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Reappearance of Metachromasia after Digestion of Hyaluronate with Hyaluronidase

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The present investigation supports the recent theories on the metachromatic reaction. It is shown that the metachromatic colour can reappear after digestion of hyaluronate with testicular hyaluronidase. A difference exists between the absorption maxima of hyaluronate and heparin.

It has long been recognized that certain macromolecular substances present in animal tissues, *e.g.* acidic mucopolysaccharides, couple with basic dyes such as azure A, thionine and toluidine blue, forming coloured complexes. The term metachromasia is used to describe the phenomenon by which these tissue structures are stained by the dye solutions to a colour which is different from the dye solutions, the colours of the dye solution and of the stained tissue or solution being called orthochromatic and metachromatic, respectively. The metachromatic reaction combined with enzymic investigations is widely used for the detection and differentiation of substances such as hyaluronic acid, chrondroitin sulphuric acids and heparins.

MATERIAL AND EXPERIMENTS

The samples of the hyaluronate employed were prepared according to Jensen [1]. The preparation of toluidine blue used was a commercial one ("Toluidinblau nach Hoyer", Riedel de Haën A.-G., Seelze, Hannover, Germany). In some experiments this commercial product was recrystallized from water. The employed preparations of hyaluronidase were commercial Invasin "Lundbeck", Copenhagen, and "Penetrase" which was kindly supplied by Dr. Jørgen Ploug, Leo Pharmaceutical Products, Copenhagen. The sample of chondroitin sulphate used was prepared by Boström, Institute of Medical Chemistry, University of Uppsala, Sweden. The used sample of heparin was kindly placed at our disposal by Novo Terapeutisk Laboratorium, Copenhagen. All experiments were performed at pH 6.5.

The absorption of aqueous solutions of toluidine blue was measured in a Beckman Spectrophotometer in the interval of 200—800 mμ, the concentration of toluidine blue being 0.01 % (w/v). In Fig. 1 curve B shows two absorption

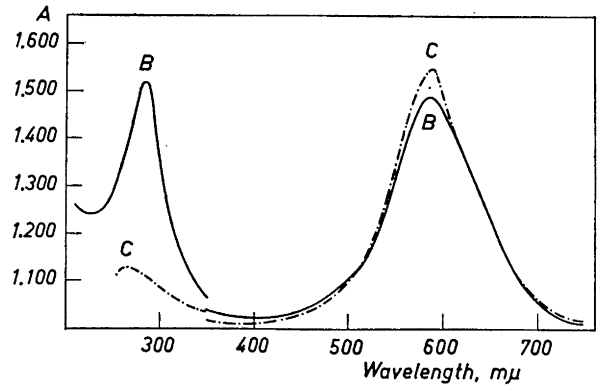

Fig. 1. Absorption spectra of commercial toluidine blue (B) and recrystallized toluidine blue (C). Ordinates are the absorbance $A = \log_{10} (1/T)$, where $T = I/I_0$. Length of cuvette 1 cm. Concentration of toluidine blue 0.1 g/l. Abscissae are the wavelengths in mμ.

maxima at 285 mμ and 590 mμ, respectively. The curve C (stippled) which is for recrystallized toluidine blue, likewise shows two maxima, at 265 and 590 mμ, respectively. It is seen that the first maximum has shifted towards a shorter wave-length after recrystallization.

Fig. 2 shows the absorption of a solution which was 0.03 % with regard to hyaluronate and 0.01 % with regard to toluidine blue, the solvent being a phosphate buffer (pH = 6.5). It is seen that the hyaluronate-toluidine blue complex has absorption maxima at 284 and 555 mμ.

It was found that the chondroitin sulphate-toluidine blue complex shows the same maxima as does hyaluronate-toluidine blue, whereas the heparin-toluidine blue complex shows a maximum at 540 mμ as seen in Fig. 3.

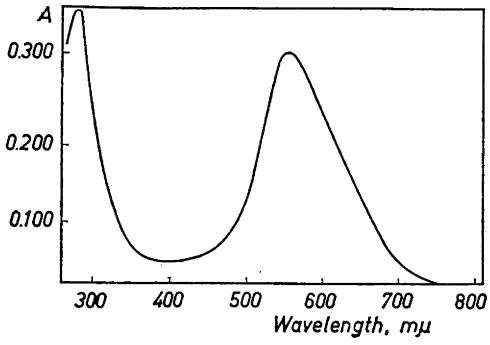

Fig. 2. Absorption spectrum of the hyaluronate-toluidine blue complex. Experimental conditions: *cf.* text to Fig. 1.

