# NEW APPARATUS

## AN APPARATUS FOR SEMI-MICRO CRYSTALLISATION

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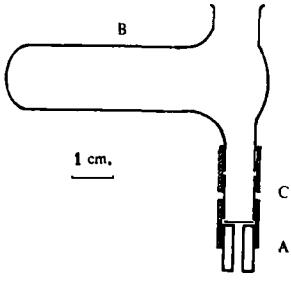
Repeated recrystallisation of quantities of material of the order of 20 to 40 mg. often presents a difficult problem. Application of the usual micro-methods or of the conventional macro-methods, using small apparatus, frequently results in disappointing losses of material owing to premature crystallisation during filtration or to repeated transferences of small quantities of solutions or solids from vessel to vessel. We have found that an apparatus, consisting essentially of a crystallisation vessel attached to a form of the Schwinger filter, is very convenient for repeated and rapid crystallisations. The principle of the apparatus is somewhat similar to that of the Bergkampf[1] filter-beaker.




The apparatus, having the dimensions shown in the figure, is convenient for crystallisations from about 0·4 to 4 ml. of solvent. The surfaces, which meet inside the rubber sleeves, are ground flat. Cell C is tared together with the filtering unit A, and the disc of hardened filter paper secured by the lower rubber sleeve. The crude reaction product is collected, washed and dried in cell C, slight positive pressure being applied to B for rapid filtration. After weighing the product, the cell C and filtering unit are again attached to the crystallising vessel B and the thick-walled capillary tube is replaced by a plug of glass rod of the same dimensions. Most of the solid is tapped down into B and dissolved in hot solvent; by tilting the apparatus, the hot solution is run to and fro into C to dissolve solid adhering to the sides. Cell C is then removed and the filtering unit is attached directly to the crystallising vessel. The solution is reheated for a short time to warm the whole apparatus and then filtered, under slight positive pressure, into a micro-beaker by turning the apparatus through 90°. If crystallisation occurs during filtration, the crystals are easily dissolved in more hot solvent run to and fro from B and then filtered into the bulk of the solution.

The crystals which separate are collected in the same manner as the crude reaction product. Since filtration of the hot solution in further crystallisations is not normally necessary, the crystals are transferred to B as before and dissolved; crystallisation is then allowed to take place in B. The crystals are collected again in cell C. If some crystals adhere to the sides of B, they are redissolved in some of the mother liquor, allowed to crystallise and collected with the main bulk in cell C. The crystals are then washed and dried.

The process can be repeated as often as necessary. After the first hot filtration, there are no losses owing to transference from vessel to vessel, and the material is accessible for weighing and melting-point determination between each recrystallisation.

In the first two trials with this apparatus, hyoscyamine picrate, obtained

from hyoscyamine sulphate solution produced in a partition chromatogram of *Atropa Belladonna*[2] was used. The results of crystallising two samples of the picrate from aqueous alcohol are shown in the Table. We have since obtained equally satisfactory results in many other crystallisations.

| Recrystallisations | Weight recovered mg. | | Melting-point °C. (uncorrected) | |
|---|---|---|---|---|
| | (a) | (b) | (a) | (b) |
| Crude precipitate ... ... ... ... ... ... | 21 | 40 | 162—3 | 162—3 |
| First crystallisation with filtration of hot solution ... | 14·5 | 34 | 164—5 | 164—5 |
| Second crystallisation without filtration of hot solution | 11·5 | 32 | 164—5 | 164—5 |
| Third crystallisation without filtration of hot solution ... | 10·5 | 30 | 164—5 | 164—5 |
| Fourth crystallisation without filtration of hot solution | 9·5 | 27 | 164—5 | 164—5 |

REFERENCES.

1. von Bergkampf, *Z. anal. Chem.*, 1926, **69**, 321.
2. Evans and Partridge, *Quart. J. Pharm. Pharmacol.*, 1948, **21**, 126.

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is less irritating; its low viscosity also makes it easier to handle, and a comparatively small gauge needle may be used. Ethiodan is specifically indicated for use in the radiological diagnosis and localisation of cord tumours, herniated nucleus pulposus, intraspinal protrusion of intervertebral discs, and any other conditions in which obstructions in the cerebrospinal canal or compression of the cord are suspected. Normally, 3 ml. is injected immediately below the level at which the obstruction is suspected. It is issued in boxes of 3 ampoules each containing 3 ml. S. L. W.

**Ferosan Tablets*** contain exsiccated ferrous sulphate 3 gr., copper sulphate 1/25 gr., and manganese sulphate 1/25 gr. Their use is indicated in all cases of hypochromic microcytic anæmia, including anæmia due to chronic or acute hæmorrhage, idiopathic hypochromic anæmia and anæmia of pregnancy or lactation. The adult dosage is 1 or 2 tablets 3 times daily after meals. Ferosan tablets are supplied in bottles of 100 tablets. S. L. W.

