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The macroanionic activity of heparins in the presence of dextrose and calcium ion

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The effect of dextrose on heparin was investigated using the heparin-azur A interaction as a measure of macroanionic activity. Dextrose solutions did not diminish heparin – azur A metachromasia but prior autoclaving of the dextrose solution resulted in a slight decrease. When calcium chloride was added to the dextrose (autoclaved or unautoclaved) there was a reduction which was greater than that caused by calcium ion in the absence of dextrose. The effects of calcium ion and dextrose acting together were each concentration - dependent. A low molecular weight fraction (8400) was more susceptible to the effects of calcium in the presence of dextrose than a fraction of mol. wt. 19 000, hence unfractionated heparins with different amounts of various fractions may respond differently to calcium in the presence of dextrose. This has not been considered in earlier studies of the anticoagulant activity of unfractionated heparins in dextrose infusion and could have contributed to reported discrepancies, particularly in view of the variety of test methods used. At present, it is not possible to predict from in vitro tests whether dextrose will modify the in vivo anticoagulant activity of heparin.

When heparin is dissolved in dextrose infusion its in vitro anticoagulant activity can be shown to be temporarily reduced (Okuno & Nelson 1975; Anderson et al 1979) but evidence has not been presented for destruction of heparin nor have the variable reductions reported for in vitro activity been fully explained. Unchanged activity after dissolving heparin in dextrose infusion has also been found (Mitchell et al 1976). In studies of heparin activity in dextrose infusion, interest has generally centred on the anticoagulant activity likely to be delivered intravenously, particularly since in many cases the heparin infusion may remain at the bedside for 24 h during administration and any instability could be important. Although autoclaved dextrose solutions of proprietary origin in a variety of containers, and containing acidic by-products, have usually been used in these investigations, the observed changes in heparin activity have been ascribed to an effect of dextrose. It has been shown by Anderson & Harthill (1982) that autoclaved and unautoclaved dextrose solutions can have different effects on the in vitro anticoagulant activity of heparin. Whether the dextrose effect is due to changes in the heparin molecules or disturbance of the anticoagulant assay system, or both, has not been determined but it is obviously important.

Most anticoagulant assay systems contain added calcium ion as a clotting factor. However, heparin has particularly affinity for calcium ion (Braud et al 1980) and calcium is capable of associating with certain sugar hydroxyls (Angyal 1980). Hence the effect on the activity of heparin of calcium and dextrose together requires investigation.

To avoid the complexities of the anticoagulant assay it is possible to study the effect of dextrose and calcium on the macroanionic activity of heparin by using certain cationic dyes; dye-heparin interactions may serve as models for the complex interactions with protein which occur in the anticoagulant assays. By use of the cationic dye azur A, the object of this study is to demonstrate, independently of a clotting system, the effect of dextrose and calcium ion separately and together on the macroanionic reactivity of heparin.

MATERIALS AND METHODS

All solutions were made in 0·02 M NaCl, except where otherwise stated; preliminary experiments revealed that 0·02 M NaCl eliminated dye-glass adsorption without affecting metachromatic reactions significantly. Azur A (MacNeal) was obtained from Gurr Ltd and purified from hydrochloric acid solution followed by washing with ethanol and diethyl ether, after Pal & Schubert (1962).

Sodium heparin and calcium heparin were given by Leo Laboratories Ltd (Lot No. 180011) and Laboratoire Choay (batches CH7081, 801M) respectively and were desalted by ultrafiltration (Amicon UM-2) followed by chromatography on Sephadex G25 in water. Amicon UM-2 membrane has a cut-off

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at around molecular weight 1000; refiltration when necessary resulted in an insubstantial loss of azur A positive material. Fractions of sodium heparin were prepared as previously described (Anderson & Harthill 1981); fractions L2 and L6 respectively had molecular weights of 19000 and 8400; S contents of 9.67%, 10.28%; uronic acid contents of 26.4%, 29·1%. The unfractionated parent had S = 10·47% and uronic acid = 26·6%. Sulphur was determined by the oxygen flask method in the Chemistry Department, University of Strathclyde; uronic acid was determined by the method of Bitter & Muir (1962).

