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INVESTIGATION OF THE TOXICITY OF A SERIES OF DEXTRAN SULPHATES OF VARYING MOLECULAR WEIGHT

BY

KENNETH W. WALTON

From the Department of Experimental Pathology, University of Birmingham

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In previous communications (Walton, 1952, 1953) it was reported that differences of behaviour were demonstrable *in vitro* between members of a series of dextran sulphates of varying molecular weight. Although all the compounds examined showed heparin-like anticoagulant activity, those with molecular weights in excess of 40,000 differed from heparin and from the dextran sulphates of smaller molecular weight in precipitating the plasma proteins, and especially fibrinogen, in causing agglutination of the platelets and leucocytes and in producing alterations in the sedimentation rate of the erythrocytes.

Grönwall, Ingelman, and Mosimann (1945), using a more limited series of molecular sizes, showed that the toxicity of dextran sulphates for experimental animals declined as the molecular weight decreased. When the *in vivo* behaviour of the present series was investigated, it was found that the compounds which precipitated plasma-protein and agglutinated blood-cells *in vitro* were toxic in the intact animal. A direct correlation was observed between the molecular weight of the dextran sulphate and the degree of toxicity (Walton, 1951). This observation has since been confirmed by Stavely, Baker, and Payne (1952).

The present communication gives the results of an investigation into the nature and mechanism of this toxicity. The findings are broadly in agreement with those of Piper (1945b) and of Astrup and Piper (1946), who examined the sulphuric esters of several other polysaccharides (cellulose, starch, chitin, etc.).

Dextran sulphate of low molecular weight, which did not interfere with the stability of the plasma proteins or of the blood-cells, was found to have a toxicity comparable with that of commercial heparin when administered to experimental animals. The preliminary therapeutic trial of one such compound has been reported separately (Ricketts, Walton, van Leuven, Birbeck, Brown, Kennedy, and Burt, 1953).

MATERIALS AND METHODS

Dextran Sulphates.—The compounds used were those previously described (Walton, 1953). They were selected members of a series prepared by Ricketts (1952a). The average molecular weight of the D size compounds was 458,000; of the E size, 47,000; and of the I size, 7,500.

Heparin.—The anticoagulant activity of the dextran sulphates was assayed by the method of Kuizenga, Nelson, and Cartland (1943) against a sample of International Standard Heparin. Commercial heparin preparations were used for qualitative comparisons with the dextran sulphates. For most purposes, the preparation used was "Pularin" (Evans), batches No. F 85020 and No. N 10090, both assaying at 90 i.u./mg. In one specified experiment, the following additional preparations were employed: (*a*) British: "Liquemin" (Roche), batch dated August 31, 1948, 122 i.u./mg.; Heparin (Boots) batch No. 3583, 102 i.u./mg.; Heparin (British Drug Houses) batch No. 30607/c, 160 i.u./mg. (*b*) American: Heparin (Lederle) batch No. NP 115–128, 100 i.u./mg. (*c*) Swedish: Heparin "Leo" (no batch number or date supplied), 100 i.u./mg.

Platelet-counting Methods.—Lempert's (1935) modification of Kristenson's (1922) method, and the method of Rees and Ecker (1923), modified by increasing the concentration of formalin to 1%, were used. Since high concentrations of heparin and the dextran sulphates formed insoluble precipitates with cresyl blue, unstained platelets were counted in some experiments with the phase-contrast microscope.

Clotting-time Methods.—In experiments on small animals, necessitating frequently repeated estimations of the clotting-time, the method of Dale and Laidlaw (1912) was employed using standardized capillary-tubes containing steel shot of uniform size. Otherwise the method of Lee and White (1913) was used. All estimations were performed at 37° C.

Histological Techniques

Fixatives.—10% neutral formol-saline, 80% alcohol and Heidenhain's "Susa" were employed as routine fixatives. A preliminary trial of 4% basic lead acetate (Holmgren, 1940) for the demonstration of metachromatic material in reticulo-endothelial cells showed no substantial improvement over these.

Staining Methods and Materials.—In addition to routine staining of all sections with Erlich's haematoxylin and eosin, the following were used :

Metachromatic Stains.—Batches 851, 948 and 1234 of Toluidine Blue G were supplied by G. T. Gurr, Ltd. Batch No. 005804 of Azure A and batch No. 5146 of Thionine were supplied by Hopkin and Williams, Ltd. All these stains gave satisfactory results with Hughesdon's (1949) technique. Mallory's phloxine-methylene blue stain, containing Azur II (Lillie, 1948), also gave satisfactory results.

Fibrin Stains.—The recognized "fibrin" stains were found not to distinguish between fibrin and fibrinogen. The methods used were: the Weigert-Gram technique; Mallory's phosphotungstic acid haematoxylin; phloxine-tartrazine (Lendrum, 1947); and the acid picro-Mallory method (Lendrum, 1949).

Other Stains.—Haematoxylin-Giemsa and the Turnbull-Jenner (Turnbull, 1931) methods were used for the identification of intravascular leucocytes and platelets. The periodic acid Schiff technique (McManus, 1946; Hotchkiss, 1948) was employed at first, but was found to give weakly positive staining reactions with dextran sulphate and fibrinogen, singly and in combination.

Animals.—Albino mice (20–45 g.), albino rats (150–300 g.), guinea-pigs (450–700 g.), and rabbits (2–4 kg.) were used. All these animals were kept on standard pellet diets during the course of the experiments.

RESULTS

The dextran sulphates were found to be effective anticoagulants by parenteral administration only. No evidence of absorption or of toxic effects was obtained on oral or sublingual administration. Oral and sublingual heparin is similarly ineffective according to most workers (see Kennedy and Brown, 1952; McDevitt, Huebner, and Wright, 1952; and Tomich and Woollett, 1952).

Effective Dose.—As with heparin, some variation of response to intravenous dosage was encountered amongst animals of the same species, but, in general, it was concluded that a single injection of 6–8 mg./kg. body-weight was sufficient to prolong the clotting-time to about three times the normal, when estimated one hour after injection (Walton, 1951). The administration of similar doses subcutaneously, intramuscularly or intraperitoneally was also effective, but absorption was slower and more uneven, and the effect obtained less reproducible, than when the material was given intravenously.

Equivalent doses of this order of the various dextran sulphates and of heparin were given intravenously to rabbits and their effects upon the plasma proteins and the blood cells compared (Figs. 1 and 2).

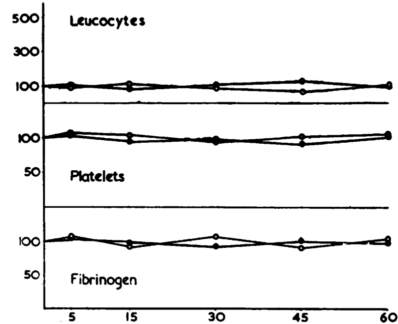

FIG. 1.—Effect of the minimal effective dose of heparin (1 mg./kg. body-weight) and of the I size dextran sulphate (6 mg./kg. body-weight) on the levels of the leucocytes, platelets and plasma fibrinogen in the rabbit. Ordinate: percentage deviation from pre-treatment level. Abscissa: min. after intravenous injection. ●——●: Heparin. O——O: I size dextran sulphate.

