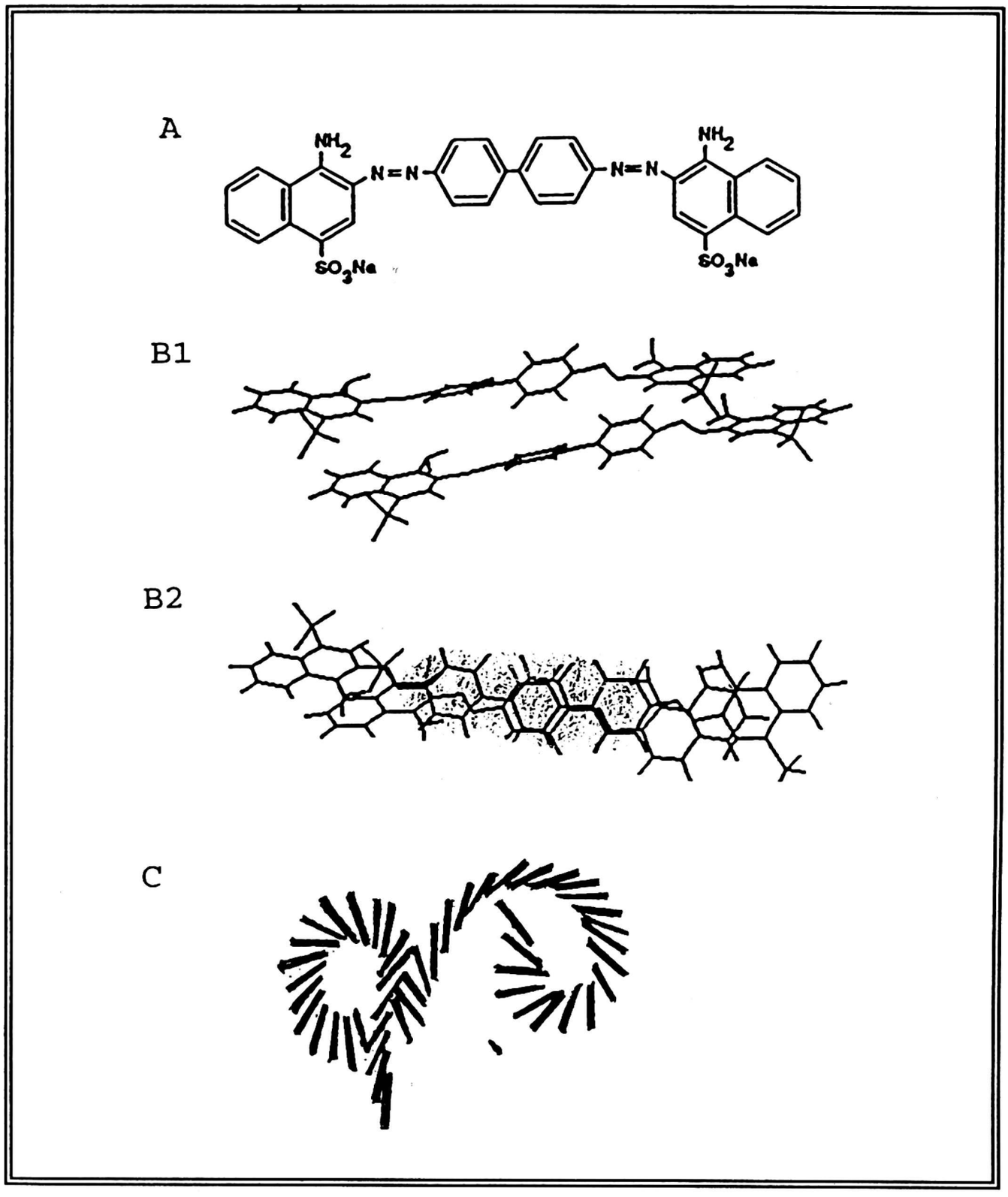


Fig. 1. Simplified drawing of Congo Red micelle organisation A — Congo Red formula, B — Two-molecule-unit of Congo Red micellar arrangement in 3-D (short-distance, stabilizing interaction area shaded) (B1, B2 — 90° rotated projections) C — Simplified model of Congo Red micellar arrangement

solution, but hydrophobicity and charge also significantly affect the insertion of the studied ligands. Also some water-insoluble molecules can be trapped by Congo Red mesophase and dissolved as a result.

