INHIBITION OF LACTATE DEHYDROGENASE EX RABBIT MUSCLE BY CIBACRON BLUE 3G-A BOUND TO WATER-SOLUBLE HYDROXYETHYLCELLULOSE

Danica MISLOVIČOVÁ[a], Peter GEMEINER[a] and Ladislav ŠOLTÉS[b]

[a] *Institute of Chemistry, Centre for Chemical Research, Slovak Academy of Sciences, 842 38 Bratislava and*
[b] *Institute of Experimental Pharmacology, Centre of Physiological Sciences, Slovak Academy of Sciences, 842 16 Bratislava*

Received March 16th, 1984

The interaction between lactate dehydrogenase and hydroxyethylcellulose — Cibacron Blue 3G-A conjugates was investigated kinetically. These conjugates were obtained by covalent binding of Cibacron Blue to water-soluble hydroxyethylcelluloses, of average relative molecular masses $2{\cdot}5 \cdot 10^4$ to $11{\cdot}6 \cdot 10^4$. The polymeric matrix (non-branched β-glucan) was, unlike the branched α-glucans, found to inhibit the action of the dye, irrespective of the molecular mass of this matrix. With increasing amount of the bound dye the interaction between the dye and the enzyme was enhanced.

The preceding paper[1] described the differences observed in the interaction of lactate dehydrogenase (LDH) with the triazine dye Cibacron Blue 3G-A (CB), free or bound to dextran or hydroxyethylstarch (HES). Unlike other authors[2-5], who determined the inhibitory effects of the free dye and the dye bound to dextran of a given $\overline{M}_w$ (usually dextran T-2000, with the commercial name Blue Dextran 2000), we used a series of dextrans T, covering an $\overline{M}_w$ range from $1 \cdot 10^4$ to $2 \cdot 10^6$, and HES (prepared from waxy maize starch) in a range $\overline{M}_w = 3{\cdot}6 \cdot 10^4$ to $2{\cdot}0 \cdot 10^6$, dyed with CB to various extents. This enabled us to study the inhibitory effect of the bound dye on LDH in relation to *a*) the amount of the bound dye, *b*) the average relative molecular mass of the polysaccharide and *c*) the structure of the polysaccharide. The results show that the dextrans and HES in a considerable range of molecular masses enhanced the interaction of CB with the muscle LDH (non-homogenous). This finding accords with the results published by Thompson and coworkers[2,3], who report more than a thousandfold increase of the interaction between LDH isoenzyme M_4 and CB after binding of the latter to dextran T-2000. The object of the present paper was to verify the previous results[1] and extend their applicability to another polymeric matrix, *viz.* water-soluble hydroxyethylcellulose (HEC), which, in contrast to dextran and waxy maize starch, is a non-branched β-glucan.

EXPERIMENTAL

Materials

Natrosol hydroxyethylcelluloses, types 250 L and 250 G, with a mole substitution MS = 2·5, was kindly supplied by Hercules BV, Hague, The Netherlands. Dextrans of series T were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Fractionated hydroxyethyl derivatives of waxy maize starch, substitution degree DS = 0·64 to 0·67, were a gift from Dr K. Granath, Pharmacia, Uppsala. Lactate dehydrogenase (L – lactate: NAD^+ oxidoreductase, EC 1.1.1.27) ex rabbit muscle (Boehringer, Mannheim, F.R.G.) was a preparation for analytical purposes (a crystalline suspension in 3·2 mol l^{-1} ammonium sulphate, starting specific activity 262·0 U mg^{-1}, protein concentration 5 mg ml^{-1}). Cibacron Blue 3G-A (C.I. Reactive Blue 2, Ciba-Geigy, Basel, Switzerland) and its dechlorated analogue[1] were purified by a twofold precipitation from ethanol.

Fractionation of Hydroxyethylcellulose

Hydroxyethylcellulose was fractionated as described by Brown[6]. It was precipitated by adding acetone to a 0·4% aqueous solution (samples up to $\overline{M}_w$ $3·5 . 10^4$) and to a 0·3% aqueous solution (samples with $\overline{M}_w$ above $3·5 . 10^4$). The fractions were reprecipitated with acetone. Water was removed by repeated washing with acetone and finally ether. The fractions were dried at room temperature. With three fractions (L–1, L–2, G–1) the mass-average relative molecular masses, $\overline{M}_w$, were determined by the sedimentation velocity method. The sedimentation constants were determined in an ultracentrifuge MOM 1370 (Hungary) and the diffusion coefficients in Semimicroelectrophorese-model 37 (G.D.R.). The intrinsic viscosity numbers $[\eta]_{25}$ were obtained by measuring viscosities of aqueous solutions of these fractions at 25°C. The determined values of $\overline{M}_w$ and $[\eta]_{25}$ were inserted into the Mark–Houwink equation; employing the least-square method we calculated constants $K = 1·02 . 10^{-3}$ dl g^{-1} and $a = 0·70$. The $\overline{M}_v$ values of the fractions were then determined by inserting the experimental values of $[\eta]_{25}$ into this equation (Table I).

