83. A COLORIMETRIC METHOD FOR THE STANDARDIZATION OF HEPARIN PREPARATIONS

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It is a fact well known to histologists that certain basic dyes undergo a change of tint when taken up by particular tissue elements, especially cartilage, mucus and the granules of the so-called 'mast cells'. The chemistry of this phenomenon, named 'metachromasia' by Ehrlich, has been studied in some detail by Lison [1935]. This investigator concluded that the change of colour in the dye is due to the formation of the tautomeric imino-base, accompanied by an increased degree of molecular aggregation; and that all the substances which show the metachromatic staining contain organically combined sulphuric acid. Any sulphuric acid ester, in fact, provided that its molecular weight is not too low, will produce the characteristic colour change when added to an aqueous solution of a metachromatic dye. Now most of the substances known to be active in delaying the clotting of blood have large molecules containing esterified sulphuric acid; and, conversely, most substances having this structure appear to be anticoagulants [cf. Huggett & Rowe, 1933; Bergstrom, 1936; Chargaff *et al.* 1936]. Thus anticoagulant activity, and the capacity for giving the metachromatic reaction, are related to the same type of chemical structure. The effectiveness of any substance in producing the colour change appears, indeed, as will be shown, to be roughly proportional to its anticoagulant potency.

Heparin contains esterified sulphuric acid [Jorpes, 1935], and gives the metachromatic reaction with toluidine blue [Jorpes *et al.* 1937]. The reaction has been used with success as a histochemical test for heparin [Jorpes *et al.* 1937; Wilander, 1939]. It has not, however, been applied hitherto for the standardization of heparin preparations; and the purpose of this paper is to specify conditions under which such an application is possible. The dye which has been chosen is toluidine blue.

The reaction between toluidine blue and heparin

If a weak (e.g. 0·01 %) solution of heparin is added to an equal volume of an aqueous toluidine blue solution of the same strength, the tint of the dye solution changes immediately from blue to red-violet. With smaller amounts of heparin, intermediate shades of purple are produced. The absorption of such mixtures for light of a given colour can be measured in a suitable instrument, and is a function of the concentration of heparin and of the dye.

This simple procedure is not, by itself, very useful as a basis for the colorimetric estimation of heparin, since (*a*) the absorption spectra of the two forms of the dye overlap, and (*b*) the heparin-dye complex is precipitated at a rate depending on the concentration of the reagents and on other factors. If, however, the mixture is shaken with an immiscible organic solvent, the heparin-dye complex is removed by adsorption at the interface, while the uncombined dye

remains in the aqueous phase, and retains its normal tint. The proportion of dye removed can now be determined by measuring the absorption of the aqueous phase, the calculated loss then serving as a measure of the added heparin.

Fig. 1 shows the effect of adding different amounts of a heparin preparation to a standard solution of toluidine blue, the heparin-dye complex having been

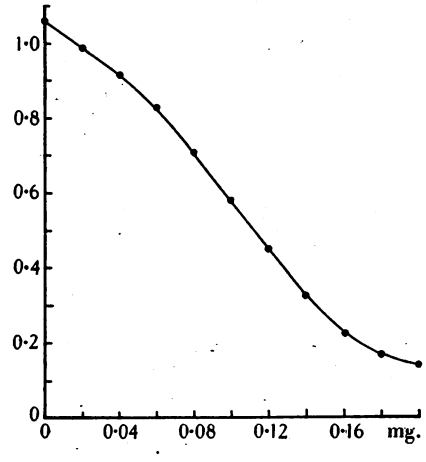

Fig. 1.

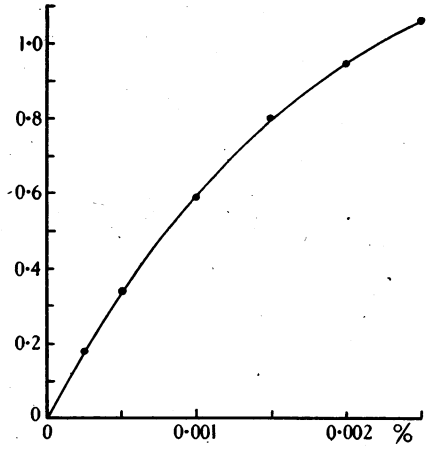

Fig. 2.

Fig. 1. Abscissae: mg. of heparin added to 10 ml. of 0·0025% toluidine blue solution. Ordinates: absorptiometer readings (orange filters) after extraction with light petroleum.

