ANTRAQUINONE-TRIAZINE DERIVATIVES OF POLYSACCHARIDES. RELATION BETWEEN STRUCTURE AND AFFINITY TO LACTATE DEHYDROGENASE

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The inhibition effects (I_{50}) of a series of dyes (Cibacron Blue 3G-A, Procion Blue MX-R, Remazo Brilliant Blue R and Ostazin Brilliant Red S5-B) covalently linked to Dextran T 70 were the criterion of the relationship between the structure of antraquinone-triazine compounds and their affinity to rabbit muscle lactate dehydrogenase (LDH). The I_{50} values indicate a substantial importance of the terminal benzenesulfonane moiety in the structure of Cibacron Blue. Also the degree of substitution of polysaccharide plays an important role in the affinity; its influence upon I_{50} can be expressed for Cibacron Blue-Dextrans T 70 by an equation of line. The influence of the structure of the polysaccharide constituent on the affinity of Cibacron Blue-(α-glucans) towards LDH is discussed, as well.

The affinity of LDH to Cibacron Blue-polysaccharides is of such an extent that it avoids the affinity elution of LDH from Cibacron Blue-bead cellulose. The LDH can easily be eluted from other three derivatives of bead cellulose under conditions of affinity elution. The separation properties of Ostazin Red-bead cellulose indicate an involvement of triazine grouping into affinity formation towards LDH.

The possibility to use bead cellulose as a matrix for affinants of antraquinone-triazine type has been mentioned in our previous paper[1]. Thus, Remazol Brilliant Blue was shown to be effective as an affinant in affinity chromatography of LDH from an extract of rat liver, whereas the hitherto frequently used affinant Cibacron Blue 3G – A proved to be less suitable.

The affinity of antraquinone-triazine compounds, especially of Cibacron Blue, to several enzymes mainly dehydrogenases and kinases is associated with the presence of an antraquinone grouping in the structure of an affinant and "dinucleotide fold" supersecondary structure of the respective enzymes[2]. As has later been shown, the antraquinone moicty of Cibacron Blue preserved its affinity towards NAD(P)-dependent dehydrogenases even after a considerable simplification of the original structure[3,4]. The function of the non-antraquinone moiety of the molecule, *e.g.* triazine grouping, remains open.

The important component, in addition to the affinant, is the polysaccharide as a matrix. Its choice has so far been rather intuitive[4]. Therefore, this paper deals, besides the study of relationships betweeen the structure of antraquinone-triazine

affinant and its affinity to rabbit muscle LDH, also with the effect of structure of the polysaccharide matrix on this affinity.

EXPERIMENTAL

Chemicals

Antraquinone-triazine compounds were commercially available: Cibacron Blue 3G-A, C.I. Reactive Blue 2 (Ciba-Geigy, Basel, Switzerland), Procion Blue MX-R, C.I. Reactive Blue 4 (ICI Ltd, Macclesfield, England), Remazol Brilliant Blue R, C.I. Reactive Blue 19 (Farbwerke Hoechst AG, Frankfurt/M, Federal Republic of Germany) and Ostazin Brilliant Red S5-B, C.I. Reactive Red 2 (Sdružení pro odbyt dchtových barviv, Prague). Dextrans series T, Blue Dextran and Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden), glycogen (Merck, Darmstadt, FRG and soluble starch according to Zulkowski (Lachema, Brno, Czechoslovakia) were also commercially available, bead cellulose grain size 20—320 μ was kindly donated by Dr J. Štamberg, Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, Prague.

Lactate dehydrogenase (L-lactate: NAD oxidoreductase, EC 1.1.1.27) of rabbit muscle, a crystalline suspension in ammonium sulfate (100 U/mg) and NAD^+ were obtained from Biochemica Boehringer, Mannheim, FRG, NADH, bis-sodium salt from Reanal, Budapest, Hungary.

The antraquinone-triazine dyes were twice crystallized from ethanol and their purity was checked by paper chromatography (Whatman No 1) in the solvent system 1-butanol–water–ethanol (40 : 32 : 28) (ref.[3]) and by elemental analysis (C, H, N, S, Cl). Their concentration was determined colorimetrically using following values: $\varepsilon_{610} = 12400\text{M}^{-1}\,\text{cm}^{-1}$ (Cibacron Blue), $\varepsilon_{590} = 5930\text{M}^{-1}\,\text{cm}^{-1}$ (Remazol Blue), $\varepsilon_{600} = 9390\text{M}^{-1}\,\text{cm}^{-1}$ (Procion Blue) and $\varepsilon_{540} = 23\,800\text{M}^{-1}\,\text{cm}^{-1}$ (Ostazin Red).

