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BIS AZO DYES — STUDIES ON THE MECHANISM OF COMPLEX FORMATION WITH IgG MODULATED BY HEATING OR ANTIGEN BINDING

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The mechanism of binding of azo dyes (bis azo) to immunoglobulin G of altered conformation, induced by heating or interaction with antigen was analysed in this work. Azo dyes: Congo Red, Evans Blue and Trypan Blue were selected for these studies. The molecules of Congo Red and Evans Blue associate readily in water and exist as polymolecular micellar species of liquid crystalline organization. Such organization of molecules appeared necessary for these dyes to interact with antibodies and to affect the formation of immune complex. It was proved by studying the properties of isomeric dyes Evans Blue and Trypan Blue, whose ability to form polymolecular conglomerates in water differs, being high for Evans Blue and low for Trypan Blue. The dyes seem to influence the formation of the immune complex generally by interacting with individual immunoglobulin molecules. The rate of exchange of dye molecules in conglomerate bound to protein varies and is the lowest for the portion of molecules which are engaged directly in the complex with protein. Electron microscopic studies also confirmed the polymolecular form of the dye in the dye-protein complex. The computational simulation of dye-dye and dye-protein interaction was performed in a model system. The micelle in the system was represented by three molecules of Congo Red. The peptide loop composed of amino acids 68—88 originating from VL IgG domain, was selected to represent protein. Amino acid side chains of this fragment were reduced to Cβ. The best fitness was found for peptide chains of twisted β conformation and independently optimized conformation of the dye in a form of „twisted ribbon" micelle.It was concluded that the IgG domains become accessible for penetration of the dye after being relaxed in the result of heating or interaction with the antigen.

Key words: *azo-dyes, binding to IgG, immunological signal, Congo Red*

INTRODUCTION

The mechanism for generating the immunological signal by antigen binding and especially the role of the individual IgG molecule in this process remains unclear in spite of many studies concerning this problem (1-4).

The notion of structural alterations occuring in single immunoglobulin molecule is still not generally accepted (5). Poor accessibility of immune complexes for standard instrumental analysis delays the solution of this problem. The finding, that molecules of immunoglobulin G may selectively interact with some azo dyes, after being structurally modulated by heating or binding to antigen (6—8), seems to offer the new entry into this problem and supports the notion that conformational alterations occur in antibody molecule upon binding to antigen. Azo dyes exist in water basically as large molecular conglomerates corresponding to structures observed in liquid crystal solutions. The polymolecular, organized form of the dye is also preserved in protein-dye complex (8).

As reported in previous paper (8), the accessibility of antibody molecules (necessary for the interation with the dye) results from their altered conformation which is forced by the binding of antibodies to antigen. The stabilization by the dye of this energetically unfavourable conformation releases the antibody for stronger interaction with the antigen and causes higher stability of the immune complex (8). The augmented stability of immunoglobulin molecules in the immune complex is indicated by additional large number of antibodies bound to antigen. These antibodies are derived for the low affinity fraction which is unable to hold the antigen with sufficient stability without the dye. Short living antigen-antibody complexes, formed by low affinity antibodies which escape the registration in experimental conditions, reach the measurable level after being stabilized by the dye. Hence the formation of immune complexes by this antibody fraction may become fully dye controlled.

The most frequently used azo dye, Congo Red, has been known for years as the specific stain for amyloid (9). Also, binding of this dye to some dehydrogenases has been reported (10). The finding, however, that it may affect the functional activites of immunoglobulins by selective binding to their activated form offers a new approach to understand the function of antibody molecules and it may perhaps be helpful in some other immunological studies. An attempt to explain the dye-dye and dye-protein interaction, in experimental approach and computational simulation is presented in this paper.

MATERIALS AND METHODS

Reagents

Human IgG was isolated from pooled sera by chromatography on cellulose DE-52 (Whatman, USA), then filtration on Sephacryl S-300 (Pharmacia, Sweden) column. This same procedure was used for isolation of polyclonal anti-SRBC IgG sera of rabbits immunized with sheep red blood

cells. Bence Jones protein (L chain dimer) was obtained from the urine of a patient with multiple myeloma by precipitation with ammonium sulphate. Homogeneity of the isolated protein was assessed by polyacrylamide gel electrophoresis.

Low affinity anti-SRBC antibodies were obtained by adsorption and the removal of high affinity antibodies from the solution of anti-SRBC IgG with sheep red blood cells in the absence of the dye. Adsorption was repeated until standard agglutination tests (with supernatant as a source of anti-SRBC antibodies) had not become negative.

Azo dyes: analytical grade Congo Red (Polish Chemical Reagents, Poland) and Evans Blue (Serva, Germany) were analytical grade. Trypan Blue (Loba, Austria) was purified by chromatography on aluminium oxide (BDH Chemicals, England) in alkalized ethanol-water solution.

Synthesis of Ag-Congo Red derivative for electron microscopic studies

Amino groups of Congo Red were thiolated with AHTL (Immunothiol — Koch Light, England) in 10% imidazole buffer, pH 8.5, for 24 hours in an excess of $AgNO_3$. The product was precipitated by acidification to pH 2.5, washed with diluted nitric acid, (pH 2.5), and dissolved in 0.1 M TRIS-NHO_3 buffer, pH 8.0. Precipitation was repeated if necessary. Silver incorporation to the Congo Red was tested by Proton Induced X-ray Emission method (performed by the Institute of Nuclear Physics, Cracow).

Electron microscopy

The images of IgG-dye complexes were obtained by TESLA BS 500 electron microscope without using standard negative staining. The contrast on the picture originates exclusively from Ag-Congo Red derivative attached to protein.

Preparation of dye-protein complexes

Human IgG or Bence-Jones protein at concentration of 20 mg/ml were heated for 20 min. at 63°C with a 100-fold molar excess of a dye. To remove free and weakly bound dye molecules the protein-dye complex was filtered through weakly adsorbing bed, Sepharose CL-6B in order to retain the possibly high number of quickly exchangeable dye molecules, in the complex or through Sephadex G-200 sf for more efficient adsorption. The number of dye molecules bound to the protein was estimated spectrophotometrically as described earlier (6).

Absorption coefficient for ligh chain $A_{1cm}^{0.1\%} = 1.2$ at 280 nm (11) was used to calculate the concentration of Bence Jones protein.

Agglutination

Agglutination tests were carried out at room temperature in barbital buffered saline pH 7.4.

Exchange between free and attached to protein dye molecules

50 µl aliquots of the sample containing the complex of heat aggregated IgG with Congo Red (dye to protein molar ratio egualed 37.1 : 1) were mixed with Evans Blue (10-fold excess to Congo Red). Samples which differed in the time of incubation with Evans Blue were then separated

by thin layer gel filtration on Sephadex G-200 sf. Portions of Sephadex containing protein-dye complex were cut off, and the complexes were extracted with 0.05 M Tris-HCl buffer, pH 8.0: DMSO (1:1,v/v) mixture. DMSO was used to ensure the complete elution of the dyes from Sephadex. Dye concentrations were determined spectrophotometrically using following absorbancy coefficients for DMSO: buffer (1:1,v/v) solutions: $A^{mM}_{1cm} = 52.1$ at 518 nm for Congo Red and $A^{mM}_{1cm} = 71.0$ at 626 nm for Evans Blue. In all tested samples the dye: protein molar ratio was the same and equaled 15:1.