**Priscol*** is the hydrochloride of 2-benzyl-4:5-imidazoline; in colourless crystals, freely soluble in water; m.pt. 171°C. Its principal action is to dilate the peripheral vessels. This effect is primarily on the arterioles and the smaller arteries, and its use is therefore followed by hyperæmia and acceleration of the blood flow in the capillaries. The improved circulation is usually accompanied by a fall in blood pressure. It is indicated particularly for the treatment of peripheral vascular disorders, by intravenous, intramuscular or intra-arterial injection; arthritic conditions are treated by peri-articular injections or the local use of an ointment. It may also be employed as a local application, combined with parenteral or oral therapy, for the treatment of slow-healing wounds and ulcers. It is claimed to be especially valuable in ophthalmic conditions where active hyperæmia is desired; for this purpose, it is employed either in the form of drops of a 10 per cent. solution or by subconjunctival injection. Priscol is supplied in bottles of 40 or 200 tablets containing 25 mg., in boxes of 10 ampoules containing 1 ml. (10 mg.), in bottles of 10 ml. of 10 per cent. solution, and in tubes containing 20 g. of 10 per cent. ointment. S. L. W.

# REVIEW ARTICLE

## ANTICOAGULANTS

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THE processes that underly the clotting of blood are still far from adequately understood, in spite of the many facts uncovered by generations of patient investigators. The classical scheme associated with the name of Morawitz[1], although neither complete nor universally accepted, is still useful as a summary of the main events. According to this scheme, the plasma contains three essential ingredients of the complete clotting system: these are ionised calcium and the proteins prothrombin and fibrinogen. The addition of a fourth ingredient, thrombokinase (or better thromboplastin, since its enzymic nature is still in doubt) completes the system, and initiates clotting. Thromboplastin is present in the tissues generally and also in the blood platelets. When blood makes contact with tissue fluid, or the platelets are injured by contact with a hydrophilic surface, prothrombin reacts with thromboplastin and with calcium ions to form an enzyme, thrombin: this is the first stage of clotting. In the second stage of clotting thrombin acts upon fibrinogen, changing it into the insoluble protein fibrin, whose threads constitute the matrix of the clot. Or in summary:

$$\text{Prothrombin} \xrightarrow{\text{Thromboplastin, Ca ions}} \text{Thrombin};$$

$$\text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin}.$$

Any substance, or treatment, which removes or inactivates any of the five clotting factors will prevent coagulation. Anticoagulants might therefore be divided into five groups, according to the factor interfered with: such a classification, however, would not be a very useful one, since some important anticoagulants affect more than one component of the system, and since a single component may be attacked by anticoagulants differing completely in their mode of action. In addition, it must be emphasised that the Morawitz scheme is oversimplified. Thus, at least one further plasma protein[2,3,4] is involved in the conversion of prothrombin to thrombin; and prothrombin itself has been regarded as a complex of two easily separable factors[5,6,7]. The velocity of both stages of clotting is related to the nature and concentration of the electrolytes present, and that of the second stage is reduced by antithrombin, of which traces are always present in plasma and further amounts are set free during clotting. Finally, the relation of the plasma proteases and their inhibitors to clotting is still poorly defined. No further mention will be made of these factors, and the action of anticoagulant substances will be discussed in terms of the Morawitz scheme alone.

## Modes of Action of Anticoagulants

A list of the possible mechanisms of anticoagulant action is given below, with a number of the most important substances exhibiting each type of activity.

*Prevention of platelet disintegration:* hydrophobic surfaces (paraffin, amber, perspex, collodion, silicone, etc.); most anticoagulants.

*Removal of calcium ions:* oxalates, fluorides, citrates, soaps of alkali metals, ion-exchange resins.

*Interference with prothrombin formation:* dicoumarol, salicylates.

*Inhibition of the conversion of prothrombin to thrombin:* heparin, other sulphuric acid esters, salts of the rare earth metals, organic bases, reducing agents, trypsin inhibitors.

*Inhibition of the action of thrombin on fibrinogen:* heparin, other sulphuric acid esters, reducing agents (cysteine, glutathione, bisulphite, etc.), 'lipid inhibitors', organic bases, trypsin inhibitors.

*Inactivation of fibrinogen:* fibrinolysin, protamines.

*Release of heparin from the tissues:* peptone, antigens (in sensitised animals), radioactive substances, nitrogen mustards, diamines, diamidines, etc.

The following discussion will be concerned chiefly with those substances that are now used with the primary aim of inhibiting coagulation *in vitro* or *in vivo*. Only brief mention will be made of substances formerly so used, but now discarded in favour of more active or less toxic materials, and of substances whose anticoagulant action is important only as a side-effect of their therapeutic employment.

## Substances Preventing the Disintegration of the Platelets

It is difficult to withdraw blood from a vein or artery without contaminating it with tissue fluid, but with good technique (clean puncture, avoidance of stasis, discarding of the first portion of effluent blood) the contamination may be kept small. The speed of clotting then depends, other factors being equal, on the rate of platelet disintegration, and this in turn depends on the surface with which the blood is in contact. Clotting is promoted by increasing the area of contact; the large contact area is the main factor in the hæmostatic efficiency of gauze and of its absorbable substitutes such as fibrin foam and gelatin foam. The chemical nature of the surface is equally important. Clotting is slower in a Pyrex glass vessel than in a soda-glass vessel, and still slower in a vessel lined with a water-repellant substance such as paraffin, amber, collodion or any of a variety of plastics. The best of all surfaces for the delay of platelet lysis is provided by the silicone film formed by the hydrolysis of dimethyldichlorosilane[8]. Blood taken with silicone-coated syringes and needles, and kept in silicone-coated vessels, may remain fluid for several hours. The silicone technique should facilitate the study *in vitro*, or in perfusion experiments of phenomena depending on the presence of normally reactive platelets: for example, the liver of a sensitised dog may be perfused with normal whole blood, and can then respond with a maximum anaphylactic reaction when the specific antigen

is added to the perfusion stream, an effect not obtained when heparinised or defibrinated blood is used for the perfusion[9].