Antraquinone-Triazine Derivatives of α-Glucans

Cibacron Blue-(α-glucans) were prepared by an orthodox way[5] except for a greater amount of Na_2CO_3. The aqueous solution (10 ml) of the polysaccharide (0·2 g) was stirred after addition of Cibacron Blue (26—200 mg) at 60°C for 30 min; after addition of NaCl (0·35 g) it was heated to 80°C, Na_2CO_3 (0·4 g) added and stirred for another 2 h at 80°C. The unbound dye was removed by a gel filtration through Sephadex G-25 (100 ml; 2·4 × 21·5 cm) equilibrated with distilled water. The aqueous fraction of the coloured polysaccharide (40 ml) was evaporated to dryness under reduced pressure. The purity of the product was verified by a thin-layer chromatography on Silufol plates (Kavalier, Votice) in the solvent system 2-propanol–acetone–water (2 : 2 : 1). The product was gel-filtered once more unless it was pure enough. The degree of substitution of the polysaccharide with dye was estimated colorimetrically.

Procion Blue-Dextran T 70 and Ostazin Red-Dextran T 70 were prepared in the same way. Solution of 0·1M-K_2CO_3 (5 ml) containing the polysaccharide (0·2 g) and dye (40 mg) was stirred at 20°C for 1 h. Further procedure after neutralization with 0·1M-HCl was the same as that given with Cibacron Blue derivatives. Remazol Blue-Dextran T 70 was prepared similarly with the exception that the reaction was carried out in 0·25M-NaOH (5 ml).

Antraquinone-Triazine Derivatives of Bead Cellulose

The starting material was the suspension of suction-dried bead cellulose (5 g, 0·61 g of dry material) in water (10 ml) (Cibacron Blue), or the respective solution. The amount of the added

dye was as follows: 50 mg (Remazol), 100 mg (Cibacron, Ostazin), 150 mg (Procion). Reaction conditions corresponded with those given for the preparation of α-glucans with the exception of the amount of the added Na_2CO_3 (80 mg) employed for preparation of Cibacron Blue-cellulose. The products were thoroughly washed with distilled water after the reaction was through, submerged in a 0·02% aqueous sodium azide and stored at 0—4°C. The substitution degree of cellulose was calculated from the absorbance of the filtrate after reaction using the appropriate ε values.

Affinity of the Antraquinone-Triazine Derivatives
of α-Glucans and Cibacron Blue Towards Lactate Dehydrogenase

The I_{50} values, expressing the concentration of the inhibitor or affinant needed for attaining a 50% inhibition at certain concentrations of substrates were the criterion of affinity. The I_{50} (μM) values were determined from the LDH activity (25°C) after addition of an inhibitor as pI_{50} ($= -\log_{10}[I_{50}]$). The pI_{50} values were obtained from a graphic interpolation of f_I *versus* $\log [I]_I$, or from an extrapolation employing the rewritten equation $I_{50} = (1 - f_I)[I]_I/f_I$, where f_I, standing for the fractional inhibition, can be expressed as $f_I = R_I/R_C$; R_I and R_C are the initial rates in the presence or absence of an inhibitor, respectively; $[I]_I$ is the total concentration of the inhibitor.

The activity of LDH was measured spectrophotometrically[6] at 340 nm in a mixture consisting of 48·3 mM phosphate buffer KH_2PO_4–K_2HPO_4 of pH 7·5, 0·7 mM sodium pyruvate, 26 μM-NADH and 0·38 mM-$NaHCO_3$. The reactions were triggered by addition of an enzyme in such a concentration as the initial rate of reaction $R_C([\Delta A_{340}/\text{min}])$ did not exceed 0·09. The inhibitors were added as solutions of antraquinone-triazine derivatives of α-glucans, or Cibacron Blue in a 50 mM phosphate buffer. The presented I_{50} values are the averages of minimum two measurements.