Anhydrous dextrose for parenteral use (B.P.) was provided by CPC(UK) Ltd. Dextrose solutions (in 0·02 M NaCl) were made with water which had been double-distilled in glass and cooled immediately. Autoclaved dextrose solutions were prepared by autoclaving (122 °C/55 min) molar dextrose solutions in water (prepared as above) and thereafter adding 0·02 M NaCl. All solutions were made on the day of use. Dextran 70 was provided by Fison Ltd.

Variation in dextrose concentration

Stock azur A solution (60 mg litre^{-1}) was diluted with volumes of M dextrose and calcium chloride solutions to give 0·05–0·5 M dextrose and where appropriate 0·01 and 0·02 M calcium chloride, and 12 μg ml^{-1} azur A. To 1 ml of these solutions 50 μl heparin solution (0·1 mg ml^{-1}) was added and absorbance read at 510 nm. Appropriate controls were included. These experiments were also conducted using the following: (i) dextrose solution which had been autoclaved (M solution; 121 °C/55 min); in this case the sodium chloride to give 0·02 M was added after autoclaving; (ii) dextran 70; molarity was calculated on a unit weight of 179.

Variation in calcium chloride concentration

Preparation of solutions was as above except that calcium chloride concentration ranged from 0·005 to 0·025 M with dextrose (autoclaved or unautoclaved as appropriate) constant at 0·5 M.

Variation in sodium chloride concentration

A similar experiment was done in which sodium chloride (up to 0·3 M) replaced calcium chloride. Only unautoclaved dextrose (0·5 M) was used.

Heparin-dye titrations

Stock solutions of azur A (60 mg litre^{-1}) and desalted heparin (ca 1 mg ml^{-1}) were diluted 1 in 5 with 0·02 M NaCl. μl volumes of heparin solution were added to 2·5 ml of the diluted azur A solution in the cuvette maintained at 25 °C and stirred. The spectrum was traced over 400–700 nm after each heparin addition. From a plot of absorbance at 625 nm against volume (μl) of heparin added, the end point was read as the intersection of the linear portions of the two limbs. The molarity of the dye at the end point (azur A = 256) was calculated and the equivalent weight of heparin (relative to moles of dye) determined as in Stone & Bradley (1967). Titrations were conducted using as solvent for heparin: 0·5 M dextrose autoclaved and unautoclaved, with and without 0·01 M calcium chloride, and 0·01 M calcium chloride. All solutions contained 0·02 M NaCl.

Critical electrolyte concentrations (CEC)

Scott & Willett (1966) defined CEC as the highest concentration of salt at which metachromasia remains apparent. In the present work a stock solution of azur A was diluted with appropriate salt solution to contain 12 μg ml^{-1} azur A and various concentrations of sodium chloride or calcium chloride. To 4 ml azur A – salt solution, 0·2 ml of heparin in appropriate salt solution was added and the absorbance at 510 nm determined. The CEC was that concentration of salt (by extrapolation) which gave zero absorbance at 510 nm, that is where metachromasia just failed to occur.

RESULTS

Effect of unautoclaved dextrose and Ca^{2+} on metachromasia

The addition of increasing amounts of desalted heparin to azur A solution containing unautoclaved dextrose resulted in a progressive absorbance band shift from 625 nm (free dye) to 510 nm, the μ band associated with metachromasia (Fig. 1). Absorbance at 510 nm of a constant amount of heparin increased slightly but consistently with increasing unautoclaved dextrose concentration, whilst in the presence of 0·01 M calcium chloride absorbance was lower at zero unautoclaved dextrose concentration and steadily decreased as the dextrose concentration was raised (Fig. 2a). Thus, only in presence of Ca^{2+} is the metachromatic (macroanionic) activity of heparin reduced in the presence of unautoclaved dextrose. At constant dextrose concentration (0·5 M) the Ca^{2+} influence is marked (Fig. 3). The limiting Ca^{2+} and dextrose concentrations, given in Table 1, are the (extrapolated) concentrations of either Ca^{2+} or dextrose required to reduce metochromasia to zero in the stated concentration of the other.