Gel Chromatography

The glass column used for gel permeation chromatography (length 215 mm, I.D. 25 mm) was packed with Sepharose 2B-CL (Pharmacia Fine Chemicals, Uppsala, Sweden), particle size 60–200 μm. The eluant was a phosphate buffer 50 mmol l^{-1}, pH 7·5, the elution rate 28 ml h^{-1}. The concentration of the applied sample was 2 mg of HEC/ml of the buffer; the sample volume was 1 ml. The concentrations in the effluent (3 ml fractions) were determined by the standard method[7]. The gel chromatography equipment was calibrated with a set of dextran fractions ($\overline{M}_w = 3·95 . 10^4$, $1·67 . 10^5$, $4·96 . 10^5$), hydroxyethylstarch fractions ($\overline{M}_w = 5·35 . 10^4$, $1·27 . 10^5$, $2·07 . 10^5$, $4·02 . 10^5$, $9·37 . 10^5$ and $1·92 . 10^6$) and HEC fractions (for $\overline{M}_v$ see Table I). The elution volume of a fraction was taken as the top of a chromatographic peak, found by the asymptote method. The plotted average relative molecular masses were the values of $\overline{M}_w$ or $\overline{M}_v$ (Fig. 1). The correction for spreading was calculated from the Tung integral equation[8]. The spreading function was approximated by a symmetrical Gaussian curve with a resolution factor h. To determine the values of the resolution factor and its dependence on v we employed the method of Balke and Hamielec[9], or a modification of Kotaka's method[10–12]. The corrected average relative molecular masses $\overline{M}_w$ and $\overline{M}_n$ were determined by the iteration procedure of Chang and Huang[13]. The results were calculated by using computers Siemens 4004 and HP 85. A crite-

rion of how the simulated $h - v$ relation fits its actual course[14] are the negligible differences between the values of $\overline{M}_w/\overline{M}_n$ specified for the fractions of dextran and hydroxyethylstarch and the calculated figures (Table I).

TABLE I

Average relative molecular masses and polymolecularities of fractionated hydroxyethylcelluloses Natrosol 250 L and Natrosol 250 G. Fractions L and G refer to Natrosols 250 L and 250 G, respectively. $\overline{M}_w$ Mass-average relative molecular mass, $\overline{M}_n$ number-average relative molecular mass, $\overline{M}_v$ viscosity-average relative molecular mass, $\overline{M}_w/\overline{M}_n$ polymolecularity

Fractions of HEC	Gel permeation chromatography		Viscosimetry	
	$\overline{M}_w \cdot 10^{-4}$	$\overline{M}_w/\overline{M}_n$	dl g^{-1}	$\overline{M}_v \cdot 10^{-4}$
L-1	2·54	1·17	1·22	2·49
L-2	4·92	1·63	1·74	4·14
L-3	10·3[a]	1·76	2·30	6·17
L-4	11·6	1·63	3·41	10·8
G-1	15·1	1·78	4·09	13·9
G-2	19·3	1·39	5·92	23·8

[a] Bimodal distribution of molecular masses.

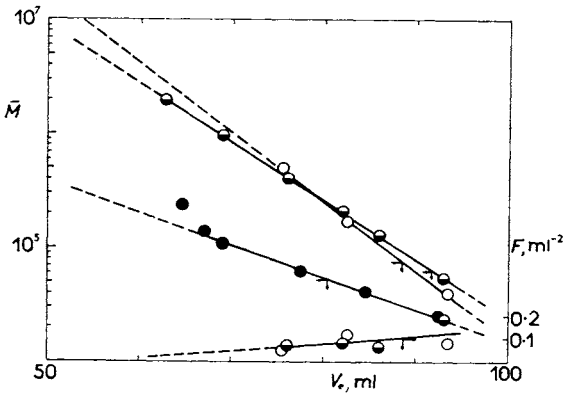

FIG. 1

Average relative molecular mass $\overline{M}$ of the polysaccharides, and resolution factor h, in relation to elution volume V_e determined by gel permeation chromatography. The values plotted as average relative molecular masses were $\overline{M}_w$ for dextrans (○) and HES (◒), and $\overline{M}_v$ for HEC (●) (Table I)