Dye-dye interaction

Samples containing Evans Blue and Trypan Blue or their mixture with Congo Red were separated by agarose gel electrophoresis (1% agarose, 0.05 M phosphate buffer pH 7.0). The migration of each dye was determined from densitograms performed at 489 nm for Congo Red and at 608 nm for Evans Blue and Trypan Blue using Gel-Scanner accessory to RESPONSE II UV-VIS spectrophotometer, GILFORD.

The computational simulation of dye-dye and dye-protein interactions

The calculation of partial charges and geometry for Congo Red molecule using the MNDO method was the first step in modelling the structure of Congo Red micelle. The symmetrical half of the Congo Red molecule was analysed. All distances in conjugated rings and all C-H distances attached to conjugated rings were assumed to be the same. The dihedral angles for the amino group and the sulphonic group, (which was assumed to have the C3v symmetry) were taken into account for optimization. All atoms were found to be coplanar except for two oxygens of the sulphonic group and two hydrogens of the amino group. The calculated Mulliken population of the optimized geometry are listed in *Tab. 1.*

The complete dye molecule was created from one half molecule by symmetry operation based on the two-fold symmetry of Congo Red molecule.

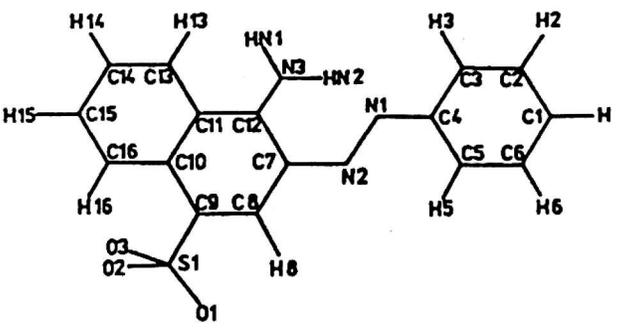
The optimal structure of the Congo Red micelle was approached by the energy minimization method. The minimized energy was calculated according to the rigid geometry force field (12, 13) including the torsional potential, (pairwise) van der Waals (14) and electrostatic interaction (12) ($\varepsilon = 4.0$ for intramolecular interaction and $\varepsilon = 40.0$ for intermolecular interaction).

The following degrees of freedom were taken into account in the search for optimal structure of the Congo Red micelle:

parameters: 1 — rotation around the bond between two benzene rings
2, 3 — rotation of amino groups
4, 5 — rotation of sulphonic groups
6—11 — rotation around diazo bonds

The molecular organization of the Congo Red micelle was represented by three molecules of the dye. The mutual orientation of molecules was defined starting from the orientation in which the single Congo Red molecule (i) was placed with its center of gravity at the origin of the Cartesian set with the molecule axis oriented along the z-axis and the upper benzene ring lying on thw yz plane. To create three molecular unit of micelle the following set of transformations were proposed (*Fig. 1.*):

Tab. 1. Partial charges and geometry of a half of Congo Red obtained using the MNDO method
[a]ecu-electronic charge unit

Atom	Partial charge (ecu[a])	Atom	Bond length (Å)	Atom	Angle (deg)	Atom	Torsional angle (deg)
							
C1	−0.098						
C2	−0.122	C1	1.38				
C3	−0.052	C2	1.38	C1	120.0		
C4	0.004	C3	1.38	C2	120.0	C1	0.0
C5	−0.064	C4	1.38	C3	120.0	C2	0.0
C6	−0.112	C5	1.38	C4	120.0	C3	0.0
H2	0.091	C2	0.97	C1	120.0	C6	180.0
H3	0.112	C3	0.97	C2	120.0	C1	180.0
H5	0.124	C5	0.97	C4	120.0	C3	180.0
H6	0.096	C6	0.97	C5	120.0	C4	180.0
N1	−0.163	C4	0.97	C3	120.0	C2	180.0
N2	−0.087	N1	1.31	C4	120.0	C3	180.0
C7	−0.074	N2	1.33	N1	120.0	C4	180.0
C8	0.013	C7	1.38	N2	120.0	N1	180.0
C9	−0.376	C8	1.38	C7	120.0	N2	180.0
C10	0.061	C9	1.38	C8	120.0	C7	0.0
C11	−0.089	C10	1.38	C9	120.0	C8	0.0
C12	0.122	C11	1.38	C10	120.0	C9	0.0
C13	−0.100	C11	1.38	C10	120.0	C9	180.0
C14	−0.099	C13	1.38	C11	120.0	C10	0.0
C15	−0.122	C14	1.38	C13	120.0	C11	0.0
C16	−0.016	C15	1.38	C14	120.0	C13	0.0
H8	0.124	C8	0.97	C7	120.0	N2	0.0
H13	0.076	C13	0.97	C11	120.0	C12	0.0
H14	0.077	C14	0.97	C13	120.0	C11	180.0
H15	0.091	C15	0.97	C14	120.0	C13	180.0
H16	0.174	C16	0.97	C15	120.0	C14	180.0
S1	1.700	C9	1.78	C8	120.0	C7	180.0
O1	−0.754	S1	1.47	C7	106.0	C8	0.0
O2	−0.803	S1	1.47	C7	106.0	C8	120.0
O3	−0.806	S1	1.47	C7	106.0	C8	−120.0
N3	−0.329	C12	1.40	C7	120.0	C8	180.0
HN1	0.168	N3	1.00	C12	110.0	C7	30.0
HN2	0.141	N3	1.00	C12	110.0	C7	150.0
H1	0.092	C1	1.38	C2	120.0	C3	180.0