Rhodamine B, Rhodamine 6G and Adriamycine were considered in these studies as model ligand molecules representing the favored geometry.

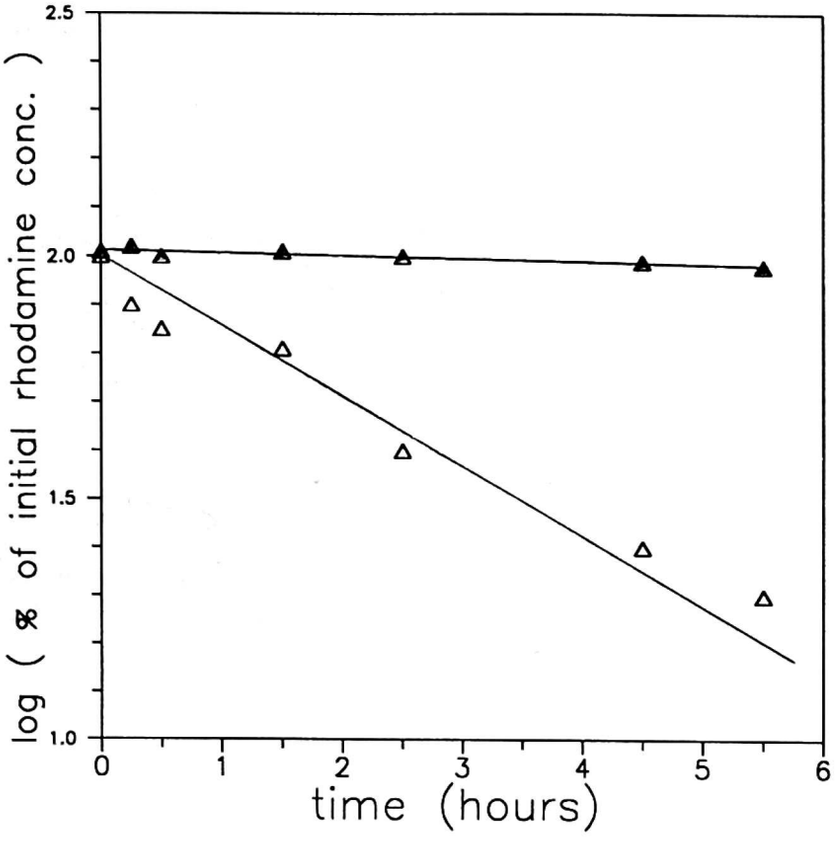


Fig. 2. Dialysis plots of free (△) and Congo Red mesophase-trapped (▲) Rhodamine B.

Of the model ligand molecules studied, Rhodamine B, which is thought to bear all the features necessary for binding, was selected as the standard molecule for these considerations. The binding of Rhodamine B by Congo Red preventing its free dialysis is shown in *Fig. 2*. The use of dialysis in this case was reliable because, unlike Rhodamine B, Congo Red above the micelle-forming concentration is not dialyzable. The affinity of Rhodamine B to Congo Red liquid crystal binding entities was evaluated by equilibrium dialysis.

Fig. 3 depicts the Scatchard plot, which yields association constant $K = 10^4$ M^{-1} and an amount of incorporated Rhodamine molecules exceeding twice that of Congo Red. This unusual realation found by extrapolation indicates surprisingly high carrying capacity in liquid crystal systems. In other experiments the relation of Congo Red to Rhodamine B was kept as values lower than 2:1, to avoid introducing dramatic changes to Congo Red mesophase.

The geometrical parameters of the liquid crystalline comicelle may be expected to change, affected by incorporation of large amounts of ligands different from Congo Red, mostly if they differ in charge (15).