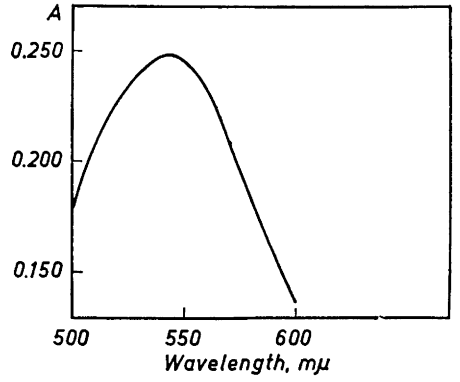

Fig. 3. Absorption spectrum of the heparin-toluidine blue complex. Experimental conditions: *cf.* text to Fig. 1.

Table 1. The changes in absorption spectrum of hyaluronate-toluidine blue complex caused by hyaluronidase digestion.

Expt. No.	Concn. of hyaluronate in %	Concn. of hyaluronidase in VRU	Time after incubation, min.	Absorption maxima, mμ	Type of enzyme
1	0.25	0	0	555	
2	0.25	10	5	585	Invasin
3	0.25	10	18	563	Invasin
4	0.25	10	5	585	Penetrase
5	0.25	2.5	2	580	Invasin
6	0.25	2.5	30	590	Invasin
7	0.25	2.5	1 290	563	Invasin
8	0.25	2.5 + 4	1 320	586	Invasin + Penetrase
9	0.25	2.5 + 4	1 410	585	Invasin + Penetrase
10	0.25	2.5 + 4	1 560	590	Invasin + Penetrase
11	0.30	7.5	9	560	Invasin
12	0.30	7.5	19	585	Invasin
13	0.30	7.5	30	585	Invasin
14	0.30	7.5	60	585	Invasin
15	0.30	7.5	90	560	Invasin
16	0.30	7.5	120	560	Invasin
17	0.30	7.5	240	560	Invasin
18	0.30	17.6	8	560	Penetrase
19	0.30	17.6	60	560	Penetrase
20	0.30	17.6	240	560	Penetrase
21	0.30	17.6	300	560	Penetrase
22	0.30	26.4	315	570	Penetrase
23	0.30	26.4	360	575	Penetrase
24	0.30	4.4	2	575	Penetrase
25	0.30	4.4	150	580	Penetrase
26	0.30	4.4	240	585	Penetrase
27	0.30	4.4	1 080	585	Penetrase

In the following experiments the hyaluronate solutions were incubated with testicular hyaluronidase. At different times aliquots were withdrawn and toluidine blue was added, whereafter the absorptions were recorded in order to follow the degradation of hyaluronate. Table 1 gives the results of these experiments. It is noted that toluidine blue has been added in all experiments, and that toluidine blue plus enzyme and buffer did not change the maximum of toluidine after 19 h.

It is evident from experiments 1,2,4 and 18—27 that the hyaluronate has been degraded by the hyaluronidase as the absorption maxima have shifted towards the maximum of pure toluidine blue during the incubation dependent upon concentration of enzyme and time. It is seen from the table that a difference exists between the two enzyme preparations. While penetrase always degrades hyalyronate at a certain (high) concentration, the invasin preparation causes only a temporary degradation (judged from the metachromatic reaction) as the absorption maximum starts moving towards the toluidine blue maximum, but later on the maximum shifts back to the hyaluronate-

toluidine blue complex maximum. (Expts. 11—17). Further it was noted that the solutions became more viscous from the time at which the maximum moved from 585 to 563 (Expts 1, 2 and 3 and 5,6 and 7 and 12—16 and 17).

DISCUSSION

According to recent theories (see, *e.g.*, McManus [2], Kinzel [3] and Larsen [4]) the reactions making the basis of the metachromatic reaction are the following: The positively charged dye-stuff ions are adsorbed to the negatively charged polyelectrolyte ions. Now, if the polyelectrolyte molecule has a sufficient size and electric surface charge density (Sylvén [5]) the dye ions will aggregate longitudinally, which means that a polymerization of the dye occurs. According to Michaelis and Granick [6] and others [7] the metachromatic colour of the dye depends on a polymerization of the dye itself.

The facts reported in the present paper seem to support the above theory. When a sample of high-molecular hyaluronate ($\overline{M}_{n\ (reduced)}$ = about 8×10^5) was used the metachromatic reaction was strong. With decreasing degree of polymerization caused by enzymic degradation the staining property or toluidine blue decreased markedly.

However, in some experiments the metachromatic colour reappeared after some time, and at the same time the viscosity of the solutions increased markedly. These observations suggest that a restructuration of hyaluronate has taken place resulting in a re-polymerization of the toluidine blue molecules.

Note added in proof: The observation that the viscosity of a solution of partly degraded hyaluronate can increase with time seems to agree with the results obtained by S. Zamenhof and E. Chargoff (*J. Biol. Chem.* **186** (1950) 207). They showed that considerably degraded DNA may have a tendency to repolymerize, attaining high viscosity. In contrast to this, undegraded DNA retains its η_{sp} constant during several hours.

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