Fig. 2. Abscissae: concentration in % of toluidine blue in aqueous solution. Ordinates: absorptiometer readings.

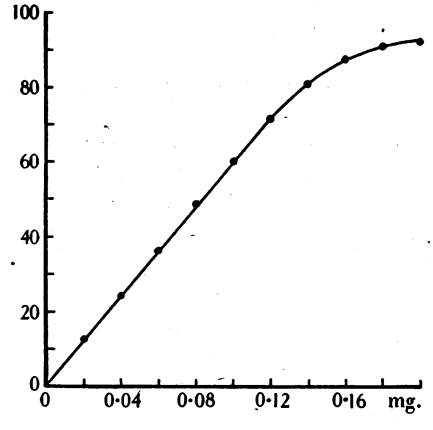

Fig. 3. Abscissae: mg. of heparin. Ordinates: % of dye removed from 10 ml. of 0·0025% toluidine blue solution. (From data of Figs. 1 and 2.)

removed by shaking with light petroleum. The extinction of the aqueous phase was measured in a Hilger 'Spekker' Photoelectric Absorptiometer with Ilford (no. 607) orange filters. The continuous curve of Fig. 2 shows, for comparison, the extinction under the same conditions of pure, aqueous toluidine blue solutions of varying concentration. From these data the relation between the quantity of heparin added and the quantity of dye removed can be calculated,

and this is represented in Fig. 3. The proportion of dye removed increases in direct ratio to the concentration of heparin, until about 90% is combined; thereafter it increases more slowly, and a large excess of heparin is necessary for complete decolorization. Since there is no sharp end-point, a simple titration of heparin against dye is impracticable.

The relationship between the concentration of dye and the absorption of light of the chosen wave-length by aqueous toluidine blue solutions is not expressed by the Beer-Lambert law. The hue of this (and of other metachromatic dyes) varies with the concentration: in the case of toluidine blue, the absorption maximum shifts from the orange toward the yellow as the strength of the solution is increased. For this reason, it is impossible to match two watery solutions of the dye in a simple colorimeter of the Duboscq type if they differ in concentration by more than a few per cent. In the presence, however, of a suitable proportion of alcohol or acetone, the extinction curve of the dye corresponds to that of a very dilute aqueous solution, and is unaffected by changes in concentration. If, therefore, an equal volume of alcohol or acetone is added to the separated aqueous phase after extraction of the heparin-dye complex, the tint of the remaining dye is practically independent of its concentration, which may thus be measured directly with the aid of an ordinary colorimeter.

Factors modifying the reaction

Heparin and toluidine blue do not react in stoichiometric proportions. The quantity of dye changed to the metachromatic form depends on several factors besides the quantity of heparin present: it is diminished by an increase in temperature, in acidity, or in the concentration of other solutes in the system. Divalent and trivalent cations are especially active in reversing the reaction. In practice, however, these factors are easily controlled: the dilution of the material to be tested is so great that, except with very crude preparations, the influence of any impurities it may contain is negligible; and the disturbing effect of temperature changes can be avoided by carrying through the procedure with a standard and the unknown heparin simultaneously, so that the determination is comparative.

In particular, it may be noted that barium ions and tricresol (the preservative commonly added to heparin solutions) do not interfere, unless present in concentration higher than that of the heparin itself. The error introduced by a difference of temperature, at the moment of separating the heparin-dye complex from the unaltered dye, is about 1% for 1° C.

Colorimetric comparison of heparin preparations

Reagents.

(1) *0·005% toluidine blue solution.* 25 mg. of toluidine blue (Gurr's is satisfactory) is dissolved with the aid of heat in 500 ml. of 0·01 *N* HCl containing 0·2% of NaCl. The presence of acid minimizes the adsorption of the dye at glass surfaces.

(2) *Heparin* is dissolved in 0·2% NaCl. For purified samples a convenient concentration for both standard and unknown heparin is 0·01%. As some heparin preparations are nearly insoluble in distilled water, all aqueous solutions contain 0·2% of NaCl: the use of higher concentrations of NaCl tends to inhibit the reaction between heparin and the dye, and somewhat diminishes the accuracy of the estimation.

(3) *0·2% NaCl.*

(4) *Light petroleum,* B.P. 40–60°.