Fig. 4 shows the effect of increased neutralization on the architecture and subsequent shape of Congo Red as seen by electron microscopy. Positively charged groups of the inserted ligands could be expected to have a great effect, since the reduction of strong repulsive forces (which disturbs the close contact and association of monomers in liquid crystal species) allows for increased packing density. Congo Red conglomerates, visualized by modified,

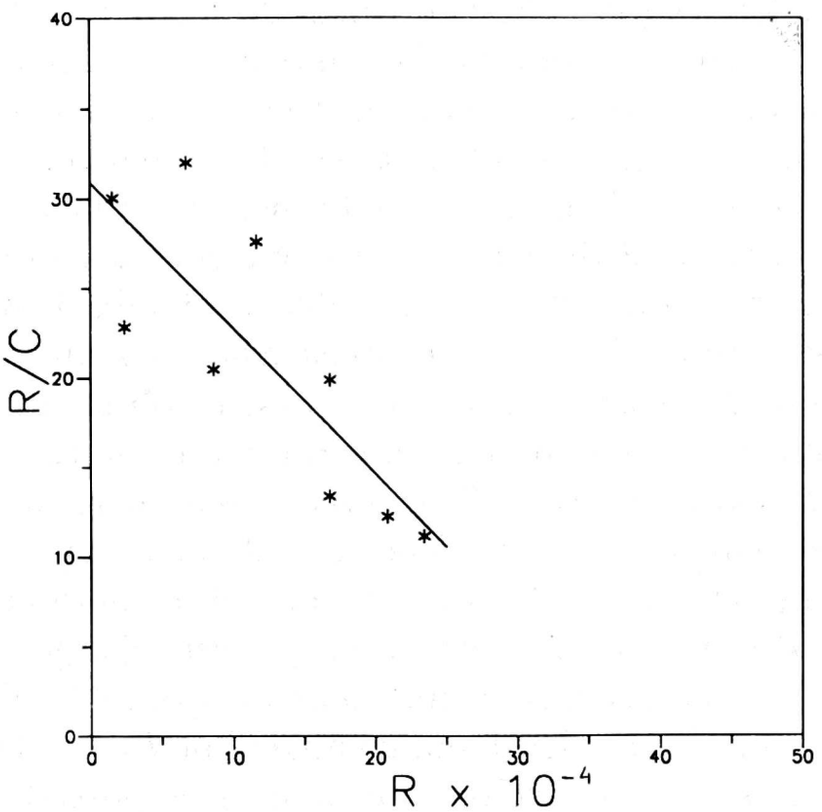


Fig. 3. Evaluation of Congo Red mesophase binding affinity and binding capacity for Rhodamine B, by equlibrium dialysis. The association constant found by the Scatchard plot was $K = 10^4\ M^{-1}$, and the maximum molar ratio of Rhodamine B to Congo Red was 2.6.