Preparation of Cibacron Blue — Hydroxyethylcellulose

The dye Cibacron Blue 3G-A (CB) was covalently bound to HEC by a procedure analogous to that reported for dextrans[15]: the dye was dissolved in 1% aqueous HEC; after 30 minutes' stirring at 60°C sodium chloride and sodium carbonate were added and the temperature was raised to 80°C. The weight ratios of the dye: HEC: Na_2CO_3 were 0·24—0·48 : 1 : 0·6—0·7 in 1·75% aqueous NaCl; the reaction was allowed to proceed for 2 h. CB-HEC complexes up to $\overline{M}_w$ *c.* 1 . 10^5 were isolated by gel chromatography[15], those above $\overline{M}_w$ 1 . 10^5 were obtained by repeated precipitation followed by centrifugation from a 0·5% aqueous solution of NaCl. The extent of the binding of the dye S_d (μmol g^{-1}) was determined spectrophotometrically, using a molar absorptivity $\varepsilon_{610} = 12\,400$ l mol^{-1} cm^{-1} for the free dye[15].

Determination of the Inhibitory Effect

The inhibitory effects are expressed by the values of I_{50}. These were obtained by graphical interpolation of the plot of f_I vs log $[I]_t$, where f_I denotes fractional inhibition and $[I]_t$ the total (free and bound) concentration of the dye (μmol l^{-1}). The catalytic activity of LDH was measured spectrophotometrically at 340 nm[16]. The blank experiments with HEC were carried out in the same concentration range as with CB-HEC.

RESULTS AND DISCUSSION

The following factors affecting the inhibitory effect of bound CB on LDH were investigated: *a*) concentration of the coenzyme NADH, *b*) extent of binding of the dye to HEC, *c*) average relative molecular mass of HEC and *d*) structure of HEC compared to the polysaccharide matrices studied previously, *i.e.* dextran and HES.

With an increase in concentration of NADH the interaction of both the bound and the free dye with the enzyme decreased (Table II). An opposite effect on the

TABLE II

Inhibition of lactate dehydrogenase by Cibacron Blue 3G-A, bound to hydroxyethylcellulose to different extents. Fraction L-4 was used, $\overline{M}_w = 11{\cdot}6 \,.\, 10^4$. I_{50} for Cibacron Blue 3G-A were 3·0 and 3·6 μmol l^{-1}, for its dechloro analogue they were 4·6 and 6·8 μmol l^{-1}

S_d μmol g^{-1}	I_{50}, μmol l^{-1}	
	a	*b*
33·5	11·1	68·0[c]
39·4	9·3	29·5
48·6	7·9	26·9
60·6	6·9	15·6
68·6	2·7	8·7

[a] 26·0 μmol l^{-1} NADH; [b] 43·3 μmol l^{-1} NADH; [c] value obtained by extrapolation.

interaction between the bound dye and the enzyme was observed with increasing the extent of binding of CB to HEC. The same effect was observed in the binding of the dye to dextrans[1,15] or HES (ref.[1]). In accordance with the previous results[1] it can be stated that *a*) CB bound to HEC also competes with the coenzyme for the binding sites of LDH, *b*) after the binding of CB to HEC the portion of the effective concentration of the dye is the higher the more CB is bound to HEC. However, at a given extent of this binding the effect on CB in HEC complexes is weaker than in the dextran

TABLE III

Inhibition of lactate dehydrogenase by Cibacron Blue 3G-A bound to fractions of Natrosol 250 L. The values of $\overline{M}_w$ were determined by gel chromatography, those of $(I_{50})_{calc}$ were obtained by calculation from the graph $(I_{50})_{exp} = f(\log S_d)$, for CB bound to different extents, S_d, to the HEC fraction L-4

Fraction of HEC	$\overline{M}_w \cdot 10^{-4}$	S_d μmol g^{-1}	$(I_{50})_{exp}$ μmol l^{-1}		$(I_{50})_{calc}$ μmol l^{-1}	
			a	*b*	*a*	*b*
L-1	2·54	53·1	9·2	30·9	7·2	22·9
L-2	4·92	62·6	4·9	12·0	5·5	14·5
L-3	10·3	49·1	9·1	24·5	7·8	26·3
L-4	11·6	60·6	6·0	15·6	6·0	15·2

[a] 26·0 μmol l^{-1} NADH, [b] 43·3 μmol l^{-1} NADH.