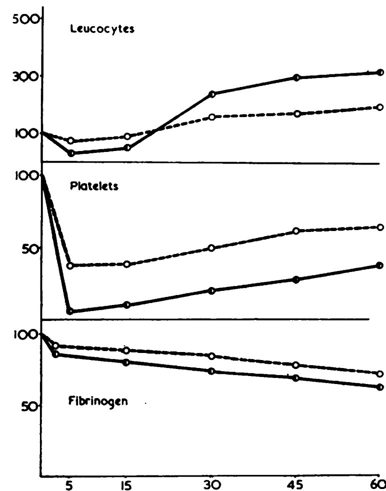

FIG. 2.—Effect of 6 mg./kg. body-weight of the E and D sizes of dextran sulphate on the levels of the leucocytes, platelets and plasma fibrinogen in the rabbit (for comparison with Fig. 1). Ordinate: percentage deviation from pre-treatment level. Abscissa: min. after intravenous injection. ◐——◐: D size dextran sulphate. O——O: E size dextran sulphate.

It will be seen from Fig. 1 that heparin and the small molecular weight dextran sulphate produced little or no alteration of the plasma fibrinogen or of the numbers of the formed elements of the blood. On the other hand, as shown in Fig. 2, the D and E size dextran sulphates produced: *(a)* A

steady fall of the plasma fibrinogen during the first hour. This was followed by a slow restoration to the normal level during the next two hours. (*b*) A sharp fall in the number of single platelets, with the appearance of large numbers of clumped platelets. These platelet-clumps varied in size, making accurate counts difficult. They showed the same general characters as those observed *in vitro* (Walton, 1953), individual platelets being enmeshed in amorphous material stained lilac in colour by the cresyl blue present in the diluting fluid. Small shreds of similar material free from adherent platelets were also present. (*c*) An equally abrupt drop in the number of leucocytes. In contrast with the behaviour of the platelet-count, however, the white-cell count was restored to normal within half an hour and often continued to rise subsequently to produce a moderate leucocytosis. (*d*) A slight and inconstant fall of the haemoglobin level. When animals showing this last phenomenon were more closely investigated, the red-cell count was found to fall proportionately and no evidence was obtained of haemolysis. Animals repeatedly injected with these compounds showed a simple normochromic anaemia with a persistently high reticulocyte count. Such animals were usually found to have one or more bleeding-points at autopsy (see below) and this was held to account for the changes inconstantly observed.

The effect upon the platelets, *in vitro* and *in vivo*, of the large molecular weight dextran sulphates, is closely similar to that shown by the cellulose, starch, and chitin sulphate esters previously investigated by Piper (1945a). Because of the eventual restoration to normal numbers and the disappearance of platelet-clumps, this author regarded the *in vivo* phenomenon as a reversible one in which the platelet-clumps could dissociate again into their individual components. No details of parallel investigations of the effect upon the leucocytes were given.

The present finding of a concomitant fall in leucocytes which is succeeded by leucocytosis strongly suggests that the thrombocytopenia and leucopenia were both corrected by the same mechanism, namely, fresh output of these elements by the marrow. This contention is supported indirectly by the work of Stetson and Good (1951), who found a similar fall of platelets and leucocytes following the administration of glycogen, agar, acacia, and other macromolecular colloids to animals sensitized to unrelated antigenic materials. These authors showed that the return to normal numbers of the platelets and leucocytes did not occur in animals in which the marrow had been rendered aplastic by nitrogen mustard.

Other evidence that the effect on the platelets was irreversible was provided by the finding that platelet thrombi were demonstrable in various organs long after the effect on the clotting-time of a single injection of the large molecular weight dextran sulphates had worn off.

Acute Toxicity of the Dextran Sulphates

In pilot experiments an investigation was made of the relation between these effects upon the blood-cells and plasma-proteins and the toxic manifestations produced by very large doses of the large molecular weight compounds. Small groups of mice and rats were given single intravenous or intraperitoneal injections of doses varying between 2 g./kg. and 100 mg./kg. Animals dying within one week were autopsied as soon as possible after death. Survivors were killed at varying intervals up to 28 days after the injection and autopsied similarly. As a result it was concluded that although the immediate cause of death varied with the level of dosage employed, the mechanism of production of the lesions found was always related directly to the effects described above.

It should be noted that with these massive doses of the anticoagulants, blood taken from the animals within an hour of the injection remained uncoagulated for 24 hours or longer and that, in surviving animals, the clotting-time was elevated —frequently to four or five times its normal value —even on the day following the injection.

Pathology

Group 1. Animals Dying Within a Few Minutes of Injection

Some animals given doses in excess of 500 mg./kg. of the D size compound, or doses in excess of 900 mg./kg. of the E size compound, died almost immediately, showing marked respiratory and circulatory embarrassment, often attended by convulsions.

At autopsy, the macroscopic findings in these animals were strikingly few, consisting merely of generalized hyperaemia and venous congestion of the thoracic and abdominal organs, with scattered petechial and purpuric haemorrhages beneath the serosal surfaces. The heart invariably showed dilatation of the right auricle and ventricle, the left chambers being relatively normal.

Histological examination of sections stained with Ehrlich's haematoxylin and eosin showed widespread embolism of vessels of varying calibre by pinkish granular or hyaline material associated with clumps of leucocytes. The process was demonstrable with greatest intensity in the lung, but was also demonstrable in other organs. In sections from material fixed and stained appropriately, clumps of platelets could also be identified. These

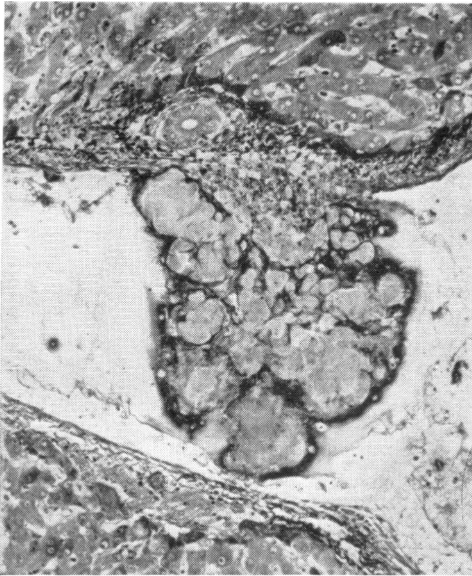

FIG. 3.—Portion of a large portal vein in the liver showing a platelet mass adherent to the wall. Note that the agglutinated platelets are enmeshed in a network of material giving a positive staining reaction for fibrin or fibrinogen. The platelet mass is being infiltrated at its point of attachment by cells derived from the sub-intimal tissues. Rabbit given 500 mg./kg. body-weight of the D size dextran sulphate. Lendrum's acid picro-Mallory method. × 228.

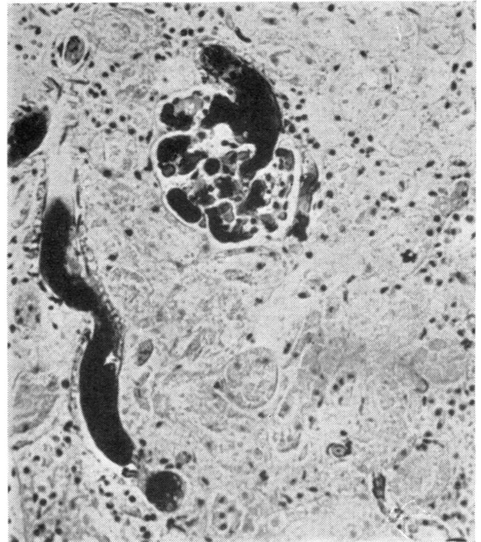

FIG. 5.—Embolism of renal arterioles and glomerular capillaries by metachromatic material. Rabbit given 100 mg./kg. dextran sulphate E. Toluidine blue. × 220.