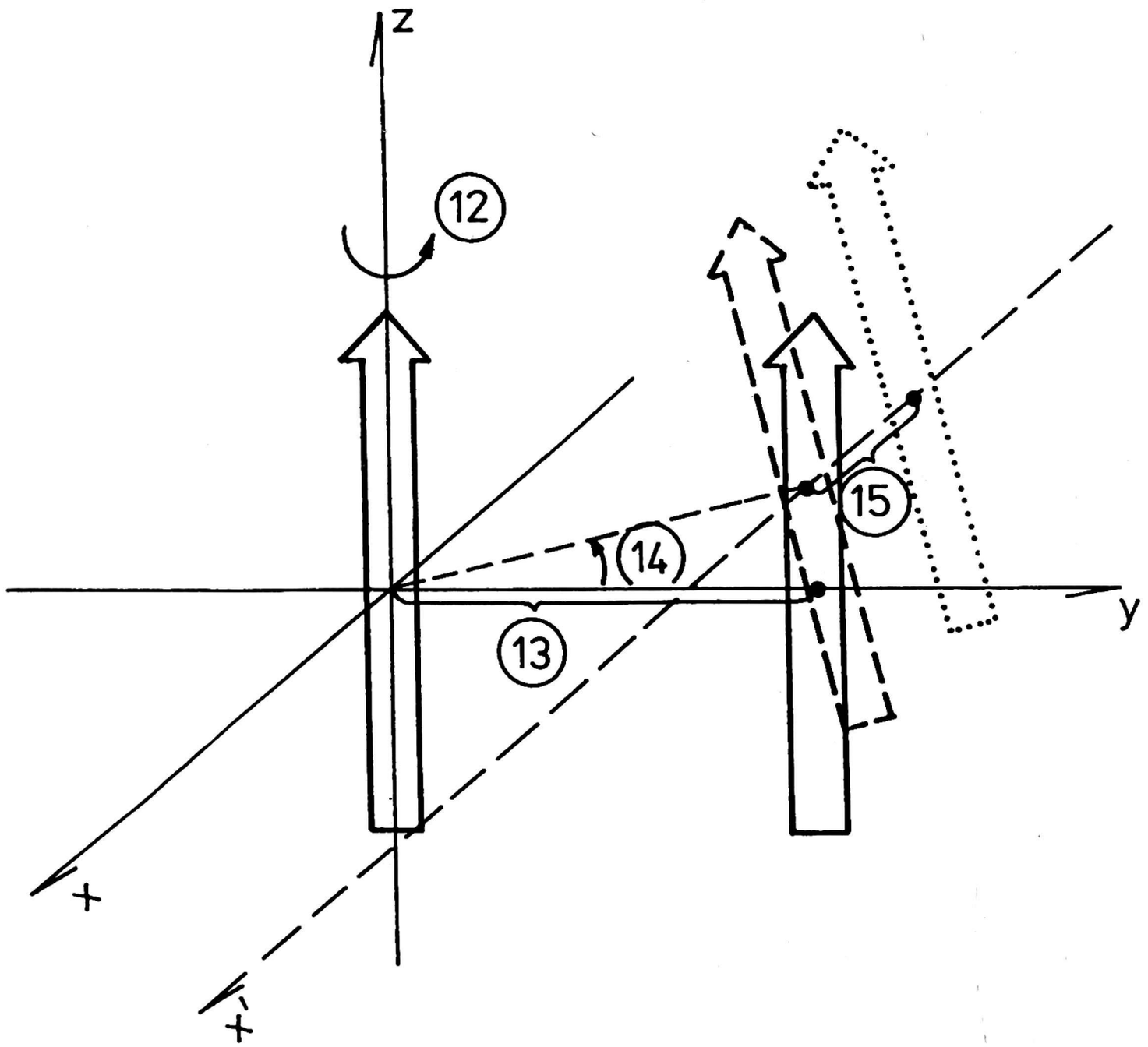


Fig. 1. Graphical presentation of symmetry operation used in the procedure creating the architecture of the Congo Red micelle. The parameters 1—11 concern the free rotations present in the individual molecule (not shown in the Fig.)

Parameters:

12 (deg) — rotation around the z-axis of the Congo Red molecule oriented initially as follows: the C1-C1′ bond placed along the z-axis and upper benzene ring placed on the yz plane

13 (Å) — translation along the y-axis equal to the radius of the micelle

14 (deg) — rotation around the a-axis

15 (Å) — translation along the x-axis

parameters: 12 — rotation of the formerly oriented molecule (i) around the z-axis

13 — translation of the molecule (i) along the x-axis

14 — rotation around the y-axis in the + (plus) direction (i+1 molecule) and − (minus) direction (i−1 molecule) to create two neighbouring molecules

15 — translation along the y-axis in the + (plus) direction (i+1 molecule) and − (minus) direction (i−1 molecule)

For the presented set of operations all possible ribbon like structures of the Congo Red micelle are potentially equally probable. The straight, non twisted, linear structure of the micelle is resulted when parameters 13 and 14 are equal to zero. For non zero values of these parameters, different helix-like structures may be formed. To find the optimal structure, the Rosenbrack minimization method was used (15). The optimization was performed stepwise with one displacement in one degree of freedom of the single Congo Red molecule at each step. The two other molecules were generated by the symmetry operations (as defined earlier). The dye-protein interaction was simulated using the same energy function as in the case of micelle with additional involvement of electrostatic and van der Waals components expressing the dye-protein interactions. The additional six degrees of freedom defining the dye micelle-protein mutual orientation, expressed by the distance (translation along the x, y, z axis — parameters 16—18) and mutual rotational orientation (rotation around the x, y and z axis — parameters 19—21) of interacting species were taken into account. The orientation of the polypeptide chain was kept fixed while the dye molecules were allowed to rotate and translate.

All calculations were performed on an IBM PC/386 using Fortran Microsoft compilator. The computing time was dependent on the starting structure and it was usually few hours.

RESULTS

The role of individual IgG molecules in binding azo dyes

Bis azo dyes, which were found to interact selectively with immunoglobulins triggered to effector activity by heating or binding to antigen have been further used to examine whether modulated conformation responsible for binding occurs in individual molecules or, may arise from aggregation contacts. The experiments designed for this aim were directed to find the existence of individual, not aggregated protein molecules, which remain linked with the dye. Congo Red was used for these studies. The protein modulation was induced by heating under conditions standard for aggregation (63°C, 20 min) or by binding of antibodies to antigen. Binding of dyes to heat modulated protein molecules was studied using the λ L-chain. λ L-chain (dimer) was used instead of IgG to prove the occurence of non-aggregated protein molecules. In the case of using immunoglobulin G, which aggregates very eagerly (and even its aggregation in the excess of the dye is difficult to be completely prevented), the interpretation of the results was not unequivocal. However, gel filtration on Sephadex G 200 sf proved that λ L-chain dimer heat denatured in the presence of Congo Red, remained in unaggregated form with the attached dye. The protein isolated after filtration still contained 8 dye molecules per 50 kD of protein molecule. It thus indicates that aggregation seems to be unnecessary for binding of the dye and suggests rather the appearance of new intramolecular structural motifs as binding elements.

The similar structural effects may perhaps explain the binding of dyes to the immune complex. Low affinity antibody molecules driven by the dye to bind to antigen, and hence controlled in their engagement to the immune complex,

were used for experiments, which concern the mechanism of binding of the dye to the antibodies. The SRBC anti-SRBC rabbit, low affinity antibody system was chosen for these studies. The low affinity antibodies were obtained from rabbit anti-SRBC IgG as fraction left in supernatant after adsorption by red cells of all those antibodies, which form the stable antigen antibody complex in the absence of the dye.

Low affinity antibody molecules obtained in this way were then used to agglutinate red cells in the presence of Congo Red (*Fig. 2*). By using only low