The mechanism of platelet lysis by contact with foreign surfaces is little understood: there is some evidence[10] that a plasma factor takes an active part. Anticoagulants generally, and notably heparin, delay the lysis, perhaps through an action on the plasma factor rather than directly on the platelets themselves. *In vivo,* platelets agglutinate on a damaged area of endothelium; *i.e.* they stick to one another as well as to the injured blood vessel wall, forming a " white thrombus " or " platelet clot." When the clumped platelets lyse, fibrin is formed locally; in addition, the clumping is itself in some way favoured by the processes giving rise to the fibrin clot. Wright[11], applying a simple quantitative test for the measurement of platelet " stickiness," found that this was reduced in animals treated with anticoagulant drugs, including dicoumarol[12], which has no important direct effect on any component of the clotting system, but owes its activity entirely to its ability to prevent prothrombin formation. A platelet thrombus may form within a vessel even when fibrin formation is completely inhibited, as for instance by the administration of heparin; if the dose of heparin is pushed still higher, platelet agglutination is stopped too. The effect of heparin on platelet agglutination begins later and lasts longer than that on the clotting time[13]. These observations are among many which emphasise the dissociability of platelet agglutination and clotting in the ordinary sense; yet the two processes are favoured or prevented by various common influences, and their interrelationships are hard to disentangle, except for the obvious fact that the platelet thrombus is a rich potential source of thromboplastin, and to that extent a likely site of fibrin formation. Further study is needed of the plasma factors affecting platelet adhesiveness and fragility. The observation of Wright[14] that the platelets become more sticky after surgical operations may perhaps be related to the finding by Macfarlane and Biggs[15] of increased fibrinolytic activity at this time, as well as to the greatly increased risk of intravascular clot formation.

## Decalcifying Anticoagulants

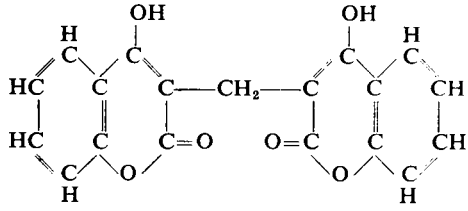
It has been known for over fifty years that calcium precipitants like oxalates, fluorides and the soaps of alkali metals can inhibit the clotting of shed blood, and that the inhibition can be removed by adding an excess of soluble calcium salt. Citrates act similarly, but without precipitating calcium, which becomes bound as part of a complex anion[16]. The cheapness and low toxicity of citrate have made it the anticoagulant of choice in blood transfusion. The injected citrate is so greatly diluted by the body fluids that it has no anticoagulant action *in vivo*: the clotting time may even be shortened somewhat[17]. When very large volumes of blood or plasma have to be transfused within a short time, the toxicity of citrate may become significant.[18]

The prevention of clotting in blood treated with ion-exchange resins, through replacement of the plasma Ca by Na, has recently been described[19].

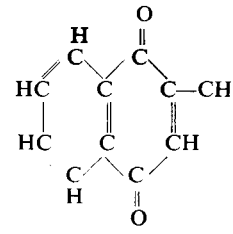
## Substances Interfering with Prothrombin Formation

It has long been believed that prothrombin is manufactured by the liver, and this belief has been fully substantiated by recent investigations. When the liver is extirpated or severely damaged (*e.g.* by carbon tetrachloride) the plasma prothrombin falls within a few days to a negligibly low level. Less extensive liver injury produces a smaller, but still readily detectable, reduction in plasma prothrombin. The formation of prothrombin can, however, be depressed without noticeably interfering with the other functions of the liver. The substance which most clearly acts in this way is dicoumarol.

*Dicoumarol.* The observations of Schofield[20] and Roderick[21] showed that the hæmorrhagic condition of cattle fed on spoiled sweet-clover hay was due to the ingestion of a water-soluble toxic principle, and made it probable that prothrombin was the point of attack. The remarkable work of Link and his colleagues[22,23,24] led to the identification of the hæmorrhagic agent as 3,3′-methylene-*bis*-hydroxycoumarin, now known as dicoumarol.