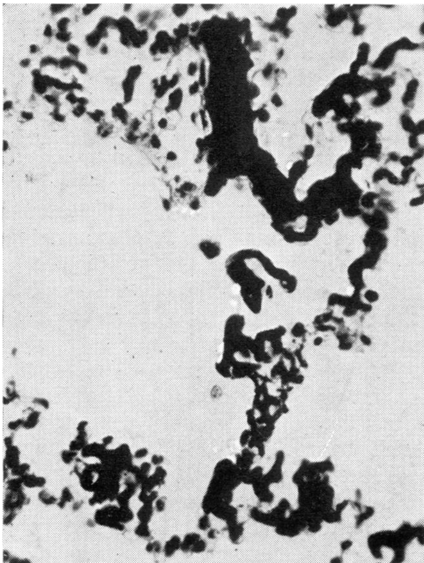

FIG. 4.—Embolism of pulmonary arterioles and capillaries by metachromatic material. Rat given 500 mg./kg. body-weight E size dextran sulphate. Toluidine blue. × 320.

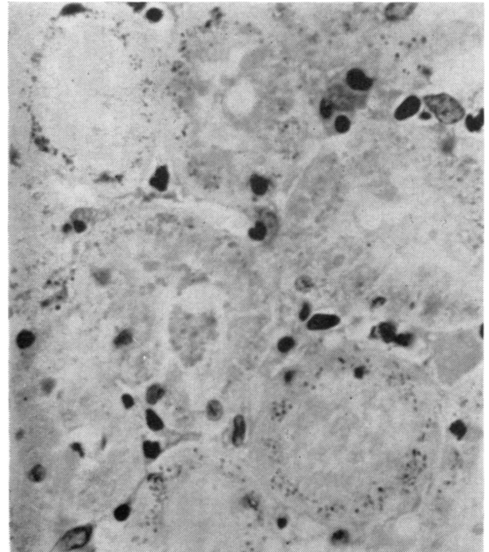

FIG. 6.—Rat kidney. The epithelium of the proximal convoluted tubules contains small granules of metachromatic material following a dose of 150 mg./kg. of the E size dextran sulphate. Toluidine blue. × 700.

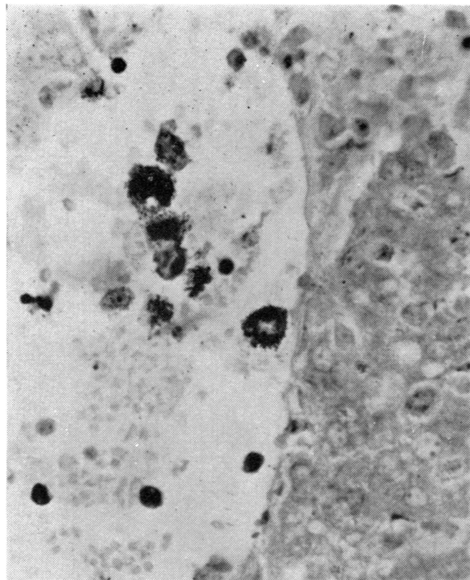

FIG. 7.—Large centrilobular vein in the liver of a mouse given 400 mg./kg. of the D size dextran sulphate. Many free phagocytic cells in the lumen of the vessel contain granular metachromatic material. Toluidine blue. × 400.

conglutination thrombi were apparently free in the lumen of a vessel, but serial sectioning frequently showed that they were adherent at some point to the endothelial surface of the vessel (Fig. 3). Sections stained with toluidine blue showed the granular material surrounding the formed elements to be strongly metachromatic—i.e., to show the same staining reaction as that given by the dextran sulphate-fibrinogen complex *in vitro* (Walton, 1953). By this means, it was possible to see that in the lung the whole vascular tree, down to the alveolar capillaries, was filled with the material (Fig. 4). Vessels of varying calibre in the kidney also contained similar material and the effect was particularly striking in the glomerular capillaries (Fig. 5). The portal veins and sinusoids in the liver contained material giving similar staining reactions, and arterioles and capillaries in the interstitial tissue of the ventricular myocardium were affected. Complete blockage of the main coronary arteries or of the primary subdivisions of the pulmonary artery was never encountered though it was looked for repeatedly. Embolism of small myocardial vessels was accompanied by patchy areas of coagulative necrosis of myocardial fibres.

Apart from the amorphous metachromatic material present in the lumina of vessels, it was noted that granules of metachromatic material were present in occasional large mononuclear cells in the blood and that similar material was present in reticulo-endothelial cells in various situations. Thus, the Kuppfer cells in the liver sinusoids, littoral cells in the splenic pulp and large mononuclear cells in the femoral bone-marrow were affected in this manner (Figs. 10 and 11). In animals injected intravenously, only occasional cells giving a similar staining reaction were observed in lymph-nodes in various parts of the body, but the abdominal lymph-nodes were found to be heavily infiltrated with these cells in animals given the material intraperitoneally. With one exception, no metachromatic material was demonstrable in the parenchymal cells of any of the organs examined. This exception was the kidney, where in rats, but not in mice, granules of metachromatic material were demonstrable in the cells of the proximal convoluted tubules (Fig. 6).

Mast-cells, in the normal rat and mouse, give a metachromatic staining reaction with toluidine blue, but are situated quite differently from the cells giving the metachromatic reaction at the sites described above. Mast-cells are distributed, for the most part, in the delicate interstitial tissue beneath the serosal surfaces of organs or in the connective tissues surrounding bronchi and other ducts, vessels, and in the portal tracts. This was verified by the examination of tissues from uninjected control animals. The only situation where confusion was likely to arise was in the bone-marrow, and here the very large numbers of metachromatic cells in treated, as opposed to untreated, animals rendered the distinction unnecessary. Basophilic leucocytes were rarely seen in the blood of controls, so, again, the marked increase in numbers of large cells containing metachromatic material in the peripheral blood was thought to be significant (Fig. 7).

Group 2. Animals Dying 1–4 Hours After Injection

Other animals given doses of the same order as Group 1 showed similar acute symptoms and signs, but recovered. A proportion of such animals died one to four hours later.

At autopsy, the most striking feature in this group of animals was an acute haemorrhagic pulmonary oedema. Frothy, blood-stained fluid could be seen to be escaping from the external nares, and the larynx, trachea and main bronchi were filled with similar fluid. The lungs were voluminous, and, on section, exuded quantities of this fluid. Numerous blotchy haemorrhagic areas were seen beneath the visceral pleura. Rarely, these showed a central portion raised above the cut surface and resembling an infarct, but diffuse haemorrhage usually extended for a variable distance into the lung parenchyma. Sometimes a

complete lobe or the whole lung was thus involved. The heart showed dilatation of the right side similar to that observed in the previous group of animals. The abdominal organs showed an intense generalized congestion. Occasional petechial and small purpuric haemorrhages were observed beneath the serosal surfaces of the stomach and intestines. The liver was congested and showed accentuation of its lobular pattern. Occasional punctate haemorrhages were present in the cortex of the markedly congested kidneys. These haemorrhages were sometimes sufficiently numerous to simulate the appearance of the " flea-bitten " kidney of human embolic nephritis.