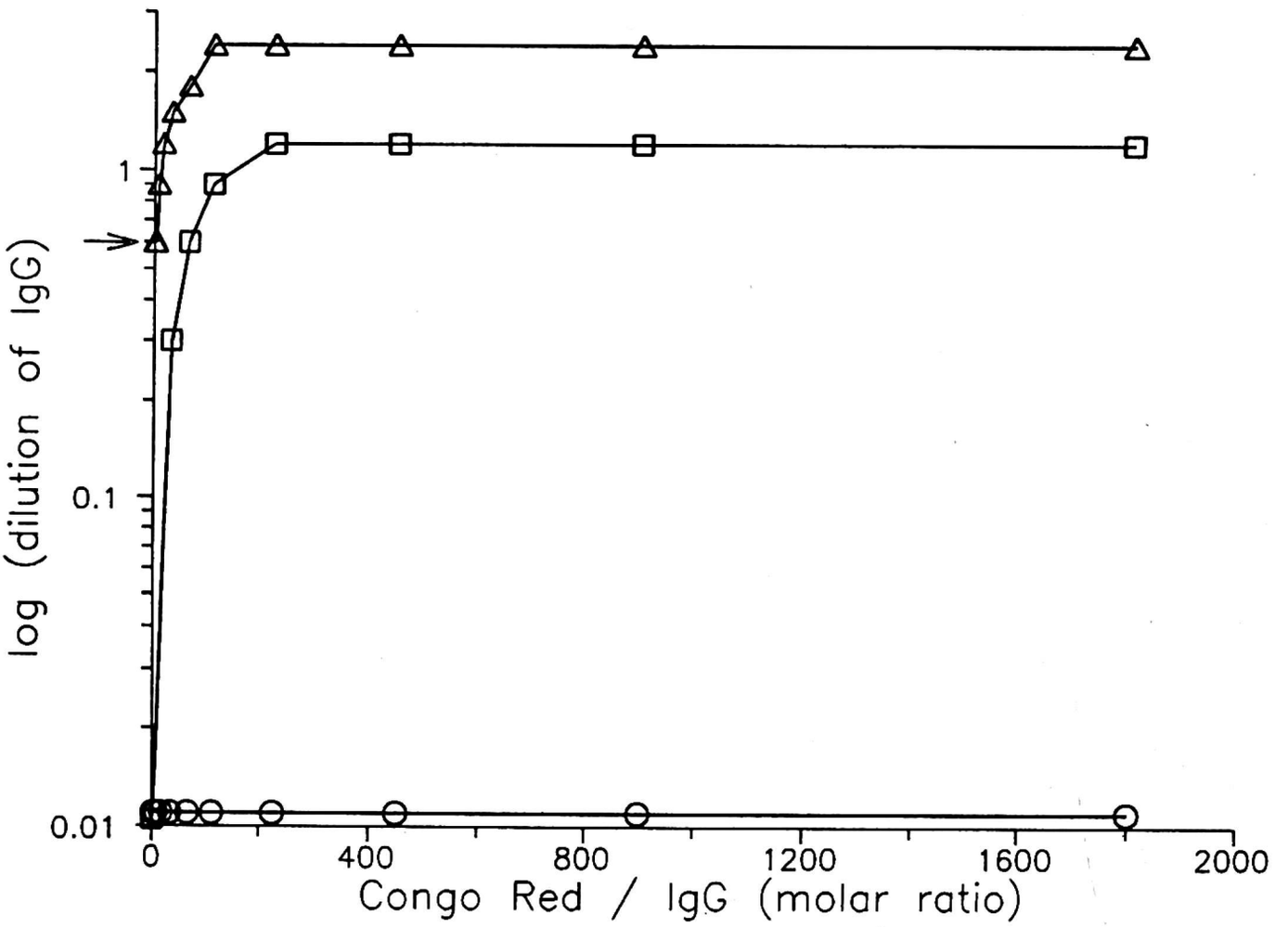


Fig. 2. The dye (Congo Red) controlled agglutination in SRBC rabbit anti-SRBC system
△—△ — agglutination of sheep red blood cells by rabbit anti-SRBC IgG (non-adsorbed by SRBC)
□—□ — agglutination driven by the dye in the case of using low affinity antibodies (left after adsorption of high affinity fraction from anti-SRBC IgG)
○—○ — zero agglutination using rabbit IgG of no anti-SRBC activity
The arrow points to the level of agglutination reached by high affinity antibodies
The ordinate on the Fig presents the dilution at which the agglutination disappears
Initial concentration of IgG anti-SRBC 0.125 mg/ml

affinity antibodies for agglutination it may be ensured that no uncontrolled agregates may appear before these which are formed under the control of the dye. It hence indicates that the immune complex is completed in this case from low affinity antibodies independently stabilized by the dye.

The dye-dye and dye-protein interaction

Evans Blue and Congo Red dyes are bound by both heat induced IgG and antibodies engaged in immune complexes, not as individual dye molecules but as polymolecular conglomerates (8). However, only a portion of dye molecules fixed to IgG are actually in direct contact with the protein, while most are attached indirectly as components of the micelle.

Structures formed by the dye molecules in water correspond to liquid crystals. Surprisingly, the ordered form of these dyes in solution appeared necessary to bind and stabilize structural motifs which are generated by

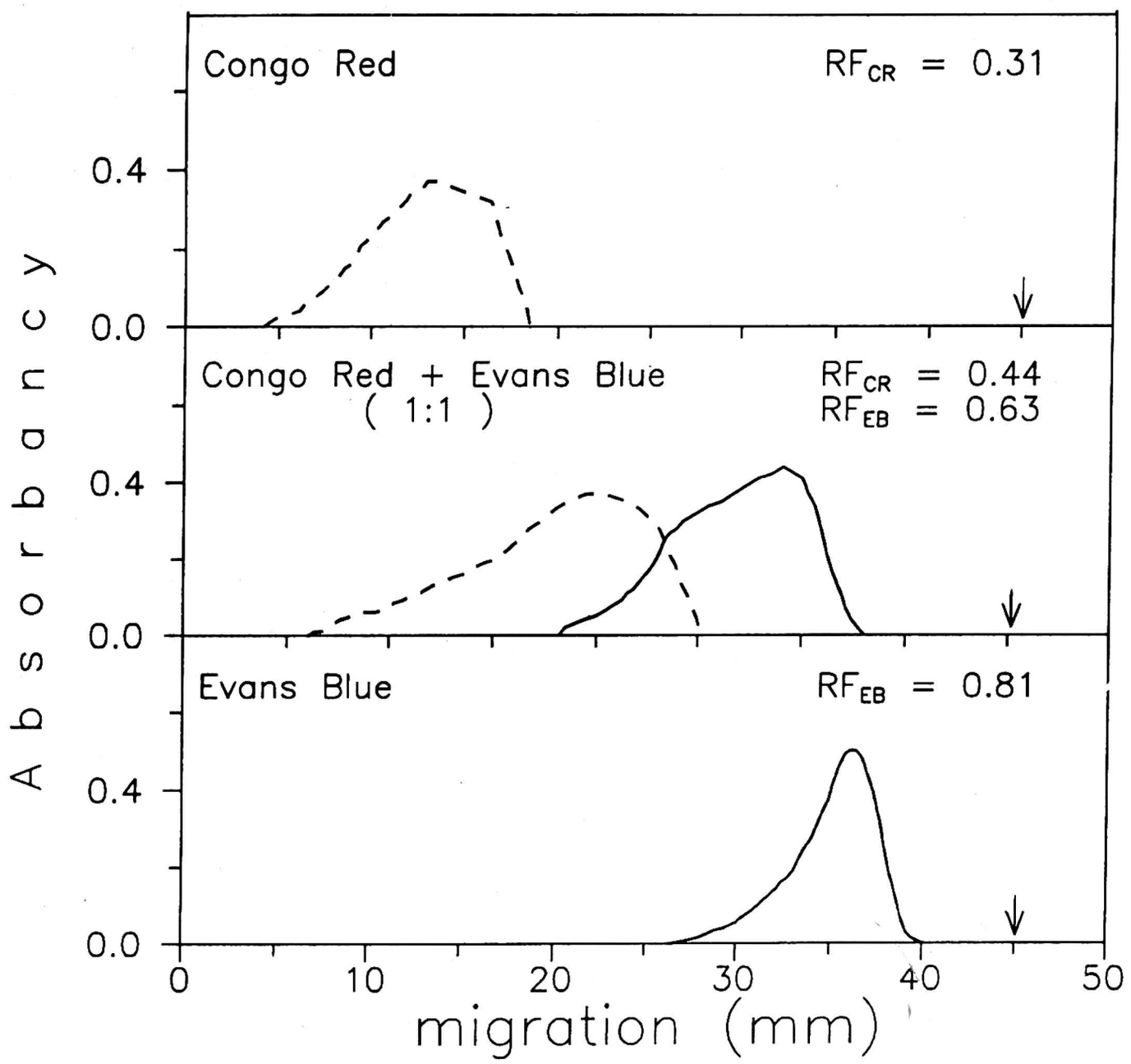


Fig. 3. Co-aggregation of Evans Blue and Trypan Blue with Congo Red as the measure of their aggregation ability. The formation of co-aggregates is expressed as modified migration in agarose electrophoresis. For simplicity, the migration of Trypan Blue, which was unaffected by Congo Red, is marked only by the arrow. Values of migration factors (RF) were calculated in relation to Trypan Blue (RF = 1.0)