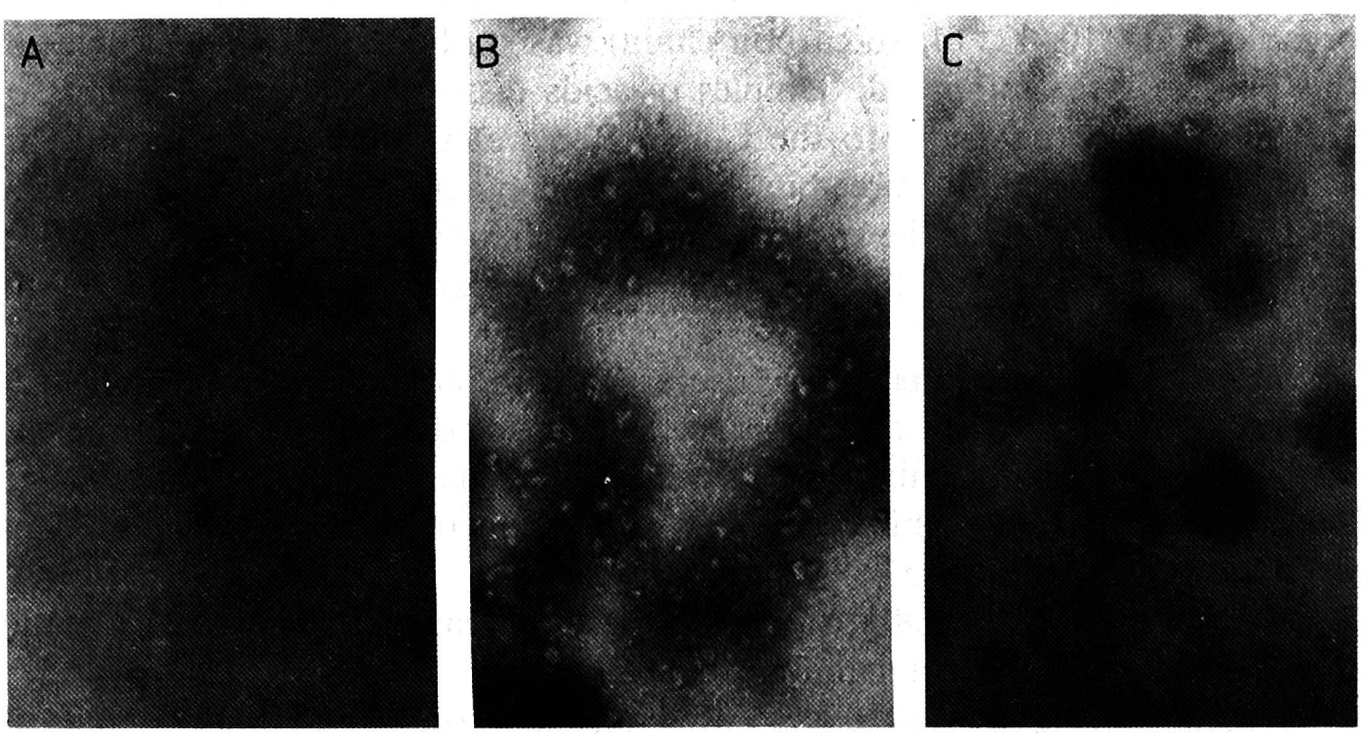


Fig. 4. The effect of charge neutralization on packing compactness of Congo Red mesophase. Immunoglobulin G induced by heating to aggregation, added to initiate dye concentration is seen as white inclusions. Silver-labelled Congo Red was used to yield the sufficient contrast. A — Congo Red B — Congo Red — Rhodamine B (20:1), C — Congo Red — Rhodamine B (10:1)

silver-labelled molecules (6), indeed revealed the altered shape and increased compactness of micellar entities as the ratio of the positive-charge-bearing component in the mesophase was increased. Charge seems to exert the essential effect on ligand incorporation into the bis azo dye liquid crystal conglomerate. The rather low stability of the complex formed by highly polar, negatively charged methotrexate, and the strong, insoluble complex formed by cationic dye fuchsine, ilustrate this effect. The stability of mixed systems depends, finally, on the structural character but most efficiently on the charge of the attached component. According to the present observations, the ligand molecules engaged in the complex do not necessarily have to be planar and rigid if they bear a positive charge. This is seen from the results, where binding of tandems of similar aliphatic (norleucine and lysine) and simple aromatic (tyrosine and tyramine) molecules were compared. In both cases the positive charge is clearly decisive for insertion into negatively charged ordered entities of Congo Red dye. The stability of the complex formed by Congo Red with a few different ligands is visualized and compared in *Tab. 1.* This stability was evaluated by dialysis or convective filtration. The possible, stronger than Rhodamine B binding, as indistinguishable in experimental conditions used, was assumed to be equal 1.

