Preparation of Dextran [^{35}S]Sulphate and Tracer Experiments in the Rabbit

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The factors influencing the blood anticoagulant activity and toxicity of dextran sulphate have been described in a series of papers (Ricketts, 1952; Ricketts & Walton, 1953*a, b*) and clinical trial of a suitable form of dextran sulphate as an anti-coagulant drug has been reported (Ricketts *et al.* 1953). The fate of this intravenously injected polysaccharide sulphate in the body is therefore of some interest. Histological staining with the dye toluidine blue shows that the larger molecules of dextran sulphate are taken up into the reticulo-endothelial cells of various tissues from which, judging by the diminishing intensity of staining, they slowly disappear. Using this method it has not been possible to show uptake of the smaller molecules and their fate has hitherto been unknown (Walton, 1954).

There are two reports of the use of the radioactive isotope ^{35}S to trace the fate of intravenously injected polysaccharide sulphates. Morrow *et al.* (1952) prepared cellulose [^{35}S]sulphate by reaction of [^{35}S]sulphuric acid with cellulose. The product contained 5–6% S and had an intrinsic viscosity of 1.5–2. Six hours after intravenous injection into cats the radioactivity of various tissues and fluids was measured. On this basis the distribution of this preparation of cellulose sulphate was urine 20–25%, blood 3.6–6.9% and liver 7.2%. Xylan [^{35}S]sulphate was prepared by Husemann, Hoffman, Lotterle & Widersheim (1952) by treatment of xylan with chloro[^{35}S]sulphonic acid and pyridine. The sulphur content of their xylan sulphates is not quoted but was probably sufficient to confer anti-coagulant activity, since this aspect was of interest to the investigators. The fate in rabbits of three preparations of xylan [^{35}S]sulphate with molecular chain lengths averaging 45, 12 and 4 xylose units was deduced from the radioactivity of tissue homogenates. The longer the chain length, the more was stored in tissues, notably liver, and the less was excreted in urine as their figures show:

Average chain length	Percentage of dose found in	
	Liver	Urine
45	21	26
12	9	27
4	0.6	66

It seemed to us that preparation of dextran [^{35}S]sulphate having a molecular-weight distribution similar to that advocated for a clinical anticoagulant would facilitate experiments settling several important features of the action of heparin-like anti-coagulants. The concentration of dextran sulphate in the blood could be correlated with the clotting time. The rate of renal excretion and nature of the material excreted could be studied. The role of renal excretion in terminating the effect of a single injection could be ascertained.

This paper describes the preparation of dextran sulphate of appropriate molecular composition labelled with ^{35}S and some experiments on the fate of this material in the rabbit, in particular the nature and amount of the excretion product. The physiological aspects will be considered in a subsequent paper.

METHODS

Intrinsic viscosity and sulphur content were determined as previously described (Ricketts, 1952).

Determination of dextran sulphate by metachromatic reaction

Dextran sulphate in urine samples was determined through its metachromatic reaction with the dye toluidine blue using the method devised by MacIntosh (1941) for determination of heparin. The metachromatic reaction appears to be the formation of an insoluble purple salt with one or two components of the dye. The principle of MacIntosh's method is that the amount of dye removed from solution is proportional to the amount of a polysaccharide sulphate added within experimentally definable limits which are constant for a given batch of dye. Ricketts & Walton (1954) found that the amount of dye precipitated is proportional to the sulphur content of the dextran sulphate over the range 2–20% and independent of molecular size for dextran sulphate molecules composed of more than four glucose units. In the determinations reported here a Unicam SP600 spectrophotometer was used to determine the amount of toluidine blue remaining in solution, measurements being made at the absorption maximum of 625 m μ . The method was calibrated with a dextran sulphate containing 17.1% S and where the sulphur content of the dextran sulphate being determined differed from this the small proportional correction was introduced. Fractionation experiments (Ricketts & Walton, 1953*a*) show that

laboratory-made dextran sulphates are nearly homogeneous in the distribution of sulphate groups among the molecules.