interaction of the antigen with antibody molecule. The necessity for the dye to occur in large conglomerates with ordered organization of molecules to affect the immunoglobulins was confirmed by comparing the properties of two isomeric dyes: Evans Blue and Trypan Blue, which differ in aggreagation tendency and capability to affect the antigen-antibody interaction. Of these two dye molecules Evans Blue, as concluded from osmotic pressure measurements and migration in polyacrylamide gel electrophoresis, aggregate eagerly, while Trypan Blue is very poor in this respect. The position of the sulphonic group (No 3) in Trypan Blue is disturbing for the intermolecular hydrophobic interaction which is essential in stabilizing the micelle. The molecules of dyes which aggregate and form micellar structures may also co-aggregate with similar dyes. This may affect mutually their electrophoretic behaviour if mixed dyes differ in charge. The migration of mixed Evans Blue and Congo Red apeared, as expected, to be significantly changed in such electrophoretic test, while the behaviour of Trypan Blue was unaffected. This is well seen in *Fig. 3*. In correlation with this behaviour, Evans Blue influences the formation of the

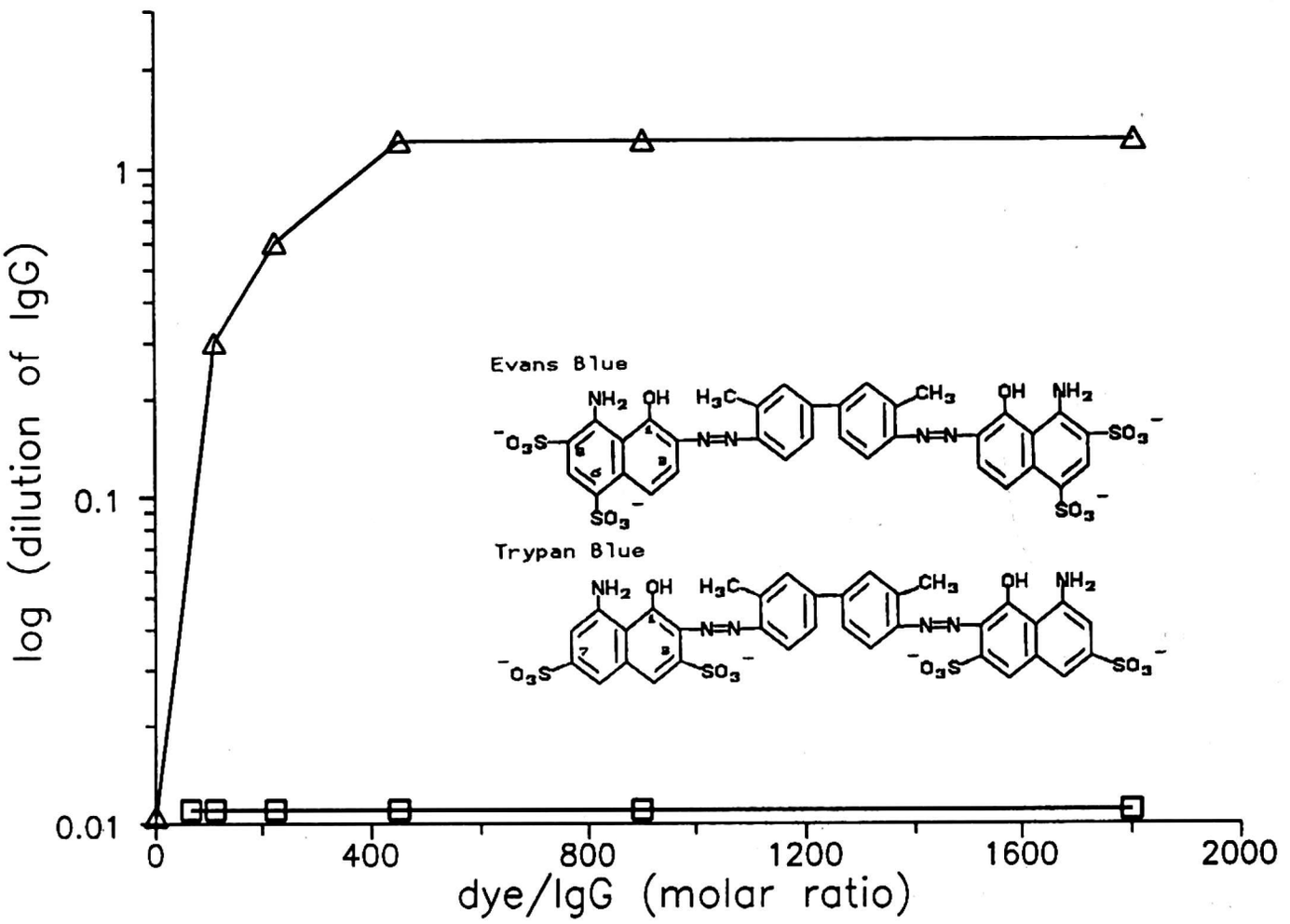


Fig. 4. The effect of Evans Blue (△—△) and Trypan Blue (□—□) on the activity of low affinity antibodies to agglutinate red cells. Initial concentration of anti-SRBC IgG 0.125 mg/ml. Formulas of Evans Blue and Trypan Blue are presented to show the different positions of sulphonic groups in these dyes. The ordinate on the Fig. presents the dilution at which the agglutination disappears.