Dicoumarol



2-methyl-1:4-naphthoquinone
(Menaphthone)

There is no doubt that the whole of the anticoagulant action of dicoumarol is due to its interference with prothrombin synthesis: it has practically no effect on clotting *in vitro*. The structural similarity of the dicoumarol half-molecule to the compounds of the vitamin K group (menaphthone is a synthetic vitamin-K analogue: the natural vitamins have branched unsaturated alkyl chains instead of methyl in the 2-position) early suggested that dicoumarol acted as an antagonist to the vitamin. Although the first attempts to counteract the action of dicoumarol by treatment with menaphthone were unsuccessful, it is now clear that the two compounds act antagonistically over a certain range: the vitamin must, however, be given in doses far exceeding the usual therapeutic ones. Ascorbic acid potentiates the anti-dicoumarol effect of menaphthone, and it has been suggested[25] that the bleeding tendency characteristic of chronic dicoumarol poisoning may be due in part to a disturbed vitamin C metabolism. Further evidence of the relation of dicoumarol to vitamin K comes from the observations of Meunier and his colleagues[26,27], who have described coumarin derivatives with vitamin K activity as well as naphthoquinones acting like dicoumarol. The view that dicoumarol acts by blocking the vitamin is now generally accepted,

but its mode of action cannot be further analysed, since the role of the vitamin itself in prothrombin formation is unknown.

Dicoumarol is a weak acid, nearly insoluble in water but forming water-soluble salts. Unlike most other anticoagulants, it is active by mouth. The clotting time as ordinarily measured does not provide a satisfactory index of the effectiveness of dicoumarol, and some form of prothrombin-time test is universally used in both experimental and clinical work to gauge the effectiveness of the drug. The action on prothrombin-time is a delayed one, since time must be allowed for the existing prothrombin to disappear: the maximum prolongation is seen in 2 to 4 days after the administration of a single dose. A rather longer time is required for the restoration of prothrombin after the effect has begun to wear off. In the presence of liver injury the effect of the drug is enhanced both in degree and in duration; the same is true when the kidney is damaged. There is no doubt that the response to dicoumarol varies considerably from subject to subject: the effect of an excessive reduction of prothrombin can be counteracted by the administration of a vitamin K preparation, or (since symptoms do not occur unless the plasma prothrombin is depleted to a small fraction of the normal value) by the transfusion of normal blood or plasma.

The toxicity of dicoumarol for both animals and man is related almost entirely to its anticoagulant action, and death when it occurs is due to hæmorrhage. Morphological changes in the liver, when seen at all, are usually secondary to local hæmorrhage, and most liver function tests reveal no impairment of the organ. The plasma fibrinogen level is, however, somewhat raised by moderate doses of dicoumarol, and reduced by large doses[28]: similar effects are produced by various agents toxic to the liver, and it may be that the abnormality in this organ is not strictly confined to the prothrombin-forming system. Plasma fibrinogen levels are, however, distinctly labile, and the degree of general hepatic injury produced by dicoumarol is at most an extremely slight one.

*Salicylates.* Link[24] has suggested that the action of dicoumarol may be an indirect one: its breakdown within the body may liberate salicylates, and these may be responsible for inhibiting the formation of prothrombin. While there is not yet enough evidence to prove or disprove this idea, the deleterious action of salicylates on prothrombin formation has been amply confirmed in both human and animal studies.

### Substances Inhibiting the Conversion of Prothrombin to Thrombin and/or the Action of Thrombin

It might be thought convenient to consider in separate sections the substances opposing the formation of thrombin, and the substances impeding its action. In practice this is difficult. Heparin, the most important of all, acts on both stages of clotting; and the same is true of some, at least, of the anticoagulants which resemble it in being sulphuric acid esters of high molecular weight. Even in the case of heparin it is very hard to assess the relative contribution made by its antiprothrombin and antithrombin activities to its overall effect on clotting systems containing

whole blood; and the same statement applies with greater force to the related anticoagulants, none of which has been investigated in such detail as heparin. Antiprothrombin activity, in turn, might be due to inactivation of either thromboplastin or prothrombin itself. In practice, the amount of anticoagulant required to prevent thrombin formation goes up if the clotting system is enriched in either thromboplastin or prothrombin. This, however, would be expected on the basis of any theory postulating a reversible combination between either of the clotting factors and the inhibitor. It is likely that both prothrombin and thromboplastin may combine with the anticoagulant and lose activity on so doing; but until the factors concerned in thrombin formation can all be isolated and studied by adequate physio-chemical methods, the relative importance of the various possible reactions can only be guessed at.

Most of the anticoagulants listed as possessing antiprothrombin or antithrombin activity have large molecules, or can form more or less stable complexes with protein constituents of the plasma. They thus remain in the blood for some time after injection, and their anticoagulant action, unlike that of the decalcifying anions, is demonstrable *in vivo* as well as *in vitro*. Many of them are compounds containing sulphuric acid in ester linkage. Of these heparin has received by far the most attention, both because it is a natural constituent of the body and may be concerned in maintaining the normal fluidity of the blood, and because its toxicity is low enough to permit its prolonged administration to patients in danger from intravascular thrombosis.

*Heparin.* The monograph by Jorpes[32] is a recent and comprehensive treatise on the chemistry, physiological action and clinical applications of the substance. Earlier summaries by Best[33,34] and Wilander[35], both of whom have made important contributions in this field, are still well worth consulting.