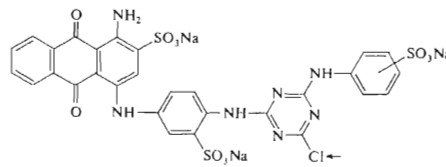
Affinity Elution of Lactate Dehydrogenase on Antraquinone-Triazine Derivatives
of Bead Cellulose

The coloured derivative of bead cellulose (1 ml) was washed with a 0·1% solution of bovine serum albumin (2·5 ml), 10 mM Tris-HCl buffer of pH 7·5 containing 1 mM of EDTA and 2 mM of 2-mercaptoethanol (10 ml of solution A), 1M-NaCl (10 ml) and finally repeatedly with solution A (20 ml). In this way prepared column (0·9 × 2 cm) was topped with LDH (0·5 mg, 50 units) in solution A (1·5 ml). The column was eluted with solution A (10 ml) and solution A containing 1 mM of NADH (20 ml, solution B). Collected were 1 ml-fractions at a 12 ml/h flow rate. The corresponding aliquots of each fraction were tested for LDH activity, in some cases for concentration of proteins, as well. The protein concentration was determined colorimetrically with bovine serum albumin as a reference[7].

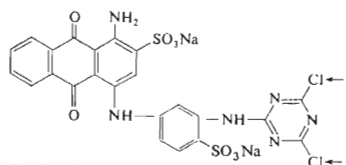
RESULTS

Four compounds were chosen (Fig. 1) for investigation of the affinity between antraquinone-triazine dyes and LDH. Cibacron Blue 3G−A was the standard for its most complicated structure; the remaining three dyes have some part of the basic structure embodied in their molecules. Ostazin Red, in contrast to *II* and *III* does not contain the antraquinone grouping, but the common part is the triazine grouping with the adjoining anilide bridge bond.

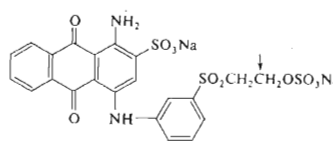
The inhibition effect (I_{50}) of those four compounds towards rabbit muscle lactate dehydrogenase (LDH) was the criterion of affinity. Each of the compounds under investigation contains, however, a reactive group, which might be responsible for the



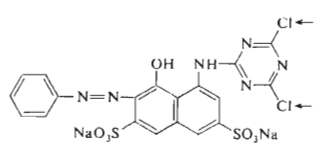
Cibacron Blue 3G-A *I*



Procion Blue MX-R *II*



Remazol Brilliant Blue R *III*



Ostazin Brilliant Red S-5B *IV*

FIG. 1

Structures of Anthraquinone-Triazine Compounds Employed
Arrow shows the binding sites of compounds to polysaccarides.

irreversible inhibition of such a type of thiol enzyme as is LDH (ref.[8]). That is why Dextran T 70 was derivatized with these affinants through this reactive group. These derivatives were prepared with the aim to obtain preparations with a similar concentration of the affinant (Table I); Cibacron Blue was shown to be more than 1000 times more effective than the others. Procion Blue- and Ostazin Red-Dextrans T 70 displayed such a low inhibition effect that it was impossible to determine their I_{50} value by the method employed.

TABLE I

Inhibition Effects of Antraquinone-Triazine Derivatives of Dextran T 70 Towards Lactate Dehydrogenase

The I_{50} for Cibacron Blue was 3·0 μM.

Antraquinone-triazine compound	Substitution degree μmol/g	I_{50} μM
Cibacron Blue	72·8	0·3
Procion Blue	55·5	>100
Remazol Brilliant Blue	101·0	380[a]
Ostazin Brilliant Red	90·7	>100

[a] Extrapolated.

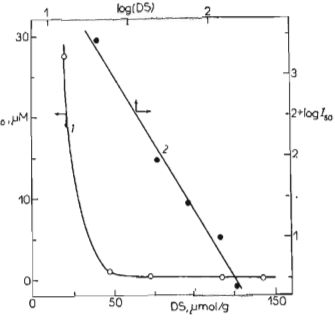


FIG. 2

Relationship Between the Inhibition Effect of Cibacron Blue-Dextran T 70 and its Substitution Degree

1 I_{50} *versus* (DS); *2* log [I_{50}] *versus* log (DS). 26, 60, 100, 150 and 200 mg of the respective dye was used for preparation of Cibacron Blue-Dextrans. Inhibition effect was studied with rabbit muscle lactate dehydrogenase.