Clearly, the metachromatic effect of heparin

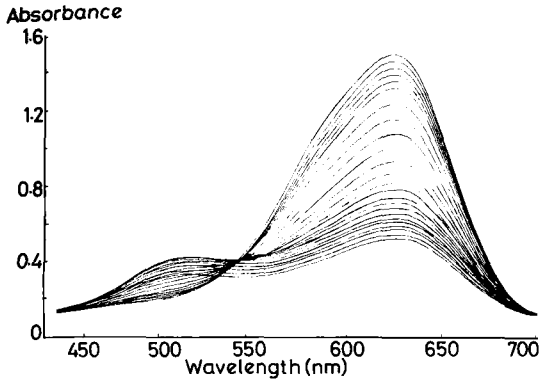

FIG. 1. Azur A titration with unfractionated desalted heparin in the presence of 0·5 M dextrose in solvent 0·02 M NaCl at 25°C. To azur A solution (9·848 μg ml^{-1}), heparin solution (198·4 μg ml^{-1}) was added in the following volumes (μl): 0, 2, 4, 6, 8, 10, 15, 20, 25, 30, 35, 40, 45, 50, 60, 80, 100, 150, 200, 300, 500, 700, 1000, 1500 and the spectrum recorded after each addition, which gave successively decreasing absorbance at 625 nm and up to about 150 μl increasing absorbance at 510 nm, the μ band of metachromasia. Additions ≥200 μl demonstrate suppression of metachromasia (decreasing absorbance at 510 nm) which occurs when excess heparin is added.

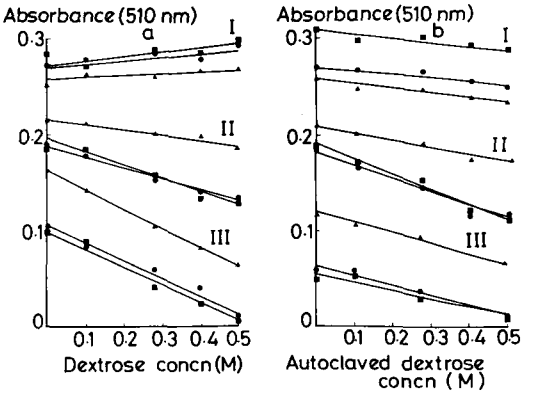

FIG. 2 a. Change in heparin – azur A metachromasia (absorbance at 510 nm) with increasing dextrose concentration in the absence and presence of Ca^{2+}. Significance of differences (unfractionated and L2 heparins): i, increase in the absence (I) and decrease in the presence of Ca^{2+} concentrations (II, III). Note that at 0·5 M dextrose, $P < 0·001$; ii, in absence of Ca^{2+} and in the absence and presence of 0·5 M dextrose, unfractionated heparin ● was more active than L2 ▲, $P = 0·02$–$0·05$; iii, in presence of both Ca^{2+} concentrations (II, III) (absence and presence of 0·5 M dextrose) L2 was more active than unfractionated heparin, $P < 0·001$. II = 0·01, III = 0·02 M $CaCl_2$.

b. As for Fig. 2a except that autoclaved dextrose solution was used. In absence of Ca^{2+} metachromasia was significantly decreased at 0·5 M autoclaved dextrose, $P < 0·001$; in the other experiments the significance of differences corresponded to those given for Fig. 2a. n = 12 in each instance.

●, unfractionated heparin; ▲, heparin fraction L2; ■, heparin fraction L6.

fraction L6 (8400) is prevented by lesser amounts of both Ca^{2+} and dextrose than that of fraction L2 (19 000) suggesting greater affinity for Ca^{2+} of L6 which also has greater affinity than L2 for the dye (at zero dextrose concentration).

Effect of dextran and Ca^{2+} on metachromasia

Dextran behaved in the same way as dextrose.

Effect of autoclaved dextrose and Ca^{2+} on metachromasia

In contrast to the small rise in unautoclaved dextrose, a small fall in heparin metachromasia occurred in autoclaved dextrose solutions (Fig. 2b).

This difference was tested at 0·5 M dextrose in the absence of Ca^{2+} and was very highly significant ($P < 0·001$) for all heparins examined. Clearly, autoclaved and unautoclaved dextrose solutions are different solvents for heparin in the metachromasia system. The small respective increase and decrease in activity in unautoclaved and autoclaved 0·5 M dextrose are each significant ($P < 0·001$). The effect on metachromasia of increasing autoclaved dextrose concentration in presence of Ca^{2+} (Fig. 2b) was similar to that for unautoclaved dextrose, including the greater susceptibility of unfractionated and L6 heparins to Ca^{2+} in presence of autoclaved dextrose, than that of L2.