Procedure. 5 ml. of the toluidine blue solution are measured into each of nine 50 ml. test tubes. Varying amounts of a solution of the standard heparin (e.g. 0·2, 0·4, 0·8, 1·2 ml.) are added to tubes 1 to 4; corresponding amounts of the solution to be tested are added to tubes 5 to 8. The total volume is brought to 10 ml. in each tube by the addition of 0·2 % NaCl, and the contents are mixed. 10 ml. of light petroleum are added to each tube, which is then stoppered and shaken vigorously for 30 sec. When separation of the two layers is complete, the aqueous phase is pipetted off from each tube, with care to avoid contamination by the heparin-dye complex precipitated at the interface. The absorption of each aqueous solution may be read immediately in the Spekker absorptiometer with orange (Ilford no. 607) filters. Alternatively, an aliquot may be diluted with an equal volume of alcohol and the reading made in an ordinary colorimeter, the toluidine blue solution containing no heparin (tube 9), and diluted with alcohol serving as a standard. Concentrations of the standard heparin are then plotted against absorptiometer (or colorimeter) readings, and a graph is drawn from which the relative affinity of the unknown heparin for the dye can be obtained. This may be conveniently expressed in terms of *colour units*, the *colour value* of the present provisional British standard heparin being taken as 110 units/mg. to correspond to its anticoagulant activity, which is defined as 110 units/mg. Thus, if 2 ml. of a solution of the unknown heparin removed the same amount of dye from solution as 1 ml. of a solution of equal concentration of the standard heparin, the 'colour value' of the unknown would be 55 units/mg.

From Fig. 3 it is evident that the colour value of a purified heparin can be measured with greatest precision when heparin is added in an amount sufficient to remove about 60–80 % of the dye. With crude specimens, however, the impurities present may inhibit to a significant degree the formation of the heparin-dye complex, and the error so introduced will increase with increasing concentration of the impure heparin. It is therefore desirable to make the comparison between standard and unknown at different levels of heparin concentration, as described above. In this way, the graph will give, for each volume t of the test solution, a corresponding volume s of the standard solution, which produces the same colour change. If the ratio s/t is the same in each case, no difficulty arises; if (as with certain impure test samples), s/t decreases as s and t increase, it can be assumed that the highest value of s/t is the most nearly correct. It may be noted that, for all heparin samples so far examined which had been prepared by a process involving crystallization as the barium salt, s/t was found constant, within a few per cent, for all values of t.

Comparison of colorimetric and biological methods

The results obtained with a number of different materials are given in Table 1. The colorimetric comparison was carried out as described above, the biological comparison by the method described in the foregoing paper [MacIntosh, 1941]. The standard material was, in each case, the provisional British heparin standard, which has been defined as containing 110 anticoagulant units/mg. It has been taken to contain, by definition, 110 'colour units' per mg.

For all the samples of purified heparin tested, with one exception, the results of the two methods agreed within the limits of experimental error. The exception (no. 9 in Table 1) was a barium salt preparation, the activity of which had been deliberately reduced by prolonged treatment with acid alcohol: this showed reduced activity by both tests, but was about twice as active by the colorimetric as by the biological test. Sample no. 5 has been referred to in the preceding paper as giving significantly different results when compared with the standard,

Table 1. *Anticoagulant activities and colour values of various materials*

(The provisional British heparin standard is taken to contain 110 anticoagulant units and 110 colour units/mg.).

No.	Description	Anticoagulant activity Units/mg.	Colour value Units/mg.
	Heparin		
1	Na salt (commercial)	106	110
2	Na salt (prepared from no. 5)	92	93
3	Na salt (commercial)	80	76
4	Na salt (commercial)	73	79
5	Ba salt (research specimen)	73	73
6	Ba salt (recrystallized from no. 5)	58	65
7	Ba salt (old sample)	30	35
8	Ba salt (from commercial Na salt)	68	72
9	Ba salt (acid-alcohol inactivated)	25	46
10	Crude dog heparin	20	23
11	Crude dog heparin	12	16
12	Crude dog heparin	12	8
	Other materials		
	'Liquoid' Roche (polyanethol-sulphonic acid)	50–60	46
	Chlorazol fast-pink	50–110	110
	Chlorazol sky-blue	30–75	75
	Bayer 205 (Germanin)	60	100
	Neosalvarsan	8–12	12
	Chondroitin sulphuric acid	<1	1
	Mucoitin sulphuric acid	<1	1

by two biological tests, the plasma-kinase and the rabbit's whole-blood methods: it is interesting to note that the colorimetric and plasma-kinase methods gave concordant results for this preparation.