Histological examination of the tissues from these animals confirmed the acute haemorrhagic pulmonary oedema. Conglutination thrombi or emboli were again visible in the pulmonary vessels. In some fields there was intense intra-alveolar haemorrhage, while elsewhere the exudate present in the alveoli was faintly eosinophilic, granular, protein-like material. In the centres of the haemorrhagic areas it was sometimes possible to distinguish areas of definite infarction. Small areas of haemorrhagic necrosis were also found in the liver, and minute necrotic foci were present in the spleen. The latter commonly involved the Malpighian follicles, but occasionally similar areas were also present in the pulp. The accumulation of metachromatic material in the reticulo-endothelial cells of the liver, spleen and bone-marrow was much more pronounced. The granules of metachromatic material in the cells of the proximal convoluted tubules of the kidneys of rats were scanty in numbers in this group of animals.

Group 3. Animals Dying 12 Hours to 7 Days After Injection

The remaining animals given doses similar to those of Group 1 were obviously gravely ill. Most of them succumbed within 24–48 hours, but occasional animals survived up to four or five days. Some of the animals given doses of the D size compound between 500 mg./kg. and 200 mg./kg., or of the E size compound between 900 mg./kg. and 300 mg./kg., also died within this period, but the numbers of survivors increased as the size of the dose decreased. Only an occasional animal died within one week when given doses of the D size compound of 100 mg./kg. or less, or of the E size compound of 300 mg./kg. or less. The fatalities at these various dosages showed lesions of similar character and will be described together.

Post-mortem examination of these animals showed that the cause of death was always haemorrhage. The site and source of this haemorrhage varied. Many animals again showed evidence of severe intrapulmonary bleeding. This was often so extensive as to involve a whole lobe or lung. Sometimes a central area of infarction, which was now more easily distinguishable, was surrounded by diffuse and irregular haemorrhage into the adjacent parenchyma. Other animals showed intraperitoneal bleeding. This was observed only occasionally in animals injected intravenously (via the tail vein) but very commonly in animals injected intraperitoneally. Other sites of intra-abdominal bleeding were the testicular omentum, the retroperitoneal tissues and the mesentery. Small petechiae were seen frequently beneath the peritoneum of the anterior abdominal wall ; in the subserous tissues on the surface of the stomach, caecum and large intestine ; beneath the visceral pericardium and pleura and occasionally scattered at random in the subcutaneous fascia. Melaena was rarely encountered. In the few animals in which it occurred, a bleeding point was seldom demonstrable in the alimentary tract, but in two animals it was associated with a haemorrhagic necrosis, due to infarction, of the duodenum. The abdominal organs were blanched and soft in consistency. The liver showed blurring of its normal pattern with small yellowish areas scattered at random through its substance. Obvious areas of infarction were present in the spleen. The kidneys showed many pin-point haemorrhages against the pale, swollen background of the cortex. Occasionally obvious areas of cortical necrosis were present. Urine collected from the bladder and centrifuged showed the presence of red-cells, pus-cells, amorphous debris and occasional granular casts.

Histologically, it was possible only occasionally to define with precision the point from which the haemorrhage had originated. The haemorrhage was usually so extensive as to obliterate pathological changes in the area. In a few instances, where the haemorrhage had been less severe, it was possible to trace the bleeding to an area showing micro-embolism and infarction. It was therefore assumed that the larger lesions had originated from involvement of larger vessels in a similar manner, and occasionally this assumption was supported by the finding of an arteriole plugged with amorphous metachromatic material, in the affected area.

In the animals surviving longest in this group, there was evidence of siderotic phagocytic activity in the lung, liver and spleen, and of the usual reactive changes at the margins of areas of necrosis in the liver, spleen and other organs.

The reticulo-endothelial cells of the liver, spleen and bone-marrow were still heavily loaded with

metachromatic material. The accumulation of metachromatic granules in the epithelium of the proximal convoluted tubules in the rat was maximal in this group of animals.

Group 4. Surviving Animals

When these animals were killed occasional small haemorrhages and haematomata were found in similar situations to those seen in the previous group, but these lesions were less severe in extent and fewer in number.

Histological examination showed hyaline material present in vessels attached to the wall and becoming endothelialized in the animals which had survived longest. This was seen most commonly in the pulmonary vessels, but was also found in the vessels of the spleen and kidney. Granular metachromatic material was still present in abundance in the reticulo-endothelial cells of the liver, spleen and marrow.

To summarize, parenteral administration of doses between 2 g./kg. and 100 mg./kg. of the large molecular weight compounds caused death either immediately, by embolism (mainly pulmonary); after some delay, by acute pulmonary oedema; or more remotely, by infarction and haemorrhage, depending upon the level of dosage employed. The toxicity of the E size compound was less marked than that of the D size compound. The doses used in this experiment were vastly in excess of the average effective dose. But surviving animals, given the smaller doses, showed lesser degrees of the process producing fatalities. For this reason, in a second experiment, the range of doses used was designed to cover the possible " therapeutic " range of the less toxic compound.

A group of twenty rats was given the E size dextran sulphate in doses varying between 80 mg./kg. and 5 mg./kg. and the animals were killed at intervals from one hour to 28 days after the injection. At autopsy, animals given doses in excess of 40 mg./kg. showed occasional lesions similar to those seen in the survivors from the previous experiment. Animals given smaller doses showed no apparent macroscopic abnormality.

On histological examination of the tissues from the latter group, diligent search was necessary to secure evidence of embolic phenomena, but it was occasionally found in the animals given the larger doses. All the animals, however, showed evidence of the deposition of metachromatic material in the reticulo-endothelial system. The maximum accumulation of material occurred between three and 24 hours after injection. The amount thereafter steadily diminished, but traces were still discernible at 28 days.

Interpretation of Histo-pathological Findings

The following tentative hypothesis was framed to collate these findings. Interaction of the large molecular weight dextran sulphates with fibrinogen occurs *in vivo* as *in vitro,* leading to the formation of an insoluble particulate precipitate in the blood-stream. Platelets and white-cells agglutinate and are enmeshed in the precipitate. If the process is of sufficient severity, arterioles and capillaries are embolized. When the material is administered intravenously, the main filter-bed is the pulmonary circulation. Abrupt blockage of a major portion of the pulmonary vascular tree produces sudden death attended by cardiac and respiratory embarrassment similar to that seen in human pulmonary embolism. More gradual occlusion of the pulmonary circulation, such as might be expected to occur with smaller doses, or doses absorbed more slowly via the peritoneum, leads to gradually increasing resistance to the output of the right ventricle. This explains the marked dilatation of the right side of the heart, the generalized cyanosis and the marked passive venous congestion of the abdominal organs in these animals.

The mechanism of production of the pulmonary oedema noted in many of these animals needs further elucidation. Pulmonary infarction caused by the blockage of arterioles is not sufficient to explain the intra-alveolar oedema found, not only adjacent to obvious infarcts, but also in areas in which no gross lesion was demonstrable. It is possible that capillary embolism may produce sufficient damage to the endothelial lining to cause increased permeability. The problem is being investigated further.