*History and chemistry of heparin.* Heparin was discovered by McLean[36] in 1916 during an investigation, under Howell's direction, of the thromboplastic action of phosphatide preparations from liver and heart. During the next ten years Howell and his colleagues studied it intensively and showed that it was not, as had been thought at first, a lipoid; their best preparations contained uronic acids and had a high ash content. In the further purification of heparin and in the elucidation of its chemical nature, the major part was played by Charles and Scott in Toronto and by Jorpes and his co-workers in Stockholm. The Canadian workers[37] devised an alkaline extraction technique which gave an improved yield of a purer product, and they showed that many mammalian tissues contained heparin, ox lung being a particularly rich source. The Swedish investigators[38,39] confirmed the presence of a uronic acid in the purified material, and identified glucosamine and ester sulphate as further constituents: the remarkably high content of sulphate explained the large proportion of ash in Howell's material. Jorpes and Bergstrom[39] concluded that heparin is a mucoitin polysulphuric acid, and this view has been sustained by subsequent investigation.

About the same time Charles and Scott[40], who had been continuing

their purification studies, reported the isolation of a crystalline barium salt of heparin having a constant composition. There has been a good deal of controversy about the significance of this material. Its isolation has been repeated and the formula $C_{26}H_{44}O_{58}N_2S_5$ has been suggested[41,42] for the corresponding heparin acid, but Jorpes[32] regards the barium salt as a mixture of compounds varying in their degree of esterification. There is little doubt that the barium salt of Charles and Scott was really crystalline and that the crystallisation procedure is a valuable method of purification. The heparin molecule is, however, such a large one (the molecular weight of the barium salt is 3462 according to the formula of Charles and Todd and the compound may be a polymer) that crystallinity alone is an insufficient guarantee of purity. There is, indeed, a good deal of evidence that the crystallised preparation does not necessarily represent a chemical individual; samples of it vary appreciably in composition[32] and have been separated into fractions of unequal potency[43]; the potency may go down with repeated recrystallisation[44]; and the barium salts obtained from different mammalian species are of quite divergent potency, although similar in elementary composition, crystalline form, and chemical behaviour[45]. It is hard to say whether heparin in its natural state ought to be regarded as a chemical individual, difficult to obtain in strictly unmodified condition, or whether a family of closely related heparins exists in the tissues: the latter is perhaps the more likely alternative, if only because so complex a substance is probably not synthesised in a single step. Nevertheless, it seems probable that the best preparations obtained by the Canadian and the Swedish workers, and by others, represent a near approach to the most potent heparin obtainable, at any rate from bovine tissue.

*The standardisation of heparin.* The difficulty of obtaining heparin of uniform quality makes it desirable that each preparation should be standardised for potency. Howell[46] originally defined a unit of heparin activity as the minimum amount which, when added to 1 c.c. of freshly drawn cat's blood, would keep it fluid for 24 hours. Experience with many drugs has shown that such a unit, defined in terms of a poorly reproducible biological system, cannot be relied on to be constant; and in accordance with the practice now generally accepted, several groups of workers set up stable reference preparations, by comparison with which the potency of other samples was determined. These have now been replaced by an International Standard Heparin[47], the bulk of which is preserved at the National Institute for Medical Research, London, N.W.3, under the auspices of the Committee on Biological Standardisation of the World Health Organisation. The International Unit represents the strength of 1/130 mg. of this preparation: it is practically identical with the Toronto unit[48], defined in terms of a sample of barium salt, and, so far as can be ascertained, roughly equal to the Howell unit. It is to be expected that the practice of labelling potency in units will become superfluous in time, because all commercial preparations will be of the same maximum strength; but meanwhile the common practice of reporting doses in mg. introduces an uncertainty of at least 30 to 40 per cent.

and is to be deprecated. It is perhaps unfortunate, as Jorpes[32] points out, that the established unit is such a small one: it is inconvenient to have to write out "30,000 units", for instance, when referring to a single human dose.

A great variety of assay methods has been suggested for the standardisation of heparin preparations: the clotting systems which have been used include fresh blood; citrated or oxalated blood or plasma, recalcified with or without the addition of tissue extract; fresh fowl plasma plus tissue extract; and whole blood or plasma plus thrombin. Reproducible results can be obtained with any of these systems, but when two samples of heparin are compared by different methods the values obtained for the relative potency may differ somewhat: this is particularly true when the samples are obtained from different species[49] or when one of them has been denatured by treatment with acid[50]. The cause of such discrepancies is presumably the varying affinity of the several active principles for different constituents of the clotting mixture. As it appears that most of the heparin now on the market is of bovine origin, and is at least two-thirds as active as the best preparation obtainable from the species, the uncertainties due to the use of varying assay methods are not very serious.

*Physical and chemical properties of heparin.* Heparin and its salts with the alkali and alkaline earth metals are colourless substances, soluble in water but not in most organic solvents; they do not dialyse appreciably through ordinary collodion membranes. The sodium salt, which is the form usually supplied, is very stable in neutral or alkaline solution and can be sterilised by heating; when heated in acid solution it slowly loses its activity. Heparin is precipitated by many organic bases, including benzidine, protamines, toluidine blue and streptomycin: this property has been employed for inactivating heparin both *in vitro* and *in vivo*. Toluidine blue changes when combined with heparin into its reddish-violet "metachromatic" tautomer[51]; a similar sort of colour change is shown by other basic dyes in the presence of heparin. The metachromatic reaction was shown by Lison[52] to be specific for sulphuric acid esters of high molecular weight: it is given by a large number of substances, both natural and synthetic, which possess anticoagulant activity. The Swedish workers, in a series of particularly elegant experiments[53,54,55], have shown that the granular material of the mast cells, which takes an intense metachromatic stain, is rich in heparin.