Table 1. Limiting calcium and dextrose concentrations required to eliminate heparin – azur A metachromasia.

	Limiting Ca^{2} concn, M			Limiting dextrose concn, M		Limiting autoclaved dextrose concn, M	
Heparin	No dextrose	0·5 M dextrose	0·5 M autoclaved dextrose	0·01 M Ca^{2+}	0·02 M Ca^{2+}	0·01 M Ca^{2+}	0·02 M Ca^{2+}
L2	0·0380	0·0238	0·0268	3·87	0·82	>4	1·09
L6	0·0267	0·0153	0·0206	1·45	0·55	~3	0·56
u/f	0·0252	0·0174	0·0218	1·64	0·61	1·6	0·60

Figures are (extrapolated) concentrations of either Ca^{2+} or dextrose required to reduce metachromasia to zero in the stated concentration of the other. All solutions were in 0·02 M NaCl, u/f, unfractionated parent Na heparin; L2, L6, fractions of mol. wt 19 000, 8400 respectively.

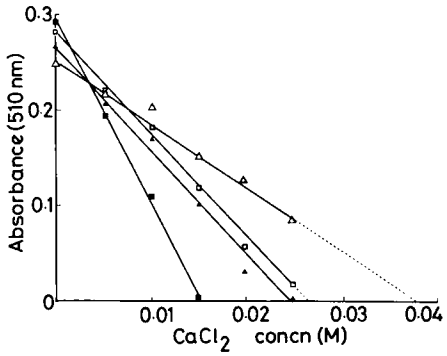

FIG. 3. Change in heparin-azur A metachromasia with increasing $CaCl_2$ concentration; ▲, ■ = heparin fractions, L2, L6 respectively in presence of 0·5 M dextrose; △, □ = fractions L2, L6 respectively in absence of dextrose.

Heparin-dye titrations

Azur A titrations were used to confirm the difference between heparins in respect of the effect of dextrose and Ca^{2+} on metachromatic activity. Preliminary experiments showed that azur A gave sharper end points than methylene blue and the former dye was therefore preferred. An example is given in Fig. 4 and equivalent weights are given in Table 2. Relatively small amounts of dye were used in titrations to the end point when dextrose or dextrose plus Ca^{2+} were included. The equivalent weights indicate the weight of heparin per mole of dye required to give maximum reduction in absorbance of free dye. Autoclaved dextrose, Ca^{2+} and the combinations caused decreased heparin-dye reactivity resulting in raised equivalent weights.

Effect of dextrose and Ca^{2+} on free dye

Dextrose and dextran (0·1 M, 0·28 M) increased the absorbance at 625 nm of the free dye and Ca^{2+} (0·01 M) decreased it; Ca^{2+} plus either dextrose or dextran together resulted in an intermediate value.

Comparison of effects of NaCl and $CaCl_2$ on metachromasia

a. CEC. Approximately eight times more NaCl (CEC = 0·25 M) than $CaCl_2$ (CEC = 0·03 M) was required to eliminate heparin-induced methachromasia. The heparin fractions L2, L6 had similar CEC.

b. NaCl plus dextrose. A plot for NaCl corresponding to Fig. 3 showed that limiting concentrations for NaCl in the presence of unautoclaved dextrose were an order of magnitude greater than those for $CaCl_2$: 0·18 M, 0·21 M respectively for L6, L2. Omitting dextrose made little difference: 0·22 M, 0·25 M respectively.

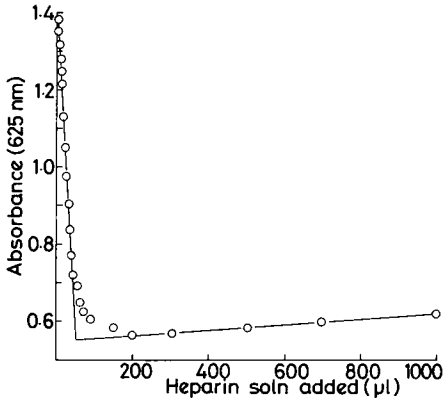

FIG. 4. Spectrophotometric titration of azur A with unfractionated desalted sodium heparin in presence of 0·5 M dextrose, effected by stepwise addition of heparin to azur A solution under standard conditions. Data from Fig. 1.