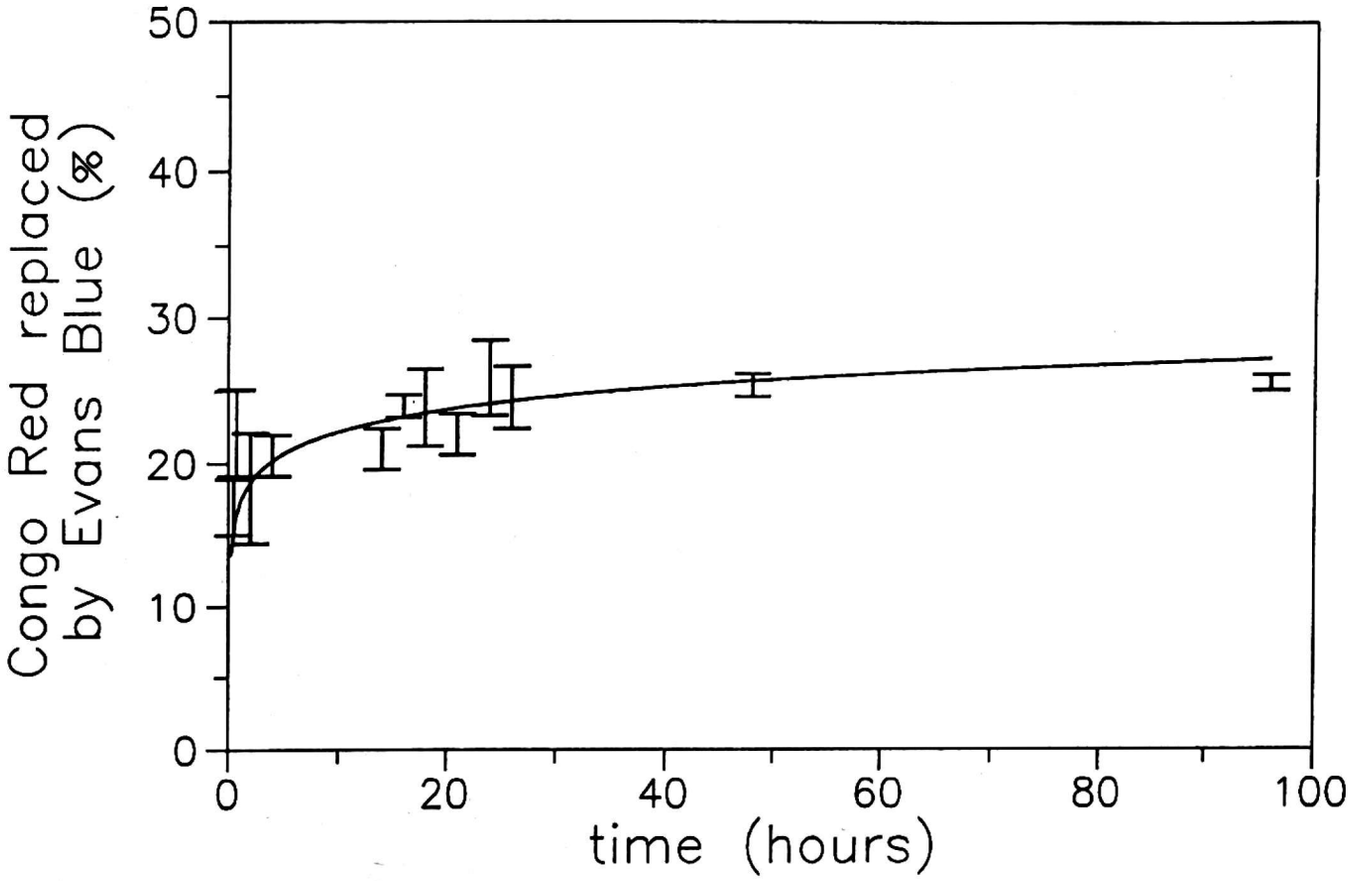


Fig. 5. The kinetics of exchange of dye molecules in complex with heat denatured IgG
The relation of dye molecules (Congo Red and Evans Blue) to protein molecule was 15 : 1 in all measured samples. This number of dye molecules was assumed to be 100%.

immune complex, while the activity of Trypan Blue appeared negligibly small which confirms that the micellar structure is necessary for the dye to interact with antibodies engaged in complex with antigen (*Fig. 4*).

The stability of dye molecules in dye conglomerates fixed to protein is not uniform since only a portion of molecules may directly interact with protein. The exchange of these and perhaps neighbour molecules which could form a more rigid portion of the dye conglomerate complex is slow and increases towards its periphery. This is shown in *Fig. 5,* which presents the exchange of dye molecules bound to heat aggregated IgG. The exchange could be followed experimentally by using two different co-aggregating dyes: Evans Blue and Congo Red. Different absorption spectra of these dyes made the evaluation of their molar ratio in the complex with protein feasible. After incubation, samples were passed through Sephadex G 200 sf to remove free and weakly bound dye in order to obtain complexes containing 15 molecules of dyes (Evans Blue and Congo Red) per IgG molecule. The molar relation of dye to protein equalling 15 : 1 was assumed to be 100% both for the control sample to which Congo Red was added instead of Evan Blue and for the Congo Red — IgG complexes incubated with Evans Blue dye. The ratio of dyes was found to change according to time of incubation (*Fig. 5*).

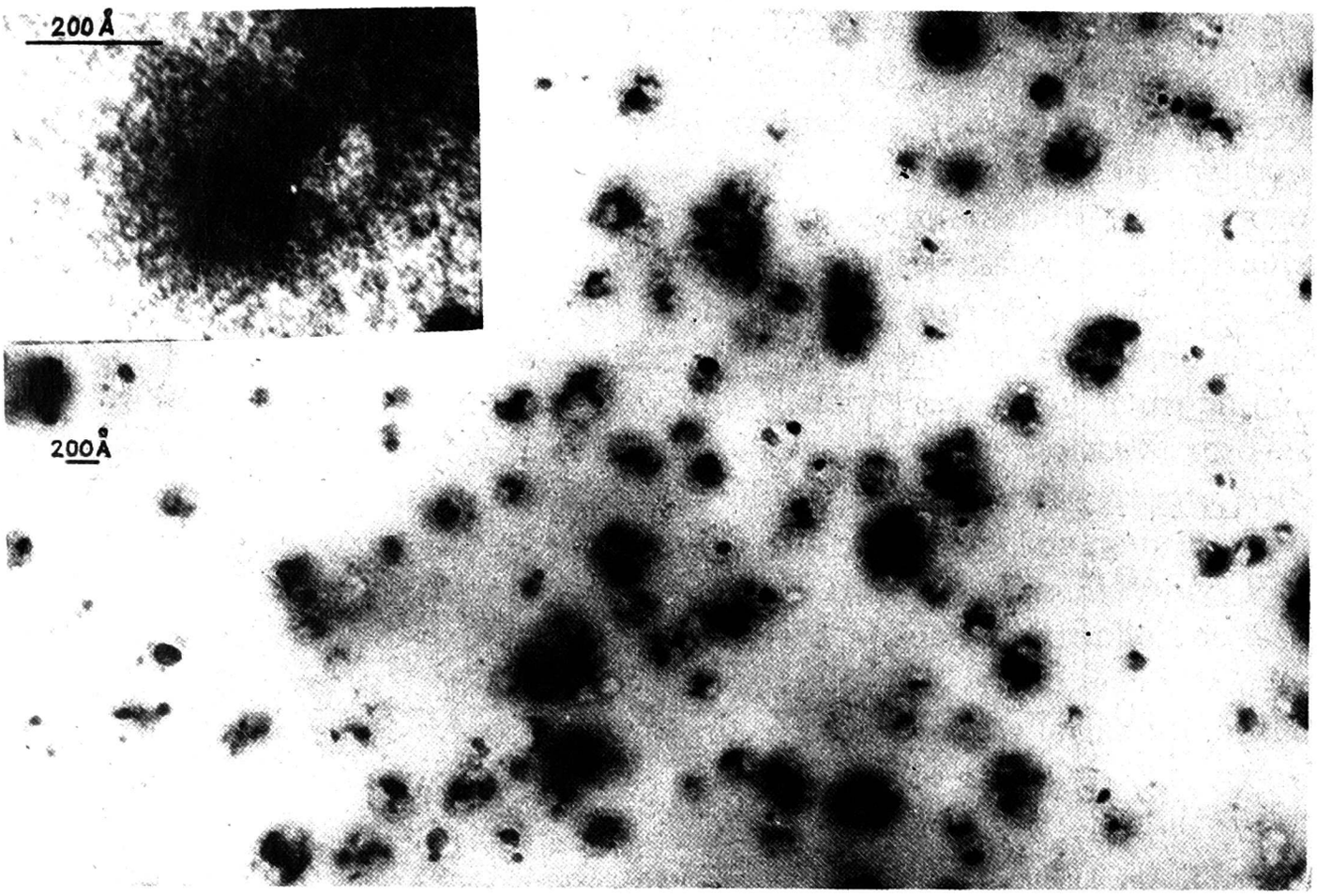


Fig. 6. Electron microscopic picture of complex of heat denatured IgG (human) with the dye (silver labelled Congo Red). High molecular weight fraction isolated by Sephadex G-200 sf thin layer gel filtration. Black irregular spots with white inclusions represent the dye particles carrying immunoglobulin molecules (mostly as small aggregates). Enlarged fragment of the dye particle is seen in the left upper corner.