The most striking property of heparin is the remarkably strong negative charge carried by its molecule in aqueous solution. Indeed, as Jorpes points out, this is almost its only important chemical property, since apart from its acidic (sulphuric and carboxyl) radicals the molecule has no reactive groups. The acidic strength of heparin enables it to react with the basic groups of proteins and other substances and is certainly the basis of its anticoagulant action. That its affinity for proteins is a general one and is not confined to the proteins concerned with clotting was shown by Fischer[55], who observed that the isoelectric point of casein was shifted to the acid side in the presence of heparin.

*Mode of action of heparin.* Although there is no doubt that the negative charge carried by its molecule is responsible for the characteristic activity of heparin, the activity is not simply a function of the proportion of esterified sulphuric acid that is present. The large size of the molecule is also involved in some way. The low potency of the heparin obtained from some mammalian species, and the reduced activity of heparin subjected to mild treatment with acid, which have been referred to, probably indicate a correlation between potency and degree of polymerisation[44]. That potency increases with molecular size is more obvious in the case of the synthetic esters of sulphuric acid, which will be referred to later.

While the key to the action of heparin must be sought in the large size and electronegativity of its molecule, these properties do not explain why heparin should act particularly on the blood-clotting system. Indeed, it is by no means certain that its physiological function has to do with the maintenance of the fluidity of the blood. Heparin does, in fact, act on other enzyme systems: it neutralises complement[56]; antagonises fumarase[57], trypsin[58], and fibrinolysin[59]; and it has some inhibitory effect on a variety of allergic reactions. When it is injected or liberated into the blood stream, its low diffusibility will tend to keep it there, and it may be in part for this reason that it has so few extravascular actions.

Heparin can prevent both the conversion of prothrombin to thrombin and the clotting of fibrinogen by thrombin. In either case the inhibitory action disappears when the isolated clotting reagents are used. Thus, heparin does not inhibit the clotting of purified fibrinogen by purified thrombin[60], or the formation of thrombin from purified prothrombin[61]. An additional factor, heparin complement[62,63,64], must be present if either reaction is to be prevented: this substance is a constituent of the albumin fraction of the plasma, but is not the crystallisable serum albumin proper. The antithrombin of normal plasma is also found in the albumin fraction, and the attractive suggestion has been made that it may be heparin-complement combined with a small fraction of its possible charge of heparin. The balance of evidence[64,65] seems at present, however, not to support this hypothesis.

*Heparin: fate in the body.* Heparin given by vein exhibits its greatest effect on clotting within the first few minutes. It is then rather rapidly inactivated or removed from the circulation. Some is excreted in the urine[66,35,57]; the greater part apparently escapes slowly into the tissues, where it may be enzymatically inactivated[68]. The duration of the anticoagulant effect is roughly proportional to the dose, but does not exceed 2 to 3 hours unless the dose is so great that its initial effect is to raise the clotting time to infinity.

*Organic esters of sulphuric acid.* The possibility of obtaining a cheap, synthetic anticoagulant suitable for use *in vivo* began to receive attention about 20 years ago. The anticoagulant action of the trypanocidal drug suramin (sodium *m*-benzoyl-*m*-amino-*p*-methylbenzoyl-l-aminonaphthalene-4:6:8-trisulphonate, germanin, Bayer 205) had been known for some time[69], and in 1930 Stuber and Lang[70] reported its successful use

in the therapy of thrombosis. In the same year Rous, Gilding and Smith[71] noted the anticoagulant effect of the dye chicago blue 6B (chlorazol sky-blue FF, the sodium salt of tetrazotised dianisidine coupled with 1-amino-8-naphthol-2:4-disulphonic acid); and Demole and Reinert[72] prepared a variety of substances of high molecular weight and found some of them active against clotting, the most potent being sulphonated aromatic polymers. One of these, the sodium salt of polyanetholesulphonic acid, was later marketed as "liquoid," and has had some popularity as an anticoagulant for *in vitro* and animal experiments: it is a rather toxic substance causing delayed death in doses not far above the effective ones. Chlorazol sky-blue, however, and some related azo-sulphonic dyes, especially Chlorazol fast-pink BKS (sodium 3:5-disulpho-diphenylurea-4:4-diazo-*bis*-2-amino-8-naphthol-6-sulphonate) were found by Huggett and his colleagues[73,74] to be relatively non-toxic, and have been used extensively in physiological experiments. They act, apparently, mainly in the first stage of clotting. In this group of dyes there is no simple relationship between chemical structure and anticoagulant activity: all have heavy molecules containing a number of $-SO_3$ groups and azo linkages; and it is of interest that they resemble heparin, and the other synthetic anticoagulants containing ester sulphate, in having a strong affinity for toluidine blue and other metachromatic dyes.