DISCUSSION

Heparin-plasma protein interactions and heparin-cationic dye interactions are principally ionic events. The latter, based on the content and arrangement of carboxyl and sulphate groups in heparin (Wollin &

Table 2. Equivalent weights for heparins in different solvents, obtained from heparin-dye titrations.

Solvent	Heparin			
	u/f	L6	L2	Ca heparin
0·02 M NaCl	183 (3·3)	171 3·5)	185 (3·2)	187 (3·2)
0·5 M unautoclaved dextrose	188 (3·2)	174 (3·4)	193 (3·1)	180 (3·3)
0·01 M $CaCl_2$	335 (1·8)	257 (2·3)*	231 (2·6)*	236 (2·5)*
0·5 M unautoclaved dextrose + 0·01 M $CaCl_2$	1248 (0·5)	687 (0·9)	451 (1·3)	454 (1·3)
0·5 M autoclaved dextrose	228 (2·6)	204 (2·9)	234 (2·6)	231 (2·6)
0·5 M autoclaved dextrose + 0·01 M $CaCl_2$	685 (0·9)	737 (0·8)	316 (1·9)*	439 (1·4)*

u/f = unfractionated sodium heparin, parent of fractions L6 (mol. wt 8400) and L2 (mol. wt 19 000); calcium heparin was also unfractionated. Equivalent weights represent g heparin per mole dye at end point. Figures in brackets represent the number of anionic sites per heparin disaccharide unit (mol. wt 600). * means of duplicate determinations; others are means of triplicates.

Jaques 1974), provide a sensitive and comparatively simple model for studying macroanionic activity. However, the relationships between the anticoagulant and dye-interacting activities of heparin are incompletely understood, the former having more specific structural requirements.

Dextrose in the absence of added calcium ion did not reduce heparin-azur A metachromasia; indeed a small increase in the metachromatic absorbance at 510 nm occurred as dextrose concentration was increased (Fig. 2a), although this change was not accompanied by a change in equivalent weight (Table 2). Dextran (polymeric dextrose) behaved similarly suggesting the involvement of the sugar hydroxyl groups. Whether this is due to an effect on the dye molecules or their aggregates is not known, but dextrose-heparin association has been indicated by viscosity measurement (Anderson & Harthill 1982). In the absence of heparin both dextrose and dextran slightly increased the absorbance of free dye (625 nm) whilst Ca^{2+} reduced it.

Ca^{2+} reduced heparin metachromasia (Fig. 3) and dextrose-increased metachromasia (Fig. 2a), the latter reduction being more pronounced as dextrose concentration increased, suggesting some sort of combined action by Ca^{2+} and dextrose. The results of the heparin-dye titrations (Table 2) in which reduction in free dye is measured, clearly show the combined effect of calcium and dextrose in increasing the amount of heparin required to reduce the free dye concentration by a given amount (i.e. greater equivalent weight).

Autoclaved dextrose behaved differently from unautoclaved dextrose. Thus, with increasing autoclaved dextrose concentration in the absence of Ca^{2+}, metachromatic activity decreased (Fig. 2b) and equivalent weight increased (Table 2), both slightly, indicating some impairment of heparin macroanionic activity. In view of the failure of unautoclaved dextrose by itself to decrease activity, this could be due to decreased ionization of iduronic carboxyls of heparin at the acid pH of autoclaved dextrose which is caused by sugar acids formed during autoclaving. As in unautoclaved dextrose, however, metachromatic activity decreased markedly with increase in Ca^{2+} concentration (Fig. 2b); also, equivalent weight increased (Table 2), showing that the combined effect of Ca^{2+} plus dextrose is much greater than that due to the acidity of autoclaved dextrose which therefore can be considered to make a minor contribution to the decrease in heparin activity. This agrees with the facts that, first, most heparin anionic activity is contributed by $-OSO_3^-$ groups, which remain ionized in strong acid, and second, autoclaved dextrose contains >95% unaltered dextrose which has a combined effect with Ca^{2+} on heparin activity. The minor influence of acidity of autoclaved dextrose confirms a similar conclusion reached on the basis of anticoagulant study (Anderson & Harthill 1982).