The described dye-dye and dye-protein interaction has been also confirmed in electron microscopis studies. For increased contrast, Congo Red was labelled with silver (see Methods). The properties of this derivative appeared very similar in all tests used to that of original Congo Red. No noticeable difference was observed in its co-aggregation with other dyes (Evans Blue) and in binding to immunoglobulins. Hence this derivative was considered as the dye which could well be used instead of the original Congo Red in studies concerning the formation of the dye-protein complex. For these studies, the complex of the dye with heat denatured human IgG was isolated from Sephadex G-200 sf after thin layer gel filtration. Protein fraction obtained in this way was then directly used for electron microscopic studies without negative staining. The aggregation of immunoglobulin molecules proceeds following the adsorption of the dye as the protein fraction migrates through the Sephadex. Therefore, the longer the distance in which the protein is allowed to migrate the more molecules became aggregated. The electron microscopic

picture *Fig. 6.* presents the dye-protein complex at an early stage of aggregation seen as a population of dye particles (black spots) carrying the protein molecules, mostly as small aggregates (white inclusions).

Computational simulation of dye-dye and dye-protein interactions

For explaining the interaction of large micellar species of the dye with immunoglobulin modified by heating or antigen binding it was assumed that tightly hitherto packed peptide β loops are growing exposed as the result of relaxed domain structure. All data concerning the protein structure were taken from the Protein Data Bank (16, 17). The file 1FB4, version Feb 1992 represents the immunoglobulin Fab λ Kol coordinates set obtained by (18). Computer simulations of the formation of dye molecule conglomerates and dye-protein interactions were performed to understand the phenomenon.

Computationally predicted structure of individual Congo Red molecule

The structure of an individual Congo Red molecule as a whole corresponded to that obtained by MNDO. The torsional angle optimal for two benzene rings was obtained after minimization and found to be near to 30 deg. Displanarity of benzidine is caused by two overlaping pairs of hydrogen atoms, which in assumption of rigid geometry excluded the planarity of this system. 30 deg was reported for this torsional angle (19) (*Tab. 2., Tab. 3.*).

Computationally predicted structure of the micelle

Forty two different, arbitrary chosen sets of starting parameters covering the probable range of each parameter were used for calculation. They allowed for the selection of the optimal structure of micelles. The linear, non-twisted, ribbon-like structure (parameter 13 = 0 and 14 = 0) appeared as energetically unfavourable. The helix-like structures were acceptable by energetic criteria. To demonstrate the influence of the value of parameters (especially 13 and 14) on the final energy the three structures were selected (*Tab. 2., Tab. 3.*).

As seen from *Fig. 7* and *Tab. 3* the energy changes are indirectly proportional to the dye-dye contact surface. Relatively small differences between energy values for different structures suggest the existence of intermediate forms of Congo Red micelles.

Tab. 2. The dihedral angles describing the structure of Congo Red molecule . The dihedral angles and parameters presenting the mutual relation of molecules in micelles: A, B, C and micelle attached to the fragment 68—88 of VL IgG domain. Each second line describes the internal degrees of freedom concerning the free rotation for the symmetrical part of the Congo Red molecule. C1' and C6' express analogs to C1 and C6 atoms present in the second half of molecule.

MOLECULE	MONO-MER	MICELLE A	MICELLE B	MICELLE C	COMPLEX
DIHEDRALS (deg)					
C3–C4–N1–N2	174.5 173.8	163.9 177.0	152.3 176.5	158.2 161.7	158.3 171.2
C4–N1–N2–C7	176.2 179.8	175.7 177.7	178.6 171.5	173.8 164.1	173.7 165.2
N1–N2–C7–C8	177.4 177.9	174.9 161.2	179.9 164.9	173.1 159.3	175.6 150.0
C6–C1–C1'–C6'	155.0	159.0	158.7	152.2	151.2
C7–C12–N3–HN1	25.2 29.0	18.2 20.1	16.7 21.1	13.9 18.9	59.2 13.8
C8–C7–S1–O1	0.5 2.1	0.0 0.0	0.1 0.3	1.1 0.2	0.8 0.2
PARAMETERS					
12 (deg)		5.9	101.5	94.9	123.5
13 (Å)		3.9	10.1	18.6	17.9
14 (deg)		90.1	59.9	8.9	9.1
15 (Å)		4.2	4.2	4.3	4.4

Computationally predicted structure of the Congo Red — protein complex

The IgG domains triggered to bind the dye were assumed to be partly unpacked and hence accessible to interaction with ordered dye conglomerates. The peptide loop of amino acids 68—88 of VL domain was arbitrarily selected

Tab. 3. The energy components (kcal/mol) of the final structures of Congo Red micelles

MOLECULE	MONO-MER	MICELLE A	MICELLE B	MICELLE C	COMPLEX
TOTAL ENERGY	−13.1	−21.6	−23.3	−27.3	−32.2
INTRA-MOLECULAR ENERGY	−13.1	−16.4	−15.0	−11.1	0.8
electr	−31.6	−35.6	−34.0	−32.3	−16.2
vdW	9.4	7.9	6.9	5.5	0.7
h. bonds	0.0	0.0	0.0	0.0	0.0
torsional	8.7	11.3	12.1	15.7	16.3
INTER-MOLECULAR ENERGY		−5.2	−8.3	−16.2	−16.7
electr		0.1	−1.1	3.5	3.6
vdW		−5.3	−7.3	−19.5	−19.6
h. bonds		0.0	0.1	−0.2	−0.7
INTERACTION WITH PEPTIDE ENERGY					−16.3
electr					−1.4
vdW					−14.9
h. bonds					0.0

as the model of protein for interaction with the dye micelle. To eliminate the possible specific interaction with amino acid side chains these side chains were reduced to Cβ atoms. The crystallographical data were completed by addition of hydrogen atoms according to the field (12) geometry. The calculations were performed starting from several different mutual orientations