Doses, insufficient to produce immediate fatalities as outlined above, still produce embolism of sufficient severity to cause widespread infarction and haemorrhage. Whether or not the site of a particular micro-infarct serves also as the site of intractable haemorrhage appears to depend upon the physical characteristics of the tissues involved. Normally it may be supposed that tissue-damage, as the result of infarction, liberates large amounts of thromboplastin which initiate clotting of the effused blood. This, and the tissue-pressure of tightly-packed parenchymatous cells, together prevent the indefinite extension of haemorrhage. In animals given the large molecular weight dextran sulphates, the clotting-mechanism is arrested (due to thrombocytopenia, fibrinogenopenia and the strong antithrombin action of the compounds), but it is nevertheless notable that lesions in, for instance, the liver, remain localized, whereas those in loose mesenchymal tissues such as the mesen-

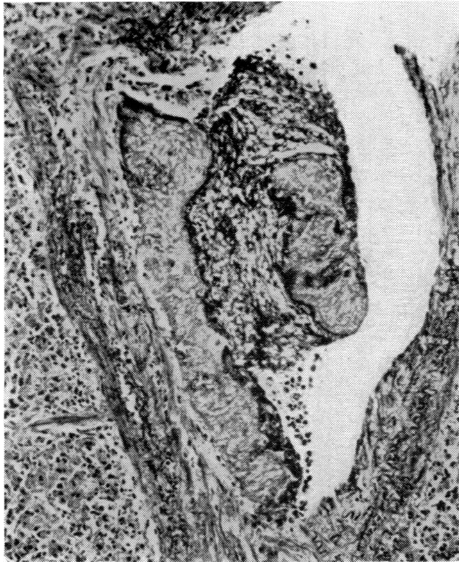

FIG. 8.—" Fibrin " embolism in rat spleen, following a dose of 200 mg./kg. of E size dextran sulphate. The splenic artery contains a laminated mass surrounded by small clumps of leucocytes. The mass stained positively with the " fibrin " stains and was also metachromatic to toluidine blue. Lendrum's acid picro-Mallory method. × 160.

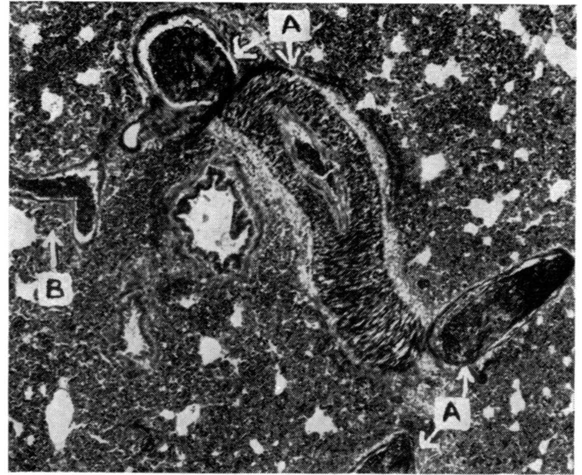

FIG. 9.—Rabbit lung following dose of 1,000 mg./kg. of E size dextran sulphate. A pulmonary artery and its branches, cut obliquely, show " fibrin " embolism. The branches marked " A " contain intensely fuchsinophil material. The branches marked " B " contain leucocytes apparently clumped to form the tail of the main embolus. Lendrum's acid picro-Mallory method. × 60.

tery, omentum and retroperitoneal tissues are characterized by massive haemorrhage.

Embolism of still less extent results in non-fatal infarction and haemorrhage. The production of minimal intravascular precipitation allows clearing of the circulation by the phagocytic activity of both fixed and free cells of the reticulo-endothelial system, and accounts for the apparently selective storage of the material in the reticulo-endothelial tissues of the liver, spleen and bone-marrow. Deposition of metachromatic material in the reticulo-endothelial cells would thus constitute the earliest indication of " toxicity " in the sense of indicating that higher dosage of a compound producing this effect might be expected to produce a bleeding tendency by the mechanism outlined.

It is necessary to point out that this interpretation is at variance with that of certain other authors. For instance, Asplund, Borell, and Holmgren (1939) reported the accumulation of metachromatic material in the reticulo-endothelial tissues of rats, rabbits, and guinea-pigs given large doses of heparin, and regarded this as the normal method of disposal of the substance. The heparin used was prepared from beef liver by the authors themselves. No details of the purification of the material nor of the chemical or physical characteristics of their final product were given. Judging from its activity, it would appear to have been a fairly crude preparation which may well have contained a substantial amount of chondroitin sulphate, which Jorpes and Gardell (1948) have shown to be a common contaminant of heparin. Piper (1945b) showed that chondroitin sulphate formed insoluble complexes with fibrinogen, and is stored in reticulo-endothelial cells in which a strong metachromatic reaction is consequently demonstrable. Personal attempts to reproduce the results of Asplund *et al.*, using commercial heparin (prepared from beef *lung*) obtained from five different sources, have been uniformly unsuccessful. Single injections of up to 3 g./kg. and thrice daily injections of 20 mg./kg. given to rats did not result in the deposition of any observable metachromatic material in the reticulo-endothelial cells of the liver, spleen or bone-marrow, though metachromatic granules were seen in the proximal convoluted tubular epithelium in this species.

Further investigation of the nature of the material deposited within reticulo-endothelial cells and upon the surface of the vascular endothelium provided support for the present hypothesis and information concerning the disposal of the material. When serial sections taken from various organs of animals given the D and E size compounds were stained alternately with toluidine blue and one or other of the various " fibrin " stains, it was found that the intravascular material not only gave a metachromatic reaction with toluidine blue, azure A or thionin, but was also Gram-positive, phloxinophilic with the phloxine-tartrazine method, fuchsinophilic with the acid

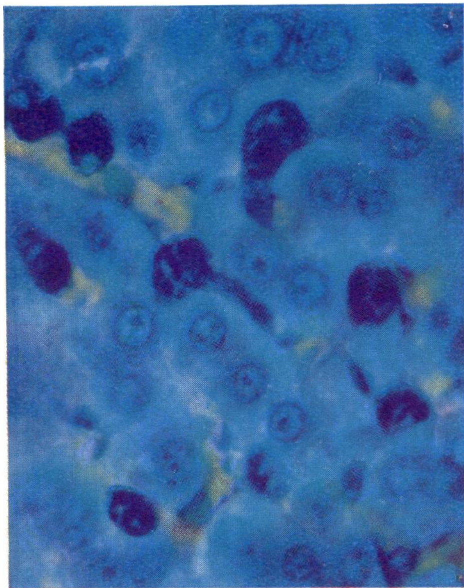

FIG. 10.—Liver, showing Kupffer cells loaded with granules of metachromatic material. Rat given 100 mg./kg. E size dextran sulphate. Toluidine blue. × 800. Kodak Ektachrome.

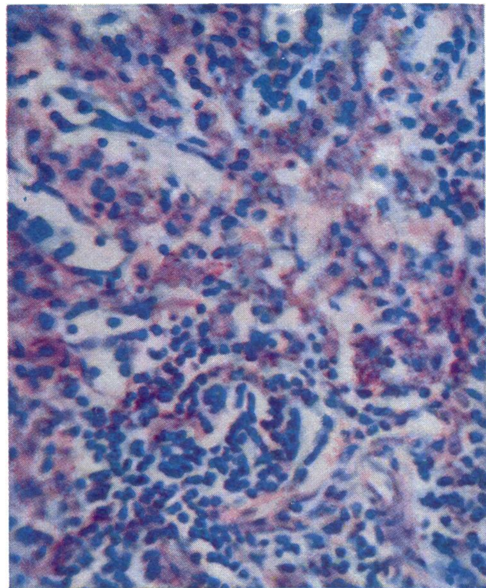

FIG. 11.—Spleen. Vacuolated phagocytes lining the pulp-sinuses contain metachromatic material. Rat given 100 mg./kg. E size dextran sulphate. Toluidine blue. × 700. Kodak Ektachrome.