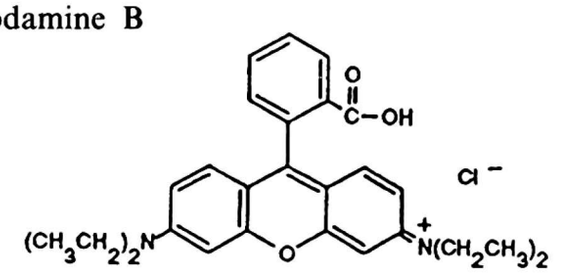
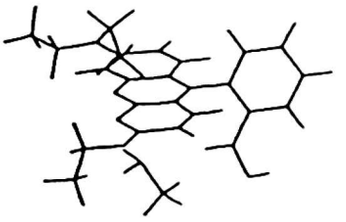
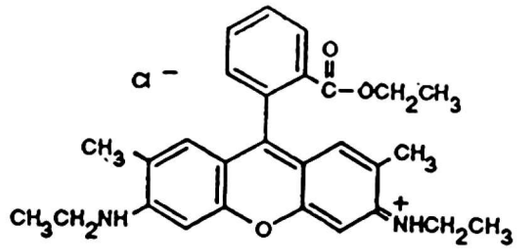
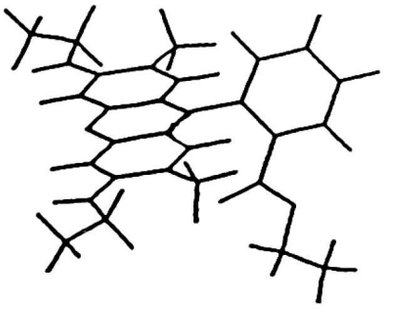
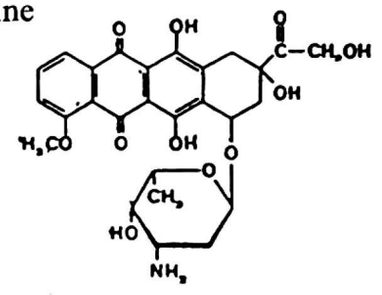
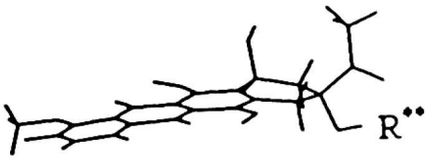
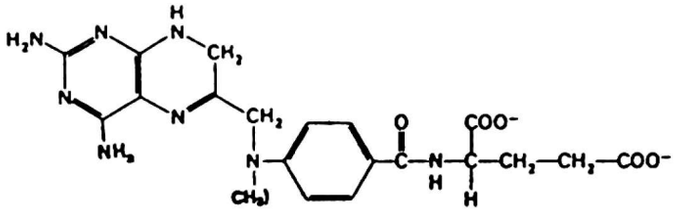
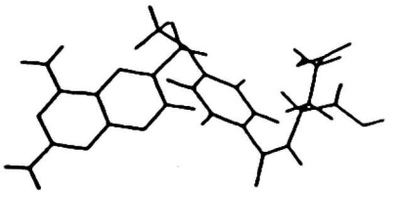
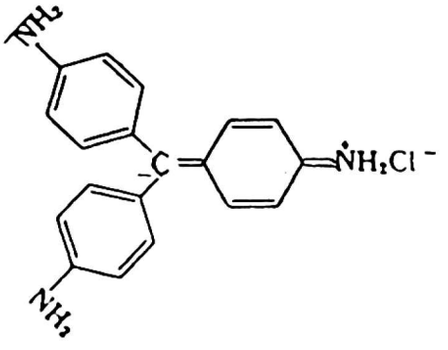
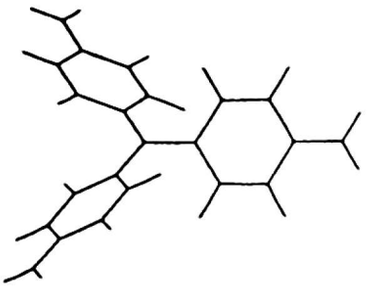
The interaction of amphiphilic liquid crystal components involves hydrophobic interaction, and hence it must be considered in predicting the ability of drug ligands to bind to bis azo dye liquid crystal conglemerates. To gain insight into this problem, the binding ability of aliphatic, homologic organic acids (10, 12, 14, 16 carbon chain) was studied and the efficiencies of this process were compared. Samples of acids dissolved in small amounts of alcohol were introduced into the Congo Red water solution as described in Methods. The unbound, precipitating portion of the acid was measured by nephelometry. As could be expected, all acids studied were incorporated by Congo Red solution as the effect of the significant hydrophobicity of the molecules.

Surprisingly, binding efficiency decreased with increased chain length. It is suggested that this effect originates from the altered range of global internal rotational freedom (number of atoms in molecule divided by number of internal free rotations) which increases with the increase in chain length. This relation is presented in *Fig. 5.*

The ability of Congo Red to bind cholesterol was also assessed and found to correspond to that of palmitic acid.