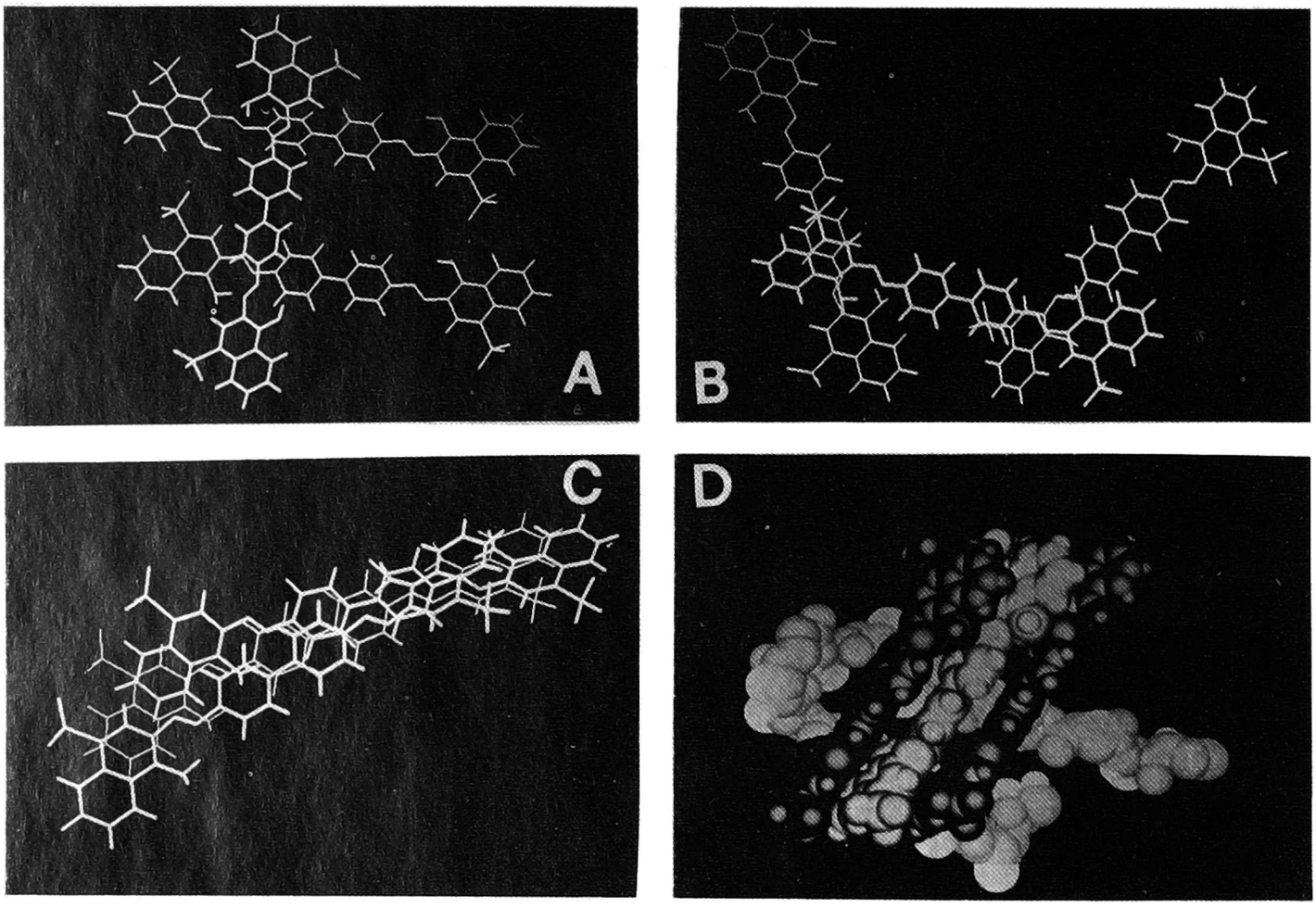


Fig. 7. The computationally predicted structure of a Congo Red micelle and dye-protein complex
The structures A, B and C viewed down the micelle axis.

A — Congo Red micelle (Micelle A) — small radius (3.9Å) of micelle and relatively large (90.1 deg) radial displacement

B — Congo Red micelle (Micelle B) — medium radius (10.1 Å) of micelle and medium (59.9 deg) radial displacement

C — Congo Red micelle (Micelle C) — large radius (18.6 Å) of micelle and small (8.9 deg) radial displacement

D — structure of Congo Red micelle in the complex with the polypeptide loop represented by the 68—88 VL IgG fragment

The axial distance between individual molecules (in all presented micelles is almost the same close to 4.2 Å) seen on the picture D.

Pictures obtained by PROMODELER New England BioGraphics

taking low energy structures of micelles obtained in the previous step of computation.

All 21 parameters were taken into account during minimization. The lowest energy structure of the complex resulting from this calculation is described in *Tab. 2, Tab. 3* and shown in *Fig. 7.* The final structure of the micelle in this case appeared to be very similar to the independently minimized structure of micelle C. In searching for the mechanism of the dye-protein interaction the model of Congo Red molecules treated independently without assumption of their symmetrical mutual orientation, was also analysed. In this approach, each

Congo Red molecule was free to rotate and translate independently versus the polypeptide (parameters 12—15 omitted). The differences in final structures in both calculations were found to be negligible. It means, that the Congo Red dye interacts with the protein as a polymolecular unit rather than as independent molecules.

As shown in *Tab. 3* the van der Waals interaction seems to be responsible for the dye-peptide interactions. No hydrogen bond was found to participate in this binding.

Different peptide conformations, which could ensure the largest possible contact for the energetically optimal form of dye micelle were considered. High structural similarity was found for micelle and twisted beta barrel polypeptide chain. The 101—109 VH (20) fragment, from among other beta barrel fragments was selected as the possible target for interaction with the micelle. The structure of this polypeptide fragment was expressed using the same set of parameters as used to define the dye micelle structure. Every second peptide bond (expressed by the position of the H atom of the amide group and the O atom of the carbonyl group) was treated as the elementary unit.

The results are presented in *Tab. 4,* where the parameters which express symmetry operations of creation of micelle and mutual orientation of peptide units in polypeptide can be compared. The similarity is obvious, though compared structures were selected independently without adaptation of micelle structure to this particular polypeptide fragment.

Tab. 4. The symmetry parameters of Congo Red micelles A, B, C; micelle attached to protein fragment 68—88 VL IgG domain and twisted beta structure of 101—109 peptide fragment of VH IgG domain

PARAMETER	MICELLE A	MICELLE B	MICELLE C	COMPLEX	BETA BARREL
13 (Å)	3.9	10.1	18.6	17.9	16.5
14 (deg)	90.1	59.9	8.9	9.1	7.5
15 (Å)	4.2	4.2	4.3	4.4	5.7

The considerable similarity of the twist was found in three independent cases: in the minimized micelle C; in the micelle attached to the 68—88 amino acids of VL IgG; and in the peptide fragment 101—109 of VH IgG. The similarities found in this model system make such overlapping and fitting contact in protein-dye complex very likely.

DISCUSSION

The correlation found between binding of azo dyes and functional activities of immunoblogulins G allows for a new approach in structure-function studies concerning antibody molecules. Unusual for this ligand-protein interaction is the multimolecular form of the dye, in which it interacts with immunoglobulins (8) and some enzymes (10, 21). Unlike the standard docking of the ligand to the binding site, here the stability of the complex is coming rather from multiple noncovalent interactions distributed across an extensive dye protein interface. The interaction of the dye with immunoglobulin is possible after the structure of the protein molecule becomes modified by heating or by binding to antigen. The structure of IgG domains loosened due to broken hydrogen bondings, makes peptide β hairpins available for interaction. The stability of the complex with the dye is based, according to theoretical consideration on multiple van der Waals contacts.

The plasticity of the micellar dye species may ensure the optimal fitness of interacting dye and peptides, and explains the resulting strong interaction. The notion of structurally modified IgG domains, which occur being forced by binding of antibody to antigen, seems to be convincingly supported by using the dyes. Unfortunately, no unequivocal conclusion concerning the location of the site of binding the dye in IgG molecule can be drawn at the moment.

The dipolar, rigid, elongated molecules of azo dyes explains their tendency to form solutions with dye molecules grouped in large species of ordered structure corresponding to solutions of liotriopic liquid crystals. The experiments designed to clarify the role and significance of the micellar character of the dye for interaction with immunoglobulins were based on using two isomeric dyes whose inclination to aggregate differs (Evans Blue and Trypan Blue). The dissimilarity of these dyes arises from the different position of the sulphonic groups. In the case of Trypan Blue the sulphonic group considerably disturbs aggregation of molecules by introducing steric and electrical repulsion. These repulsions interfere with the hydrophobic interaction of the non-polar fragment of the dye molecule which is responsible for the formation of the micellar structure in water. This is correlated with the different effects of these two dyes on the formation of immune complex, which is substantially lower in the case of Trypan Blue (*Fig. 4*). At low ionic strength the aggregating tendency of Trypan Blue, as concluded from the experiment presented on *Fig. 3,* is reduced almost completely. However, some slight effects, indicating the micellar character of this dye appear at higher salt concentrations. This behaviour may result from shielding of ionic repulsion forces. The experiments designed to identify the location of dye binding peptide loops, and clarify the biological role of the fenomenon are in progress and will be presented later.

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