The next series of synthetic anticoagulants to be investigated represented, chemically at least a nearer approach to heparin. Chargaff and his colleagues[75] and Jorpes' collaborator Bergstrom[76] independently reported the activity of carbohydrates esterified with sulphuric acid by treatment with chlorosulphonic acid. The esters derived from mono- and di-saccharides were inactive, but all the polysaccharides tested gave rise to active compounds. The treatment with chlorosulphonic acid, if unduly prolonged, yielded less active products, presumably because the materials were being depolymerized[76]. These compounds probably act on both stages of clotting, and the best of them[77,78,79] approach heparin in potency: the ratio of their activity to that of heparin cannot be exactly stated, since it varies greatly with the conditions of assay. All are too toxic for clinical use. It is of interest that their toxicity is due, at least in part, to their effect on clotting factors: they either cause the platelets to agglutinate, or precipitate fibrinogen, or both. According to Karrer and his colleagues[80] the toxicity is reduced by the introduction of other acid groups into the polysaccharide molecule before the treatment with chlorosulphonic acid.

Many high-molecular-weight esters of sulphuric acid occur naturally in both animals and plants; some of these are anticoagulants and some are not: activity is correlated at least roughly with ester sulphate content. Mucoitin and chondroitin sulphuric acids are not active, but become so when further esterified[76]. The mucus of the mollusc *Charonia lampus* contains a potent anticoagulant[81] with many chemical similarities to heparin. Two anticoagulants formerly popular with physiologists may possibly belong to this group, but little is known of their chemistry except that they seem to be acidic in nature: these are hirudin[82,83], from

the buccal glands of the medicinal leech, and novirudin[84], a melanin-like substance of vegetable origin. A number of marine algae contain polysaccharides esterified with sulphuric acid. Agar-agar, the best known of these, is not an anticoagulant; but related substances from *Chrondrus crispus*[85] and *Irideæ laminarioides*[75] are moderately active.

*Basic dyes and other basic substances.* All the substances just discussed presumably act in virtue of their strongly acid $SO_3H$ groups and the size of their molecules. The possibility that basic substances of high molecular weight are also anticoagulants has been investigated. This too is the case: protamine and histones[86,87] have well-marked activity, as have a number of basic dyes[87], including methylene blue, crystal violet and Janus green. Even so simple a base as diethylamine has some anticoagulant activity[88]. The mode of action of these substances is supposed to be analogous to that of the synthetic heparin analogues, but in a reverse direction: *i.e.* they displace the isoelectric point of protein clotting-factors toward the alkaline side, and so reduce their reactivity. The antagonism between compounds of this group and the ester-sulphate anticoagulants has already been mentioned; it is due, however, not so much to a cancelling-out of opposed actions on the electric charge of protein clotting factors, as to a simple co-precipitation of the acidic and basic anticoagulants. None of the bases so far investigated appears likely to have any practical value in delaying coagulation.

*Reducing substances.* A number of reducing agents, both inorganic and organic, have anticoagulant activity; they appear to act on both stages of clotting. Sodium bisulphite[89] and thiosulphate[90], cysteine[91] and glutathione[92] may be mentioned.

*Salts of rare earth metals.* The trivalent cations of neodymium[93,94] praseodymium, lanthanum and other elements of this group[95] are extremely active anticoagulants both *in vitro* and *in vivo*. They appear to act mainly on the first stage of clotting[94,96], but the mechanism of their action is not understood. The compounds are too toxic to be of practical value[97].

"*Lipid inhibitors*" of coagulation have been detected in phosphatide fractions of tissue extracts by Chargaff[98] and de Sütö-Nagy[99]. Their mode of action has not been studied in detail, and whether they have any physiological significance for the prevention of clotting is unknown.

*Trypsin inhibitors.* The purified trypsin inhibitors obtainable from pancreas and from soya beans are fairly active in delaying coagulation[100,101,102,103]. Their mode of action is obscure.

## Substances Inactivating Fibrinogen

The fibrinolytic enzyme of normal plasma, discovered forty years ago by Nolf[104], should be listed for the sake of completeness, since it can digest fibrinogen as well as fibrin[105]. It is usually present in an inactive form, but can be activated *in vitro* or *in vivo* by a number of different ways. Whatever the relation of this enzyme to the clotting system, it is doubtful whether it ever, *in vivo*, destroys fibrinogen so rapidly as to make the blood incoagulable.

Fibrinogen is precipitated more or less selectively by both protamines and certain anticoagulants of the sulphonic ester group, but this is certainly not the main reason why such substances inhibit clotting.

## Substances Causing the Release of Heparin

The blood of dogs thrown into shock by the injection of large amounts of Witte's peptone, or of an antigen (in previously sensitised animals), becomes incoagulable; and it has been conclusively demonstrated that the inhibition of clotting seen in such animals is due to circulating heparin[107,108]. The liver is the principal source of the released heparin. A similar effect is produced by large doses of ionising radiation[109], by the radiomimetic drugs of the nitrogen mustard series[110], and by certain simple basic drugs, particularly diamines and diamidines[111]. Evidence that these stimuli release heparin in the same way from human tissues is lacking, except in the case of ionising radiations.