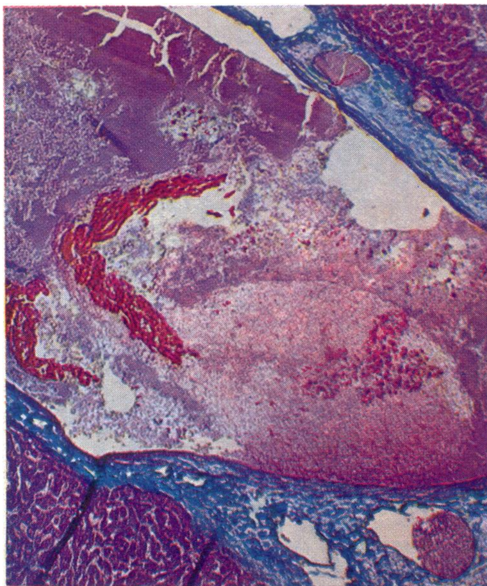

FIG. 12.—Rabbit liver following dose of 1,000 mg./kg. of E size dextran sulphate. A large portal vein contains a " fibrin " embolus consisting of a condensed, coarsely fibrillar, intensely fuchsinophil portion continuous with a more delicate network of fuchsinophil material enmeshing leucocytes. Lendrum's acid picro-Mallory method. × 1770. Kodak Ektachrome.

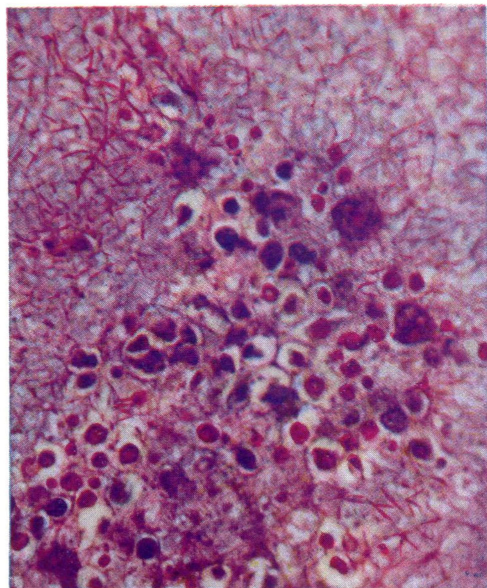

FIG. 13.—High power view of portion of section shown in Fig. 12, showing the nature of the open network enclosing leucocytes and platelets. Lendrum's acid picro-Mallory method. × 320.

picro-Mallory method and stained dark blue with phosphotungstic acid haematoxylin—that is, gave the staining reactions of fibrin or fibrinogen (Figs. 8 and 9). Occasionally, in large vessels which were not completely occluded, the intravascular mass could be seen to consist of a coarsely fibrillar portion continuous with a more delicate fibrillary network containing, enmeshed within it, clumps of leucocytes and faintly-staining granular material presumed to be the debris of disintegrated platelets (Figs. 12 and 13). In animals surviving for a week or longer, the intravascular material was seen to become endothelialized and to undergo organization in a manner closely similar to that which occurs in a thrombus. In the later stages the appearance was very like that of an atheromatous deposit.

The granular material within the reticulo-endothelial cells also gave both a metachromatic reaction and a positive staining reaction for fibrin or fibrinogen, if the animal had been killed within $\frac{1}{2}$–4 hours of injection. But in animals allowed to survive for 24 hours or longer the material no longer gave a positive staining reaction for the protein, although, as already indicated, a metachromatic reaction with toluidine blue persisted for 28 days or longer. An experiment kindly performed by Mr. Henry Finck, under the supervision of Professor Isadore Gersh, in the Department of Anatomy of the University of Chicago, indicated that this result was probably not due to diffusion of the protein component owing to the action of the solvents, etc., used during the fixation and embedding processes.

A rat was injected intraperitoneally with a dose of 10 mg./kg. of the D size dextran sulphate. Twenty-four hours later the rat was killed and portions of the liver and spleen prepared by freezing and drying. Paraffin sections were made. Material was visible in many tissue phagocytes, in some of them as granular accumulations. Examination under the ultra-violet microscope and measurement of the absorption at a wave-length of 2,800 Å gave no indication of the presence of protein in the phagocytozed material. According to Gersh (personal communication) this should not be taken to mean that no protein was present, but simply that not enough was present to be detectable by this procedure.

The reticulo-endothelial cells are known to contain active proteolytic enzymes and the dextran sulphate-fibrinogen complex has previously been shown to be easily dissociable, even *in vitro* (Walton, 1952). The above results were therefore interpreted as indicating that the protein moiety of the phagocytozed material was split off and digested quickly, leaving the charged polysaccharide component to undergo much slower catabolism. This is in keeping with the findings of Kaplan, Coons, and Deane (1950) and of Coons, Leduc, and Kaplan (1951), who showed that even heterologous and foreign proteins disappear considerably more quickly from reticulo-endothelial cells than similarly phagocytozed polysaccharides.

The material in the cells of the proximal convoluted tubules of the kidneys of rats treated with the D and E size dextran sulphates gave the staining reactions of the sulphated polysaccharide and of protein but not of fibrinogen. The significance of this observation will be discussed elsewhere.

Relation Between Molecular Weight and Toxicity

These histological findings were taken to confirm the theory that the apparently different toxic manifestations seen at different dosage levels all derived ultimately from the formation of insoluble complexes between these compounds and fibrinogen. From this it followed that one might expect lowered toxicity as the molecular weight of the compound decreased and the tendency to precipitate fibrinogen lessened. It will be seen that this has already been demonstrated, since it was shown that larger doses of the E size compound were necessary to produce changes equal in severity to those evoked by any given dose of the D size compound.

It also followed that " contamination " by large molecules of a sample of dextran sulphate of low *average* molecular weight might be expected to add appreciably to its toxicity. This has now been shown to be so (Ricketts and Walton, 1953). For this reason, the sample of the I size dextran sulphate used for the quantitative and qualitative comparisons described below was carefully refractionated to ensure the maximum possible homogeneity of molecular size.

The relation between molecular weight and toxicity was given rough numerical expression by assessment of the LD50's of the dextran sulphate in relation to heparin. The results derived from probit analyses of the survival of groups of mice during the seven days following intravenous injection are shown in Table I. 240 mice were used, in groups of 60 for each compound and of 10 for each level of dosage.

Grönwall *et al.* (1945) had previously demonstrated decrease of toxicity with decrease of the molecular weight of the dextran sulphates. Direct comparison with their results is not possible, since they expressed the toxicity of their compounds in terms of an undefined " lethal dose to rabbits." Nevertheless, in general terms, the present observations seem to agree with theirs.

TABLE I

COMPARISON OF THE TOXICITY OF INTRAVENOUS HEPARIN AND DEXTRAN SULPHATES IN MICE

Compound	Average Molecular Weight	LD50* (g./kg.)	Fiducial Limits of LD50	Slope of Curve †	Standard Error of Slope
D size	458,000	0·154	0·19–0·12	5·1834	1·206
E size	47,000	0·573	0·64–0·52	8·3083	2·416
I size	7,500	2·120	2·72–1·66	3·6396	0·862
Heparin	? 17,000	2·030	2·70–1·53	3·7488	0·833

* Derived from probit log. dose relationship which was approximately linear.

† Comparison of the significance of the differences between slopes (at 5% level), taken in pairs, showed:—

I size/Heparin: t = 0·1; p = 0·9
D size/I size: t = 1·04; p = 0·3–0·2
D size/E size: t = 1·16; p = 0·3
E size/I size: t = 1·82; p = 0·1–0·05

The differences were therefore not regarded as statistically significant.