Though normal rabbit urine often contains metachromatic substances, none was present in samples obtained after the commencement of saline infusion but before injection of dextran sulphate. The samples were also free from protein which was found to interfere with the determination.

Determination of dextran sulphate by radioactivity

In determining dextran sulphate by its radioactivity the technique employed by Rowley, Rowlands & Stuart (1948) for [^{35}S]penicillin was followed. Portions (0.1 ml.) of suitably diluted solutions were dried at 120° on Ni planchets, allowed to cool in a desiccator and counted using a thin end-window Geiger-Müller tube (type EHM2). It was necessary to correct for the absorption of radiation by other solutes. This absorption decreases with dilution. Dilution is limited by the corresponding reduction in counting rate. A dilution of 1 in 4 with distilled water was found to give a satisfactorily low correction factor without unduly prolonging the counting period.

Paper chromatography of dextran sulphate

Ascending chromatography with 25% (v/v) *n*-propanol in $\text{M}/15$ sodium phosphate buffer (pH 6) and Whatman no. 1 paper, as used by Kerby (1953) for heparin, was found to be useful for sodium dextran sulphate. Reproducibility was improved by equilibration of the paper with the vapour phase for an hour before commencing development of the chromatogram. After development and drying, chromatograms were immersed in a nearly saturated solution of toluidine blue in ethanol for 5 min., then transferred to 40% (v/v) ethanol for a further 5 min. The aqueous ethanol allows the metachromatic reaction to take place without dissolving the dextran sulphate off the paper. Chromatograms were dried by immersion in ethanol and subsequently in warm air. Spots appear purple on a pale blue background. A series of preparations of similar sulphur content (17–19% S) was available from previous work, and it was quickly established that molecular size was the feature determining the R_f value of such preparations. The mechanism of this chromatography is not fully understood, but it seems probable that the same factors are involved as in fractional precipitation of dextran sulphate from aqueous ethanol solution (Ricketts & Walton, 1953*a*). The mobile phase of the chromatogram is analogous to the supernatant solution in fractional precipitation.

Preparation of dextran [^{35}S]sulphate

The molecular-weight distribution advocated for a clinical anticoagulant may be obtained either by fractionation of the dextran or by fractionation of the dextran sulphate itself. The latter procedure would be wasteful of ^{35}S . The dextran fractionation described in a previous paper (Ricketts, 1952) was therefore modified to remove a little more of the high-molecular material.

Partial hydrolysis and fractionation of dextran. To 1 l. of a 6% (w/v) solution of a partial hydrolysate of *Leuconostoc mesenteroides* dextran with intrinsic viscosity 0.32, 100 ml. $\text{N-H}_2\text{SO}_4$ were added and the solution was boiled under reflux for 4.5 hr. After cooling, the solution was neutralized with NaOH and the volume was made up to 1200 ml. Acetone (1032 ml.) was added with thorough stirring, and

after standing overnight at 21° the syrupy ppt. containing the larger molecules was separated (1.55 g. dextran). The supernatant solution was then cooled at 6° overnight and again the syrupy ppt. was separated and rejected (3.85 g. dextran). More acetone (1968 ml.) was added, the syrup separated at 21°, poured into ethanol, ground to a powder and dried *in vacuo* over P_2O_5 , yielding 21 g. dextran of intrinsic viscosity 0.033.

Trial sulphation and tests of molecular composition. A trial sulphation in the same apparatus and on the same scale (0.53 g. dextran) as described below for the radioactive preparation gave a satisfactory yield (91%) of sodium dextran sulphate (1.0 g.) containing 16.4% S. This material was an effective anticoagulant *in vivo*, since an intravenous injection of 10 mg./kg. into a rabbit produced a satisfactory elevation of blood clotting time, i.e. the clotting time was elevated to 3 times the normal value 1 hr. after the injection of this dose. When injected intravenously into two guinea pigs no anaphylactoid response occurred (Ricketts & Walton, 1953*b*). No precipitation of fibrinogen occurred when tested according to Walton (1952). It was therefore assumed that this dextran was of appropriate molecular composition for conversion into radioactive dextran sulphate. A larger scale preparation of dextran sulphate (S) from this same dextran was carried out to provide material for control experiments; it contained 17.1% S and had intrinsic viscosity 0.027.