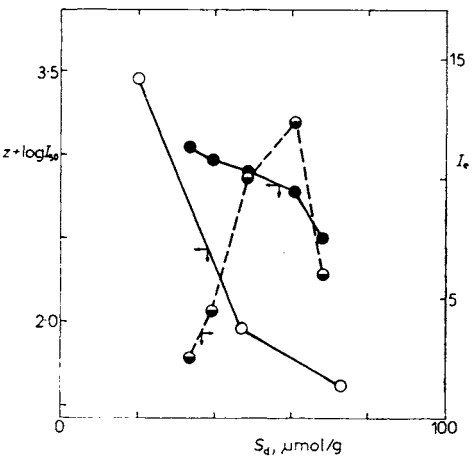

FIG. 2

Effect of the extent of binding (S_d) of CB to dextran T 70 (○) and HEC fraction L 4 (●) on inhibition of LDH, expressed by I_{50} (○, ●) and by index of efficacy (◒). The indices of efficacy, I_e, are expressed by ratios of I_{50} for CB-HEC to I_{50} for CB-Dex at equal values of S_d

complexes. The ratios of I_{50} (CB-HES) to I_{50} (CB-Dex.) for equal S_d, expressed by an efficacy index I_b (Fig. 2), are higher than 10 in the range $49 < S_d < 64$ μmol . g^{-1}.

Unlike dextrans and HES (ref.[1]), HEC diminished the interaction between the bound CB and LDH (Table II). Elongation of the HEC chain had little effect on the CB-LDH interaction (Table III). The rather narrow range of average relative molecular masses of the CB-HEC conjugates is evident from poor solubility of CB-HEC prepared from HEC of $\bar{M}_w > 1 . 10^5$.

To conclude it can be said that the interaction between the bound dye and LDH decreases in the order dextrans – HES – HEC. In accordance with our previous results[1] we ascribe this fact to different conformations of the polysaccharides in the solutions. These may cause differences in occlusion of the bound dye by the polysaccharide and in selective adsorption of LDH on the polysaccharide macromolecules. Then, in the order dextrans – HES – HEC, *a*) the effective concentration of the bound dye decreases (at a constant analytical concentration) and *b*) the differences between the analytical and the effective concentrations of the bound dye increase.

Acknowledgement is due to Dr D. Berek for criticism of the manuscript.

REFERENCES

1. Gemeiner P., Mislovičová D., Šoltés L., Tichá M., Barthová J.: Biológia (Bratislava) *39*, 763 (1984).
2. Thompson S. T., Stellwagen E.: Proc. Nat. Acad. Sci. U.S.A. *73*, 361 (1976).
3. Thompson S. T., Cass K. H., Stellwagen E.: Proc. Nat. Acad. Sci. U.S.A. *72*, 669 (1975).
4. Haff L. A., Easterday R. L.: *Theory and Practice in Affinity Techniques* (P. V. Sundaram, F. Eckstein, Eds), p. 23. Academic Press, London 1978.
5. Ashton A. R., Polya G. M.: Biochem. J. *175*, 501 (1978).
6. Brown W.: Ark. Kemi *18*, 227 (1961).
7. Dubois M., Gilles K. A., Hamilton J. K., Rebers P.A., Smith F.: Anal. Chem. *28*, 350 (1956).
8. Tung L. H.: J. Appl. Polym. Sci. *10*, 375 (1966).
9. Balke S. T., Hamielec A. E.: J. Appl. Polym. Sci. *13*, 1381 (1969).
10. Kotaka T.: Angew. Makromol. Chem. *56*, 77 (1976).
11. Kotaka T.: J. Appl. Polym. Sci. *21*, 501 (1977).
12. Šoltés L., Berek D., Mikulášová D.: Colloid Polym. Sci. *258*, 702 (1980).
13. Chang V. S., Huang R. Y. M.: J. Appl. Polym. Sci. *13*, 1459 (1969).
14. Tung L. H., Runyon I. R.: J. Appl. Polym. Sci. *13*, 2397 (1969).
15. Gemeiner P., Mislovičová D., Zemek J., Kuniak Ľ.: This Journal *46*, 419 (1981).
16. Bergmeyer H. U.: *Methoden der enzymatischen Analyse*, 2. Auflage, s. 531. Verlag Chemie, Weinheim 1970.

Translated by J. Salák.