The order of capacity to decrease heparin macroanionic activity measured by metachromasia (510 nm) or by free dye interaction (625 nm) is dextrose $+0{\cdot}01$ M $Ca^{2+} > 0{\cdot}01$ M $Ca^{2+} >$ autoclaved dextrose. The overall anionic content ($-COO^-$ and $-OSO_3^-$) per disaccharide unit (Table 2) has also been calculated, although a tetrasaccharide unit may be the fundamental anticoagulant moiety.

Calcium heparin behaved like sodium heparin in these experiments, thus ruling out simple Na^+–Ca^{2+} exchange in sodium heparin as a general basis for the effect of Ca^{2+}. Although Ca^{2+} and dextrose have a combined effect on heparin, the results with each alone suggest dominance of the Ca^{2+} effect. Interaction of Ca^{2+} with heparin could possibly be facilitated by a conformation change induced by dextrose as a result of loose association already indicated (Anderson & Harthill 1982) by viscosity determinations. The substantial loss of macroanionic activity in the presence of Ca^{2+} and dextrose together, suggests that the principal anion, $-OSO_3^-$, is involved, possibly actively.

Clearly, investigation of effects of drugs in general on heparin in solution requires consideration of Ca^{2+} concentration.

Autoclaved and unautoclaved dextrose have different effects on the anti-factor Xa activity of heparin (Anderson & Harthill 1982), the former causing a rise and the latter a temporary fall in activity. The effects therefore differ in nature from those seen in the present metachromasia study—a reflection of the different interactions occurring in the two systems. Unautoclaved dextrose in the absence of Ca^{2+} did not reduce metachromatic activity but appeared to reduce anti-factor Xa activity, but the present results suggest that the Ca^{2+} used in the anti-Xa assay, in addition to its role in the blood clotting process, could explain the apparent discrepancy in that it forms a heparin-dextrose-Ca^{2+} system.

The greater CEC for NaCl than for $CaCl_2$ is in agreement with Jooyahdeh et al (1974) who found divalent cations with their greater ionic radii more powerful than monovalent cations in dissociating heparin-methylene blue complexes. The CECs were similar for heparins L2 and L6 suggesting that, in

terms of the salt effect, the molecular weight difference was unimportant. The limiting concentrations for Na^+, higher by an order of magnitude than those for Ca^{2+} and of similar magnitude in presence or absence of dextrose, showed that heparin was less sensitive to Na^+ than to Ca^{2+} and that involvement of dextrose did not occur as with Ca^{2+}. This is in accord with the finding that in vitro heparin anticoagulant activity, in general, is not affected by saline (Jacobs et al 1973; Anderson et al 1977). In the presence of dextrose, the two heparin fractions exhibit differing sensitivities to increasing Ca^{2+} concentration (Table 1), the lower molecular weight fraction being more sensitive. This fraction has slightly more sulphate and iduronate than L2, probably resulting generally in closer proximity of the anionic sites and, in particular, more favourable conditions for Ca^{2+} – iduronate interaction for which Boyd et al (1980) stipulate pairs of iduronate. The shorter molecule, L6, binding Ca^{2+} more readily, will thus undergo greater flexibility loss than L2, which could favour dextrose involvement in the presence of Ca^{2+}.

The general importance of intersite distance in heparin-dye interactions has been emphasized (Lawton & Phillips 1976), and heparins fractionated on the basis of antithrombin III affinity which reflects specific binding site availability rather than molecular size alone, have been found to differ in methylene blue titration (Villaneuva & Danishefsky 1981).

The effect of Ca^{2+} in the presence of dextrose is much greater than that of Na^+ for which 0·1 M in conjunction with 0·5 M dextrose gave only a small reduction in metachromasia for the more sensitive L6 but none for L2. It appears therefore that Ca^{2+} in the presence of dextrose has a somewhat specific effect on the macroanionic activity of heparins, involving molecular properties additional to those involved in the simple salt effect measured by CEC.