Whether the ability of Congo Red to incorporate and bind ligands is preserved despite its immobilization by proteins was checked by following the capture of Rhodamine 6G to Congo Red that was first immobilized by attachement to heat-aggregated immunoglobulin and/or immunoglobulin engaged in the formation of an immune complex. Previous studies showed that

Tab. 1. The stability of Congo Red — ligand complex

Chemical formula	3-D structure	CS*
Rhodamine B 		1.00
Rhodamine 6G 		1.00
Adriamycine 		1.00
Methotrexate 		0.09
Fuchsine 		precipitation
Lysine		0.08
Norleucine		0.00
Tyramine		0.05
Tyrosine		0.00

* complex stability in relation to that of Rhodamine B
** — R denotes carbohydrate omitted in calculations

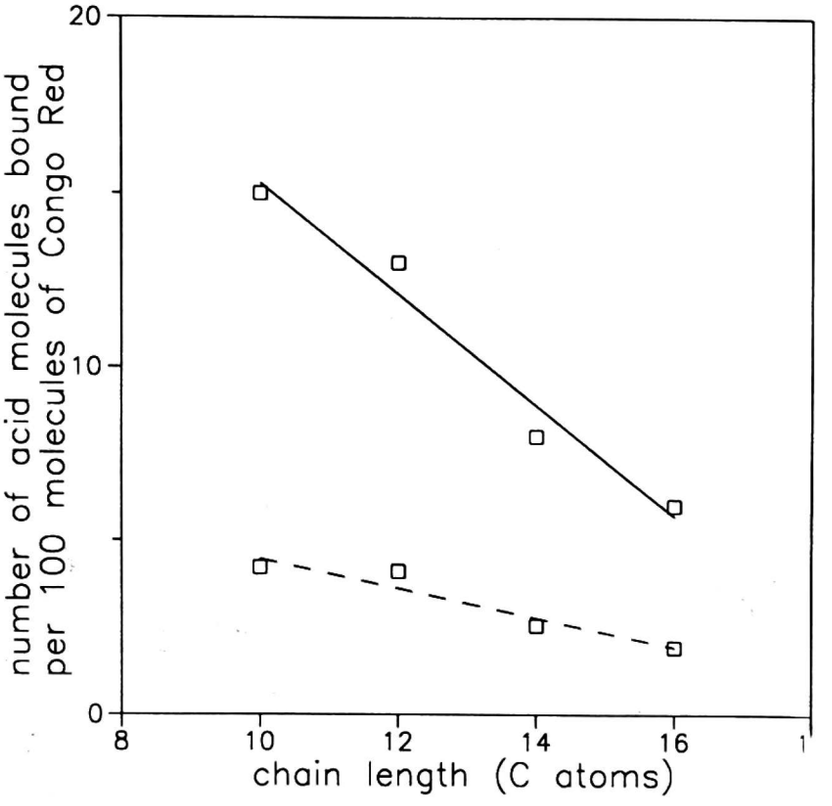


Fig. 5. Chain length related solubility of homologic organic acids driven by Congo Red water solution (——) and the same values, corrected for internal rotational freedom (experimental values divided by number of internal rotational freedom) (— — —).