A considerable role in affinity evaluation of the above-mentioned preparations of Dextran plays the substitution degree (DS) of the polysaccharide expressed by μmol of the affinant/g. Thus, with Cibacron Blue-Dextran T 70 the original functional dependence can be linearized in a logarithmic arrangement to a relationship $\log (I_{50}) = -3{\cdot}35 \log (\mathrm{DS}) + 5{\cdot}72$ ($n = 5$, $r = 0{\cdot}99$, Fig. 2).

Evidently, the substantial part of the affinity of the complex affinant–polysaccharide towards LDH bears the affinant. So far, there is not clear to what extent the polysaccharide is involved in this affinity[4]. The I_{50} value for Cibacron Blue, showing a 10 times lower inhibition effect towards LDH than that of Cibacron Blue-Dextran T 70, evidences (Table I) that this involvement is not negligible. Significant differences in inhibition effect $(I_{50})_{exp}$ of Cibacron Blue derivatives of these polysaccharides towards LDH (Table II) were found in a series of three water-soluble polysaccharides of the α-glucan series. In addition to molecular weight of the polysaccharide also the type of glycosidic bond could play a considerable role. Thus *e.g.* the improved affinity properties can be observed with Cibacron Blue-Dextran T 500 $[(I_{50})_{exp} \ll (I_{50})_{calcul}]$; on the other hand, Cibacron Blue-glycogen and Cibacron Blue-starch have a noticeably lowered inhibition effect.

Differences in the structure of affinants are also seen in the dissociation of the enzyme–affinant complexes, the matrix of which is bead cellulose. The measure of dissociation was the desorption degree of LDH with an equilibration buffer, or equilibration buffer combined with 1 mM-NADH (Table III). The elution diagrams of the remaining three derivatives of cellulose are similar with the exception

TABLE II

Inhibition Effects of Cibacron Blue-(α-Glucans) Towards Lactate Dehydrogenase

Cibacron Blue-glucans were prepared from 30 mg of dye. $(I_{50})_{exp}$ are values determined for the given Cibacron Blue-glucans, $(I_{50})_{calcul}$ are hypothetic values calculated from equation of line (Fig. 2), *i.e.* I_{50} values displayed by Cibacron Blue-Dextran T 70 of a substitution degree given for the respective Cibacron-Dextran.

α-Glucan	Substitution degree μmol/g	$(I_{50})_{exp}$ μM	$(I_{50})_{calc}$ μM
Dextran T 70	72·8	0·27	0·32
Dextran T 500	74·4	0·05	0·28
Dextran T 2000[a]	64·8	0·98	0·45
Starch	122·0	0·73	—
Glycogen	112·0	1·78	—

[a] Blue Dextran (Pharmacia Uppsala, Sweden).

of Cibacron Blue-cellulose. The desorption of LDH during elution with an equilibration buffer containing 1 mM-NADH is immediate and quantitative. The high affinity of Cibacron Blue towards LDH results in a low desorption of LDH from Cibacron Blue-cellulose. Ineffective eluents were found also the 1M salts as *e.g.* ammonium sulfate and sodium chloride. An important parameter remains the substitution degree of cellulose with the affinant; this makes it possible to regulate the binding capacity towards the separated enzymes. Results obtained with Ostazin Red-cellulose indicate a certain affinity even with a triazine grouping. Bead cellulose does not show any nonspecific sorption towards LDH.

DISCUSSION

Lactate dehydrogenase is reported to be one of the enzymes with the greatest affinity towards Cibacron Blue[3,9], further antraquinone-triazine compounds[3] and towards Blue Dextran[9,10]. The character of interaction responsible for this affinity[2] was possible to clear just on the basis of achieved degree of knowledge on its structure[8] and that of further enzymes displaying affinity towards antraquinone-triazine compounds. This also enables to convey the knowledge attained when studying these phenomena with *e.g.* LDH to further enzymes, as glucose-6 phosphate, alcohol and glutamate dehydrogenase, alanine aminotransferase *etc.*[11].