Calcium ion is present in most anticoagulant test systems in which it is recognized and bound by proteins and these, as a result, undergo structural adjustment (Williams 1980). Calcium ion also has conformation-determining effects on heparin at a biologically significant level, heparin flexibility being decreased (Boyd et al 1980). Braud et al (1980) found calcium to act as a condensed counter ion for heparin, and the L-iduronic carboxylate, essential for heparin anticoagulant activity (Lindahl et al 1979), has particular affinity for calcium (Braud et al 1980; Boyd et al 1980). Calcium ion-sugar associations are well known (Angyal 1980) and even a weak Ca-dextrose association could modify the calcium interaction with both protein and heparin. In an anticoagulant test system this could lead to altered clotting times and an apparent change in activity. Anticoagulant activity of heparin can change when the calcium ion content of the test system is changed (Anderson & Harthill 1981). Thus differences in concentrations of interacting substances in various anticoagulant test systems could easily account for the variations in heparin activity which have been observed (Hodby et al 1972; Jacobs et al 1973; Okuno & Nelson 1975; Mitchell et al 1972; Raper & Johnson 1976; Anderson et al 1977, 1979; Joy et al 1979) in dextrose solutions.

The different effects of dextrose in the presence of Ca^{2+} on heparin fractions L2 and L6 which have different molecular weights suggest that this could be reflected in corresponding differences between unfractionated pharmacopoeial heparins having different contents of various molecular sizes.

Finally, whether a dextrose effect on heparin demonstrated in vitro will persist after infusion into blood is scarcely predictable, for amongst many obscurities, it is still not clear, for example, to what extent the anticoagulant action of injected heparin is attributable to the injected molecules as opposed to molecules released from an endogenous pool in response to the arrival of the injected molecules. Also, in vitro associations between Ca^{2+}, dextrose and heparin, whilst effective in influencing macromolecule interactions in the relatively steady state in vitro, are weakly based and could be reversible in vivo.

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REFERENCES

Anderson, W., Harthill, J. E., Couper, I. A., Stafford, S. (1977) J. Pharm. Pharmacol. 29: 31P

Anderson, W., Harthill, J. E., Hawthorn, G. (1979) Ibid. 31: 55P

Anderson, W., Harthill, J. E. (1982) J. Pharm. Pharmacol. 34: 90–96

Anderson, W., Harthill, J. E. (1981) Thrombosis Res. 21: 557–564

Angyal, S. J. (1980) Chem. Soc. Rev. 9: 415–428

Bitter, T., Muir, H. M. (1962) Anal. Biochem. 4: 330–334

Boyd, J., Williamson, F. B., Gettins, P. (1980) J. Mol. Biol. 137: 175–190

Braud, C., Villiers, C., Vert, M. (1980) Carbohydrate Res. 86: 165–175

Hodby, E. D., Hirsh, J., Jones, C. A. (1972) Can. Med. Ass. J. 106: 562–564

Jacobs, J., Kletter, D., Superstine, E., Hill, K. R., Lynn, B., Webb, R. A. (1973) J. Clin. Pathol. 742–746

Jooyahdeh, F., Moore, J. S., Phillips, G. O., Davies, J. V. (1974) J. Chem. Soc. Perkin II 2: 1468–1471

Joy, R. T., Hynech, M. L., Berardi, R. R., Ho, N. F. H. (1979) Am. J. Hosp. Pharm. 36: 618–621

Lindahl, U., Backstrom, G., Hook, M., Thunberg, L., Fransson, L., Linker, A. (1979) Proc. Nat. Acad. Sci. U.S.A. 76: 3198–3202

Lawton, J. B., Phillips, G. O. (1976) J. Chem. Soc. Perkin II 38–43

Mitchell, J. F., Barger, R. C., Cantwell, L. (1976) Am. J. Hosp. Pharm. 33: 540–542

Okuno, T., Nelson, C. A. (1975) J. Clin. Path. 28: 494–497

Pal, M. K., Schubert, M. (1962) J. Am. Chem. Soc. 84: 4384–4393

Raper, C. G. L., Johnson, E. (1976) J. Clin. Path. 29: 306

Scott, J. E., Willett, J. H. (1966) Nature (London) 209: 985–987

Stone, A. L., Bradley, D. F. (1967) Biochim. Biophys. Acta 148: 172–192

Villaneuva, G. B., Danishefsky, I. (1981) Thromb. Res. 21: 191–199

Williams, R. J. P. (1980) Chem. Soc. Rev. 9: 325–364

Wollin, A., Jaques, L. B. (1974) Thromb. Research 2: 377–382