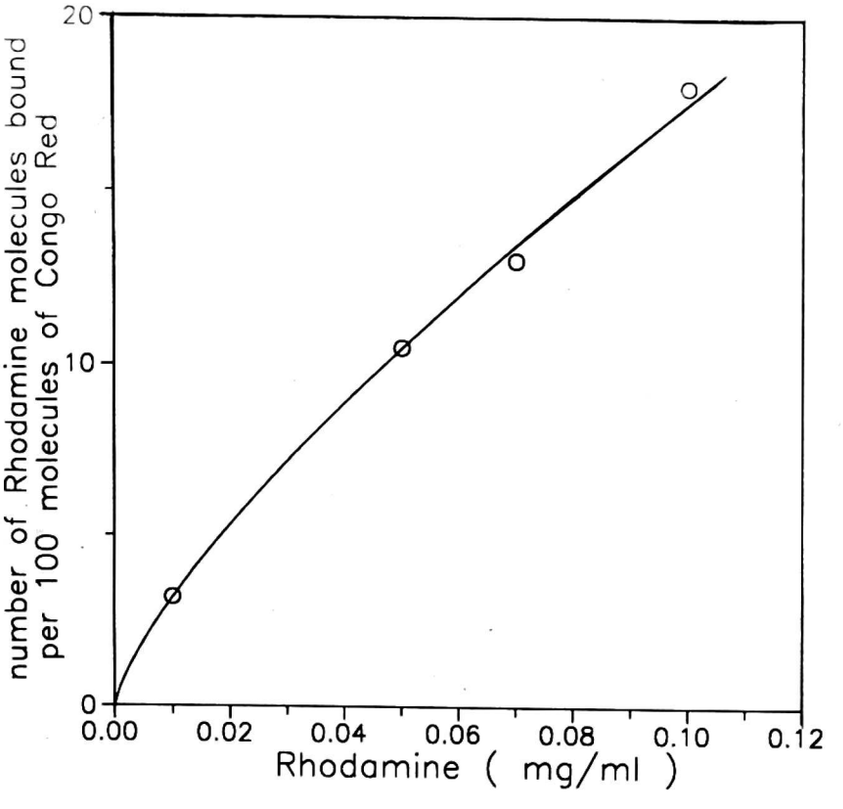


Fig. 6. The capture of Rhodamine 6G from the solution by Congo Red immobilized to a rigid bed through heat-aggregated IgG.

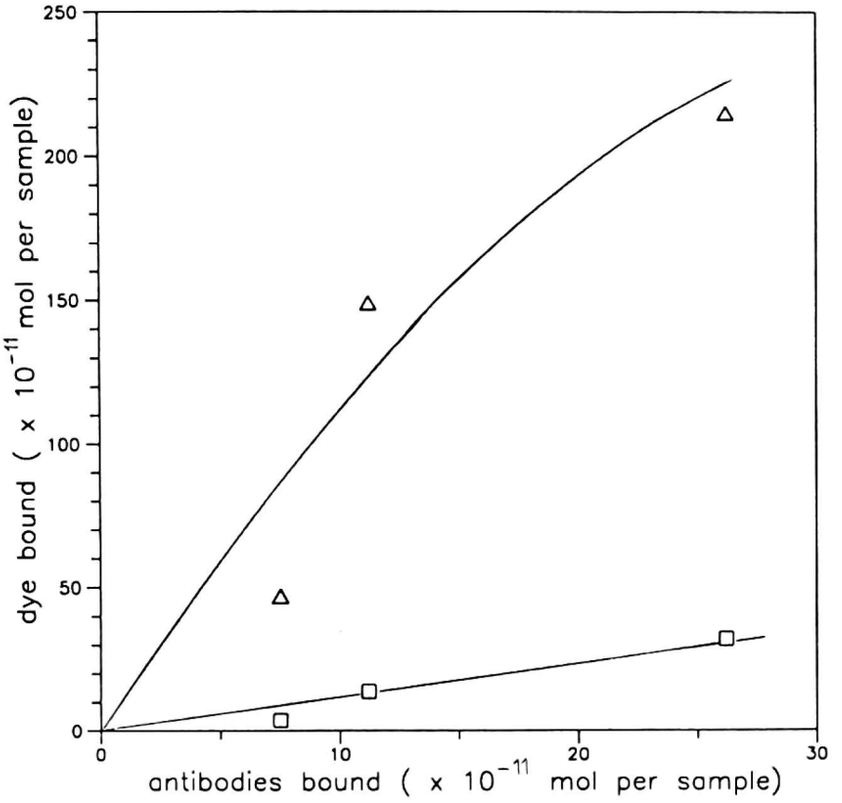


Fig. 7. The binding of Rhodamine 6G to Congo Red attached to the immune complex △ — Congo Red attached to IgG, □ — Rhodamine 6G,

dye molecules bound to protein dye complex can exchange with free dyes in solution (6). Here, the ability of Congo Red — still fixed to the protein — to form mixed systems was confirmed by measuring the uptake of Rhodamine 6G from the solution (*Fig. 6*). Congo Red in this experiment was immobilized by heat-aggregated IgG fixed to a rigid bed (see Methods).