Although the LD50 of the I size dextran sulphate was roughly equal, weight for weight, to that of the heparin sample used, this was not taken to imply equal toxicity of the two compounds. The lower anticoagulant potency of this dextran sulphate necessitates the use of a larger absolute weight of material to obtain a quantitatively equal effect on the clotting-time. Its toxicity might therefore be regarded as correspondingly greater than that of heparin. But there are certain objections to accepting this method as a satisfactory means of measuring the toxicity of these compounds.

Although a quantal response test was employed here for comparative purposes, it is clear that this technique was not an informative guide to the true toxicity of the E and D size dextran sulphates. Even at dosage levels between one-twentieth and one-fiftieth of their respective LD50's, these compounds could be shown to set in train some or all of the pathological effects described above. Nor was the test satisfactory as a means of assessing the toxicity of the small molecular weight dextran sulphate relative to that of heparin. The deaths produced by doses of 2 g./kg. or more of heparin, or the I size dextran sulphate, all resulted from haemorrhage. No evidence was obtained that this was initiated by embolism or infarction (see below). Animals given doses in excess of 1 g./kg. of either compound developed subcutaneous and occasional intramuscular haematomata at points where they had been subjected to comparatively minor traumata. Subcutaneous, intramuscular, and intraperitoneal injections were often attended by the formation of haematomata around the needle track, and these were sometimes sufficient to produce exsanguination.

In some instances haemorrhage occurred in animals given very large doses of heparin or the I size dextran sulphate without any obvious externally applied injury. It is generally agreed that, under normal conditions, many breaches of continuity of the vascular endothelium occur as the result of "wear and tear," and that these are quickly sealed by the ordinary haemostatic mechanism. The interference with this mechanism by enormous doses of any anticoagulant may result in prolonged oozing from minute injuries. Jaques, Charles, and Best (1938) pointed out that in small laboratory animals, such as the rat and the mouse, such lesions are poorly tolerated since, in relation to the small total blood volume of these animals, a seemingly small blood-loss may be sufficient to produce profound haemodynamic effects. At very high levels of dosage of these compounds the mortality rate in mice is probably, therefore, an index of the incidence of chance encounters with trivial injury rather than a measure of the "toxicity," in the accepted sense, of the compounds.

Single intravenous doses in excess of 25 mg./kg. of heparin are rarely, if ever, administered to man, in whom the therapeutically effective dose is about 1–2 mg./kg. (Jorpes, 1946; Duff, Linman, and Birch, 1951). The effective dose of the I size dextran was found to be 6–8 mg./kg., so the main efforts were directed to assessing its toxicity at doses between this level and 200 mg./kg., on the assumption that this represented an ample therapeutic range.

Investigation of the Small Molecular Weight Dextran Sulphate

It has already been shown that this compound did not affect the stability of plasma fibrinogen *in vitro* and that no significant alteration of the levels of the formed elements of the blood or of the plasma fibrinogen occurred *in vivo* (see above). When doses between 5 mg./kg. and 200 mg./kg. were administered intravenously to mice and rats, no observable alteration of physiological function occurred, other than an increase in the clotting-time. Doses between 20 mg./kg. and 1,000 mg./kg. were equally well tolerated by rabbits. Doses between 20 mg./kg. and 80 mg./kg., given intravenously to an anaesthetized dog by Dr. J. A. Barclay, caused no alteration of blood-pressure, pulse-rate or respiratory rate. Doses between 40 mg./kg. and 80 mg./kg., similarly administered to two baboons by Dr. P. L. Krohn, produced no untoward effects. Histological examination of tissues from these animals did not show any storage of metachromatic material in reticulo-endothelial tissues.

Examination of sections taken from the liver, spleen, kidneys, skeletal and cardiac muscle, skin, bone-marrow, lymph-nodes, thyroid, adrenals, pancreas, gonads and brain of mice, rats and rabbits treated with the small molecular dextran sulphate showed no evidence of parenchymal damage, though this was looked for after single injections, repeated injections and over periods

TABLE II

EFFECT OF FOUR DAILY INTRAVENOUS INJECTIONS OF 10 MG./KG. OF I SIZE DEXTRAN SULPHATE IN RABBITS

	Platelets per c.mm.	Leucocytes per c.mm.	Haemoglobin %	Fibrinogen g./100 ml.	Urine		Coagulation-time*	
					Albumen	Deposit	Maximal	Minimal
Before injections	320,000	9,800	110	0·34	Nil	Nil	3 min. 10 sec.	2 min. 5 sec.
1st day ..	362,000	10,600	110	0·36	Nil	Nil	75 min.	3 min. 50 sec.
3rd day ..	334,000	8,400	109	0·32	Nil	Nil	90 min.	4 min. 15 sec.
5th day ..	376,000	10,200	112	0·35	Nil	Nil	82 min.	4 min.

* Coagulation-times performed by Dale and Laidlaw's method at 37° C.
" Maximal " clotting-times taken 5 minutes after injection.
" Minimal " clotting-times taken immediately before next injection.

from one hour to six months after the last injection. These results are illustrated by the following typical experiment, designed to simulate a short therapeutic course of injections:

Three rabbits were given four-hourly injections during the daylight hours (four injections daily) of 10 mg./kg. for five days. Before the injections were started the following base-line observations were made—estimation of fibrinogen and total plasma proteins, platelet and white-cell counts, haemoglobin estimation, clotting-time, examination of urine for albumen, and of the centrifuged deposit microscopically for red-cells, casts, etc. These estimations were repeated at intervals throughout the experiment with the results shown in Table II. During the course of the experiment the animals showed no untoward reaction to the injections, and at its conclusion they were alive and well. One animal was killed then, and the remainder were killed at three-week intervals subsequently. Post-mortem examinations showed no macroscopic abnormalities. Examination of sections taken from the liver, spleen, lungs, heart, kidneys, endocrines, and brain showed no histological abnormalities.

Attempts to sensitize guinea-pigs to the I size dextran sulphate were unsuccessful:

Twelve adult male guinea-pigs weighing between 450 g. and 600 g. were injected daily with 0.5 ml. of a 2% solution of an I size dextran sulphate, intraperitoneally, for six days. A fortnight was allowed to elapse and then each animal was given 0.2 ml. of the same solution intravenously. None of the animals showed any evidence of an anaphylactic reaction. Intracutaneous injection of the same material did not evoke whealing.

On the other hand, the parenteral administration of the D and E size compounds or of unusually polydisperse samples of low average molecular weight to guinea-pigs was attended by a transitory anaphylactoid reaction. The further investigation of this phenomenon and its possible practical significance has been reported elsewhere (Walton and Ricketts, 1954).

DISCUSSION

The toxicity of chondroitin sulphate and of synthetic sulphuric esters of chitin, cellulose, starch, xylan, and alginic acid has been investigated by Astrup, Piper, and their colleagues (Astrup, Galsmar, and Volkert, 1944 ; Astrup and Galsmar, 1944 ; Astrup and Piper, 1945, 1946 ; Piper, 1945a, 1945b, 1946a, 1946b, 1947 ; Barsøe and Selso, 1946 ; and Astrup, 1953) with results in substantial agreement with the present findings. All these compounds were found to agglutinate the platelets and some of them, in high dosage, were reported to produce embolic phenomena and haemorrhages in experimental animals. The detailed mechanism of production of such changes is suggested by the present work on dextran sulphates of varying molecular size. In agreement with Astrup (1953) the toxicity of polysaccharide sulphates is held to be dependent ultimately upon the aggregation of blood-cells which some of them produce. With the dextran sulphates, this process has been shown to be dependent upon insoluble complex formation with the plasma proteins, since none of the dextran sulphates produced any effect upon platelets or other suspended particles in the absence of protein from the suspending medium, and since homogeneous samples of small molecular weight, which failed to precipitate fibrinogen, produced no effect upon the blood cells even in whole plasma (Walton, 1953).