The other anticoagulants tested were mostly synthetic preparations chemically very unlike heparin, and it was, consequently, not surprising to find that, in their effects on clotting, they differ qualitatively from heparin and from one another. Such differences were particularly noticeable with the dyes chlorazol fast-pink and chlorazol sky-blue [Huggett & Rowe, 1933], for which the slope of the curve relating concentration to clotting time was less steep than that found for heparin. The behaviour of Liquoid, Bayer 205 and neoarsphenamine in clotting experiments was not closely examined, but their anticoagulant action appears, likewise, not to be identical with that of heparin. Similar observations have been made by Astrup [1938]. The anticoagulant potencies given in Table 1 for these substances are therefore approximations only. As to the colorimetric comparison, it should also be noted that the activity of the synthetic anticoagulants, relative to that of heparin, was determined in only one set of conditions of temperature, salt concentration etc., and that different values might possibly have been found in other conditions.

Neoarsphenamine forms with toluidine blue a colourless complex which, like the red heparin-dye complex, is removed from aqueous solution by shaking with light petroleum.

Discussion

In the preceding paper it was pointed out that highly purified specimens of heparin, prepared by crystallization of the barium salt, are not necessarily identical in their specific anticoagulant activity. Not only are some preparations superior to others, to a degree not explained by differences in water or ash content, but the exact margin of this superiority varies, according to the test which is used to determine it. Heparin as ordinarily prepared is therefore not a

single substance, but contains two or more individuals whose activity appears to be unequally directed toward the different stages of the coagulation process.

These considerations make it obvious that the value of a colorimetric method for standardizing heparin can only rest on an empirical basis. It is obvious enough, from the results summarized in Table 1, that there is some sort of correlation between anticoagulant activity in general and the capacity for forming a complex with a metachromatic dye. What is not so clear is, whether a value found by the colour test may be taken to measure, with any degree of accuracy, the degree of a particular sort of anticoagulant activity, such as platelet-stabilizing, antiprothrombin or antithrombin. Still less is it clear—and a similar doubt attaches to the significance of the several biological assays—whether the particular activity, or combination of activities, measured by the colour reaction is actually that of most importance in the therapeutic use of heparin. While these points remain undecided, it is difficult or impossible to say that one assay method is better than another. So far as the available data go, one of the biological methods, the plasma-kinase test, appears to give results which agree remarkably well with those found by the colorimetric method, and better with these than with the results obtained by another biological method, the rabbit's whole-blood test. It may be that the first two measure chiefly one sort of anticoagulant activity and the latter another sort. It would not be unreasonable to suppose, for example, that an important factor in the delay by heparin of the clotting of the whole shed blood is due to its stabilization of the platelets, and the consequent delay in the liberation from these of thrombokinase. In the plasma test, on the other hand, this factor is eliminated, few if any platelets being present and kinase being, in any case, added artificially in excess; so that the delay by heparin of clotting in this system must be due to inhibition of the formation, or the action, of thrombin. Further speculation, however, along these lines is hardly profitable.

From the practical standpoint, the position is, perhaps, not so unsatisfactory as might appear from this discussion. It may probably be fairly described as follows (see also the preceding paper). If the standard preparation of heparin is of good quality (i.e. has suffered no appreciable denaturation during its isolation), then any good quality heparin may be compared with it, by any method, with the same result; if, however, the unknown preparation is of inferior quality, then its unit value will probably be higher by the whole-blood method than by the plasma-kinase or colorimetric method. Such experience as is now available suggests, however, that the various methods will rarely give results for samples of purified ox heparin differing by more than 25%. Differences of this magnitude are of little importance, except from the standpoint of cost; and, from any other point of view, there is a certain advantage in choosing the method giving the lowest value to a sample which has lost activity. Specimens prepared from other than ox tissues may be either more or less active [Jaques & Waters, 1940; Jaques, 1940], and it is possible that larger discrepancies in the results obtained by different methods may occur when these heparins from other species are compared with ox heparin. Until more information is available, however, there is much to be said for standardizing heparin by the simplest and most easily reproducible method yet available, and this would doubtless be a colorimetric method of the type here described.

SUMMARY

1. A simple colorimetric method for the standardization of heparin preparations is described, and its limitations are discussed. It gives results in good agreement with those obtained by the biological (plasma-kinase) method described in the preceding paper.

2. A number of synthetic anticoagulants have been compared with heparin by these two methods. In general, the relative potencies of different anticoagulants cannot be stated exactly, because of qualitative differences in their action.

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