## The Clinical Use of Anticoagulants

The utility of a non-toxic anticoagulant in the therapy and prophylaxis of thrombosis in man has long appeared probable, and was confirmed as soon as purified heparin became available in quantity. The first favourable clinical reports from Stockholm[112] and Toronto[113] have been followed by some hundreds of papers describing the successful use of both heparin and dicoumarol in a variety of thrombo-embolic conditions. It is impossible to give a brief adequate summary of this work; an excellent account will be found in the monograph by Jorpes[32], who seems, however, to emphasise unduly the toxic action of dicoumarol on the liver. Both drugs appear to have an established place in therapy. As compared with heparin, dicoumarol has the advantages of cheapness and of effectiveness on oral administration: its drawbacks are its slow onset of action, which makes it useless in emergencies unless supplemented by heparin, and the considerable variability in the response of individual patients. All anticoagulant therapy involves the risk of hæmorrhage, and this risk can only be minimised by close supervision of the patient and frequent checks of clotting time (in the case of heparin) or prothrombin time (in the case of dicoumarol).

The numerically most important field of usefulness for these drugs has been the prevention and treatment of post-operative thrombosis, particularly after pelvic operations. The incidence of this complication is notoriously variable, and the availability of an effective therapy should not distract attention from the importance of simpler measures, especially active and passive movement of the limbs. Treatment is usually begun on the second day, when the risk of bleeding at the site of operation is small, and continued till the patient is ambulant. Opinions vary as to whether anticoagulant therapy should be used routinely after pelvic and abdominal surgery or reserved until signs of clot formation appear. Early diagnosis of latent thrombosis is naturally of the greatest importance, and

phlebography of the lower extremities[114] and tests revealing hypercoagulability of the blood, such as the heparin tolerance test of de Takats[115], have been found useful for this purpose.

Other forms of active venous thrombosis respond equally well to anticoagulant therapy, which undoubtedly reduces the incidence of embolic complications. Thrombosis of the mesenteric veins, the retinal veins and the cavernous sinus, have all been treated successfully, in addition to the more common condition in which the initial site of clot formation is one of the deep veins of the lower leg. The status of the anticoagulant drugs in the treatment of occlusive coronary artery disease is still uncertain. They are quite useless in subacute bacterial endocarditis. Overdosage with heparin is treated by withdrawal of the drug, when the blood regains its normal clotting power within a few hours, or in emergency by the intravenous injection of protamine, which has an instantaneous effect. Dicoumarol overdosage can be corrected by the administration of massive doses of vitamin K preparations, or more rapidly by the transfusion of fresh blood or plasma.

Finally it should be mentioned that heparin is a valuable adjunct to vascular surgery, and has some advantages over citrate as an anticoagulant in blood transfusion.

The expense and inconvenience of heparin therapy have undoubtedly restricted its field of usefulness. While intravenous administration, either several times a day or by continuous drip, is still the method most commonly used, a number of menstrua for the incorporation of heparin have been devised[116,117], which permit a prolonged effect to be obtained with a smaller number of intramuscular or subcutaneous injections.

## References


1. Morawitz, *Arch. klin. Med.*, 1904, **79,** 1.
2. Owren, *Lancet,* 1947, **252,** 446.
3. Ware, Guest and Seegers, *J. biol. Chem.*, 1947, **167,** 231.
4. Fantl and Nance, *Nature,* 1946, **158,** 708.
5. Quick, *Amer. J. Physiol,* 1943, **140,** 212.
6. Nolf, *Arch. int. Pharmacodyn.*, 1945, **70,** 5.
7. Feissly, *Schweiz. med. Wschr.*, 1945, **79,** 696.
8. Jaques, Fidlar, Felsted and MacDonald, *Canad. med. Ass. J.*, 1946, **55,** 26.
9. Rocha e Silva, Scroggie, Fidlar and Jaques, *Proc. Soc. exp. Biol., N.Y.*, 1947, **64,** 141.
10. Brinkhous, *Fed. Proc.*, 1947, **6,** 389.
11. Wright, *J. Path. Bact.*, 1941, **53,** 255.
12. Wright, *ibid.*, 1945, **57,** 382.
13. Solandt and Best, *Lancet,* 1940, **238,** 1042.
14. Wright, *J. Path. Bact.*, 1942, **54,** 461.
15. Macfarlane and Biggs, *Lancet,* 1946, **251,** 862.
16. Hastings, McLean, Eichelberger, Hall and Da Costa, *J. biol. Chem.*, 1934, **107,** 351.
17. Ferguson, *Physiol. Rev.*, 1936, **16,** 640.
18. Adams, Thornton, Allen and Gonzalez, *Ann. Surg.*, 1944, **120,** 656.
19. Steinberg, *Proc. Soc. exp. Biol. N.Y.*, 1944, **56,** 124.
20. Schofield, *J. Amer. vet. med. Ass.*, 1924, **64,** 553.
21. Roderick, *Amer. J. Physiol.*, 1931, **96,** 413.
22. Campbell and Link, *J. biol. chem.*, 1941, **138,** 21.
23. Stahmann, Heubner and Link, *J. biol. Chem.*, 1941, **138,** 513.
24. Link, *Fed. Proc.*, 1945, **4,** 176.
25. Overman, Stahmann and Link, *J. biol. Chem.*, 1942, **145,** 155.