In vivo, the large molecular weight dextran sulphates have been shown to cause thrombocytopenia, leucopenia, succeeded by leucocytosis and fibrinogenopenia. With increasing dosage, the formation of intravascular deposits, consisting of agglutinated platelets, leucocytes and fibrin has been demonstrated. Since these deposits occurred in spite of virtually complete arrest of the normal clotting mechanism, they were assumed to arise from the same process of non-specific complex formation with the plasma proteins which had previously been reported to occur *in vitro* (Walton, 1952, 1953). This formation of apparent thrombi or emboli by a mechanism independent of the ordinary clotting-process is an unfamiliar concept in pathology and is apparently paradoxical behaviour for material showing anticoagulant acti-

vity. Whether this has any relevance to the general problem of thrombosis and embolism is conjectural. There is, however, a considerable body of evidence to indicate that such behaviour is not confined to sulphated polysaccharides but occurs even with uncharged polysaccharides and other macromolecules.

It has been shown by Ricketts (1952b) that neutral dextrans, if of sufficiently large molecular size, form insoluble complexes *in vitro* with fibrinogen in plasma and in pure solution. Fletcher, Martin, and Ratcliffe (1952) have shown that polyvinylpyrrolidone, acacia and laminarin also interact with fibrinogen in a similar manner.

Staub, Mezy, and Golondas (1938) described the occurrence of leucopenia and agglutination of leucocytes in rabbits given glycogen and gum acacia. This was confirmed and extended by Bucher (1939), who observed that platelets were also affected. He noted that rabbit liver glycogen was less effective in producing these changes than glycogen derived from pig's liver, speculating on whether these differences could be correlated with the known differences in molecular weight of the preparations.

A thorough investigation of the pathogenicity of macromolecular substances was carried out by Hueper (1942, 1943). He found that gum arabic and gum acacia, pectin, methyl cellulose and polyvinyl alcohol, glycogen, gelatin and ovalbumin all produced what he called the " macromolecular haematic syndrome." This comprised (1) primary transitory leucopenia, (2) secondary myeloid leucocytosis, (3) simple anaemia, (4) an accelerated erythrocyte sedimentation rate, and (5) in some instances, an increased coagulation time. Intravascular deposition of these materials was demonstrable, in many cases, as emboli occluding pulmonary, renal and other vessels in the acute lesions and as endothelialized plaques, resembling atheromatous deposits, in the chronic ones. The non-proteinous compounds were demonstrable in reticulo-endothelial cells in the liver, spleen and bone-marrow. The close resemblance between these and the present findings needs no stressing.

Miscellaneous macromolecular colloids have been used to provoke the so-called " generalized Schwartzman reaction " by Thomas and Good (1952), Good and Thomas (1952), and Thomas, Denny, and Floyd (1953). These materials included glycogen, acacia and bacterial polysaccharides, and they produced clumping of the platelets and leucocytes with the arrest of the clumps in the capillary beds of internal organs. The process was attended by acute circulatory collapse with widespread embolism and infarction. The eosinophilic material producing the lesions gave the staining reaction of " fibrinoid " and was thought to be mucopolysaccharide in nature. From these authors' published descriptions and illustrations, the lesions of the generalized Schwartzman reaction appear to resemble closely those obtained with the large molecular weight dextran sulphates.

This " family resemblance " between the lesions produced by widely differing macromolecular materials leads one to conclude that the toxic manifestations produced by some of them may be closely related, in mechanism of production, to that suggested for the large molecular weight dextran sulphates. These last, and other toxic polysaccharide sulphates, probably only differ from other macromolecular materials in that their additional antithrombin action also arrests the normal clotting mechanism, thereby adding the further hazard of haemorrhage to an organism already imperilled by embolism and infarction.

The present finding, that reduction of the molecular size of a sulphated polysaccharide reduces or abolishes the train of pathological events produced by its analogues of greater molecular weight, is probably of importance in the design of heparin-substitutes. It should be stressed that although it has proved possible to produce a substitute for heparin of low toxicity and qualitatively similar clinical efficacy (Ricketts, Walton, *et al.*, 1953), it is not claimed that this is the only or the best starting material which can be employed. The physical characteristics distinguishing a dextran sulphate of low toxicity are probably not transferable directly to other materials. However, since the few crude unfractioned polysaccharide sulphates (prepared from other starting materials) which have been personally examined have shown undesirable properties similar to those of the large molecular weight dextran sulphates, it seems necessary to urge again the advisability of biological screening and of definition of the physical characteristics of other compounds of this kind proposed for therapeutic use.

Summary

1. The toxicity of sulphuric esters prepared from three dextrans of widely differing molecular weight is compared with that of commercial heparin.

2. The dextrans of large molecular weight—average above 40,000—are more toxic than heparin. The toxicity increases with increase of molecular weight.

3. The toxicity is considered to depend on the effects of these compounds upon the plasma-proteins and the formed elements of the blood.

4. At low dosages, the large molecular weight dextran sulphates form insoluble complexes with fibrinogen. The particles of this complex are removed from the circulation by the phagocytic activity of reticulo-endothelial cells without evidence of serious harm to the organism in acute experiments.

5. At higher dosages, agglutinates of the formed elements of the blood are demonstrable as conglutination thrombi or emboli in blood-vessels. As a result, infarcts of micro- or macroscopic dimensions can be demonstrated in various organs. Simultaneous interference with blood-clotting by the antithrombin action of these compounds possibly lessens, but does not prevent, such changes, and sometimes leads to intractable haemorrhage from the sites of infarction. Still greater dosage produces a rapidly fatal termination from embolism itself.

6. Attention is drawn to the behaviour of other macromolecular materials which have been reported to produce a sequence of events similar to that described above, suggesting a common mechanism of action.

7. On the other hand, the small molecular weight dextran sulphates—compounds with an average molecular weight of about 7,500—show qualitatively similar anticoagulant action to that of heparin and comparable low toxicity.

8. These compounds do not affect the plasma proteins or the formed elements of the blood and produce no evidence of embolism in experimental animals. As with heparin, massive doses are required to produce death from spontaneous bleeding. They therefore appear likely to be equally safe for therapeutic use as anticoagulants.

It is a pleasure once more to acknowledge the advice, assistance, and collaboration of my colleague, Dr. C. R. Ricketts, in various aspects of this investigation, and to thank Professor J. R. Squire for his encouragement, his helpful criticisms as the work progressed, and for his assistance with the manuscript. I am grateful to Professor G. R. Cameron, F.R.S., for his opinions upon some of the pathological problems which arose and for his general interest in this project. Thanks are due to Drs. J. A. Barclay and P. L. Krohn for kindly consenting to use dextran sulphate in the course of their own experimental work on dogs and baboons respectively. I am indebted to Professor I. Gersh and Mr. H. Finck for preparing and examining freeze-dried preparations of tissues from animals given dextran sulphate. A sample of Heparin "Leo" was kindly supplied by Mr. E. G. Tomich, of Glaxo Laboratories, Ltd., a sample of Heparin (Lederle) by Professor M. Stacey, F.R.S., and a sample of "Paritol" by Mr. T. B. Wallace, of Smith, Kline, and French, Inc. The dextran from which the preparations examined were made was a gift from Dextran, Ltd. The photomicrographs were the work of Mr. F. Bradley. I am grateful to Miss A. Fisher for the probit analyses and statistical calculation of the LD50's of the various compounds.

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