The corresponding model system with Congo Red immobilized by an immune complex is presented in *Fig. 7*. TNP-nized CM-Sephadex was used as the antigen for anti-TNP antibodies. In these experiments, TNP haptens were attached through disulphile-containing spacers (cysteamin — see Methods), allowing splitting and independent measurement of all components bound in the complex. The amount of Rhodamine 6G incorporated by the Congo Red attached to the antibodies increased when the amount of antibodies engaged in the immune complex was increased. This confirms the expected behaviour of Congo Red and makes its use as a carrier for immunotargeting more plausible.

DISCUSSION

Intensive efforts have been undertaken recently to control the transport and delivery of drugs *in vivo* (4).

Herein we report a possible new drug carrier for immunotargeting. It employs lyotropic liquid crystal mesophases, here represented by Congo Red bis azo dye. Dyes of this group involve long, rigid amphiphilic molecules with

central internal symmetry. Short-range van der Waals interaction and long-range repulsive forces compete in stabilizing liquid crystal micellar moietes by determining the specific orientation of the interacting molecules and the shape of the micelles (13, 14, 16—19). Congo Red molecules form ribbonlike entities with extended, twisted, and tangled shapes, whose packing density increases at higher concentrations and/or as the amount of incorporated ligand molecules bearing the positive charge increases. Electronmicroscopic studies confirmed that reduction of electrostatic repulsion indeed favors the formation of compact, defined shapes.

Supramolecular entities of Congo Red are organized in a way that allows for the best possible separation of hydrophobic fragments from the water. As a result, Congo Red forms a separate milieu in the water, a milieu which may trap some molecules containing fragments attracted by specific forces that stabilize the mesophase organization or even solubilize insoluble hydrophobic substances. The mechanism of mixed systems (15, 20, 21) formation was previously analysed by studying the miscibility of similar amphiphiles — Congo Red and two isomeric dyes, Evans and/or Trypan Blue (6). The studies revealed largely reduced capability of Trypan Blue to form micellar entities in contrast to its structural isomer Evans Blue. Clearly the negatively charged sulphonic group, which in the case of Trypan Blue overlaps the short-range interaction area, prevents binding, in contrast to Evans Blue dye.

However, the main reason for pointing to bis azo dyes as possible drug-carrying species is their, recently discovered, unusual capability to form specific complexes with antibodies, bound to antigen. In these complexes with antibodies, bis azo dyes preserve their liquid crystalline property, being attached to antibody molecules as polymolecular entities with their ability to form mixed mesophases retained (5, 6).

Another Congo Red property which favors its use as a carrier of drugs targeted by antibodies is its unique ability to enhance antigen — antibody interaction and stabilize the immune complex (5, 22).

Also, the low toxicity of colloidal bis azo dyes makes their use additionally promising. Highly charged micellar entities are prevented from penetrating the cell interior and are easily removed from the organism. This is why these dyes have been used safely for years, for blood volume measurement (8). Quick elimination may also help lower the toxicity of carried drugs, as the efficient removal of unbound toxic drug excesses may be expected to proceed together with dye excretion.

The incorporation to Congo Red mesophase concerns mostly planar rigid molecules. However the strong negative repulsion present in ordered amphiphile arrays also sets charge requirements for the incorporated molecules. The positive charge present in incorporated ligands largely favors insertion.

Studies on the incorporation of aliphatic organic acids supplied convincing evidence that standard insertion is obligatory for binding, even for non-rigid ligands, since the increasing global freedom of internal rotations (measured as the sum of free rotations of bonds in the molecule) disturbs binding. Finally, planarity and rigidity seem not obligatory for binding. This means that a large group of drugs may be listed as ligands that penetrate and bind to azo dye amphiphilic mesophase directly and/or after suitable chemical adaptation.

The present preliminary studies were limited to model laboratory systems. The functioning and the effect of the system on living cells, including delivery and cellular uptake of therapeutic agents, will be presented later.

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