Previous papers[2,9,10] judged the affinity of Cibacron Blue towards enzymes by means of Blue Dextran (Dextran T 2000 derivatized with Cibacron Blue). Results obtained in these experiments were compared with those achieved when separating

TABLE III
Elution of Lactate Dehydrogenase from Antraquinone-Triazine Derivatives of Bead Cellulose

Column	Substitution degree μmol/g	Percentage of total activity eluted; elution with	
		10 mM Tris	1 mM-NADH
Cibacron Blue	67·2	0	7·4
Procion Blue	57·0	4·6	87·4
Remazol Brilliant Blue	56·2	3·4	104·0
Ostazin Brilliant Red	81·3	1·6	98·4
Control[a]	—	97·6	0

[a] Bead cellulose.

enzymes on Cibacron Blue derivatives of water-insoluble polysaccharides. These derivatives were prepared, in contrast to Blue Dextran, mainly *via* the cyanogen bromide activation[2,10]. Whereas Cibacron Blue is linked to dextran through a triazine grouping, in the cyanogen bromide activation probably amino groups of the antraquinone grouping take part in the linkage. This paper is aimed to avoid misconceptions associated with the cyanogen bromide method by making use of the reactive groups of antraquinone-triazine compounds, since the mechanisms of reactions of antraquinone-triazine compounds with polysaccharides are (Fig. 1), however, sufficiently described[12].

Attempts to express the affinity of products of these reactions towards LDH by means of inhibition constants K_i failed. Thus, in graphic plots according to Lineweaver–Burk or Dixon no significant linear relations were obtained. A like scattering of points in calculation of K_i was reported also in preceding papers with Blue Dextran[9,10]. In spite of this failure the competitive type of inhibition was considered. Neither this fact is sufficient for determination of the mechanism of interaction of the inhibitor with enzyme-bicatalyzed of substrate reaction. In respect to this, there is no possibility to compare[13,14] the preceding K_i data with I_{50} values ascertained in this paper.

Quite significant seems to be the involvement of the terminal benzenesulfonane in the basic structure of Cibacron Blue-Dextran T 70 in the affinity towards LDH. As long as this part of the molecule of affinant was omitted (Procion Blue) a substantial decrease of the inhibition effect was encountered (Table I). Had the centre of affinity be in the antraquinone moiety[2–4], the affinity of Procion Blue- and Remazol Blue-Dextrans T 70 should not have decreased in such an extent. The terminal benzenesulfonane does not form, however, any spacer for the antraquinone moiety (Fig. 1). In the series of affinants *I–IV*, evidently not even other parts of their structures play the role of a spacer in relation to the antraquinone grouping. Remazol Blue-Dextran T 70 shows *e.g.* with a shorter spacer a greater affinity than Procion Blue-Dextran T 70. The obtained results indicate that the terminal benzenesulfonane in Cibacron Blue-Dextran T 70 displays an individual, very important centre of affinity towards LDH.

The importance of a triazine grouping is evidenced by results obtained with Ostazin Red; its derivative with bead cellulose preserves satisfactory separation properties towards LDH (Table III). The term triazine grouping involves in this case the neighbouring substituted aminonaphthalene, which can simulate one of the anilide bridge bonds in the basic Cibacron Blue structure (Fig. 1).

α-Glucans used in this paper (Table II) differ from each other by a main glycosidic bond, polymerization degree and branching, secondary structure and conformation of chains. The influence of the polymerization degree on the affinity of the affinant–polysaccharide complex towards LDH is evident.

The low degree of desorption of phosphofructokinase from Cibacron Blue-cellulose, described with powder cellulose[5], was manifested even with LDH desorbed

from Cibacron Blue-bead cellulose; cellulose evidently differs in this direction from agarose (Sepharose) and crosslinked dextran (Sephadex) with the use of which frequently quantitative desorptions of enzymes were achieved[4,5]. On the other hand, substantial interference into the structure of these polysaccharides, *e.g.* crosslinking to various extent, also leads in some cases to the loss of a good desorption properties[4]. It could be, therefore, anticipated that these and further facts concerning the participation of the polysaccharide structure on the affinity of affinant–polysaccharide complexes towards enzymes might result in their exploitation for studying the supermolecular structure of polysaccharides.

A little attention has hitherto been paid to cellulose as a matrix for this system of affinity chromatography. Our preceding results[1] have already shown that the macroporous bead cellulose can be a suitable matrix for antraquinone-triazine affinants. Excellent hydrodynamic properties, regular geometric shape, high porosity[1,5] and finally the low price make it possible to favour bead cellulose as a perspective material also in the affinity chromatography.

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