Sulphation with chloro[^{35}S]sulphonic acid. Chlorosulphonic acid (0.7 ml.) was added to pyridine (7.2 ml.) with cooling by solid CO_2 in ethanol, followed by chloro[^{35}S]sulphonic acid (0.06 ml.) containing 1 mc of ^{35}S which was washed with more chlorosulphonic acid (0.4 ml.). The temperature was raised to 60° when all the pyridinium salts dissolved. The above dextran (0.53 g.) was added and stirring was continued at 70–80° for 4 hr. After cooling, water (28 ml.) was added, followed by NaOH (40% w/v) until pyridine separated. To the aqueous phase, ethanol (30 ml.) was added, precipitating sodium dextran sulphate as a syrup together with some sodium sulphate. The syrup was dissolved and dialysed (72 hr.) until the dialysate gave no ppt. with BaCl_2 . A sample of the final dialysate was set aside for counting and volumes were measured to enable contamination of the product with $^{35}\text{SO}_4^{2-}$ to be estimated. The solution (170 ml.) was neutralized, concentrated under reduced pressure and freeze-dried in 8 ampoules giving a total yield of 1.09 g., containing 18.0% S. On this basis 86% of the product had been recovered containing 36% of the available isotope. Under standardized conditions, 20 μg . gave 1567.0 counts/min., or 1 μg . 78.35 counts/min. A sample (0.1 ml.) of the final dialysate gave a count of 57.1/min. and from this it was estimated that 0.114% of the counts was due to contamination of the product with $^{35}\text{SO}_4^{2-}$. The background count was 11/min.

Tracer experiments in rabbits

The purpose of these experiments was to find out how much dextran sulphate was excreted and whether the excreted material was identical with that injected. Dextran [^{35}S]sulphate was injected intravenously in doses of 20 and 40 mg./kg. into rabbits during continuous infusion of physiological saline in experiments of the type described in detail by Piper (1947) for heparin. In these experiments urine was collected for six successive 10 min. periods. Dextran sulphate in the urine samples was estimated by the

metachromatic method of MacIntosh (1941) and from the radioactivity as described. After the experiment the rabbits were placed in metabolism cages and urine was collected for 2 or 4 days in an attempt to estimate the total excretion of dextran sulphate.

RESULTS

The results of analysing the urine samples for dextran sulphate are shown in Table 1. The amount excreted in successive 10 min. periods decreases and it was found that the logarithm of the weight of dextran sulphate in each period was linearly related to the time after injection as shown in Fig. 1.

The nature of the dextran sulphate in urine was investigated by paper chromatography. Fig. 2 shows a chromatogram comparing dextran sulphate excreted in urine with dextran sulphate (*S*) identical in molecular composition with that injected. The 'trailing' appearance of spot *S* is believed to indicate the dispersion of molecular weight in this compound, the components of lower *R_F* value being those of higher molecular weight. The dextran sulphate in urine was characterized by the absence of components with low *R_F* values as compared with the control. Fig. 3 shows another chromatogram comparing dextran sulphate in urine with control preparations. The control preparation (*OC*) having the same chromatographic behaviour as the dextran sulphate of urine is a mixture of the sulphuric esters of penta- and hexa-saccharides derived from dextran (Ricketts, 1954). Both this control preparation and the dextran sulphate in

urine diffused through cellophan membranes. A dialysate of one of the urine samples is included in this chromatogram (Fig. 3). These and similar experiments on the other urine samples showed that the dextran sulphate in the urine up to 1 hr. after injection was not identical with the material injected but appeared to be a fraction of low molecular weight derived from it.

Fig. 1. Renal excretion of dextran [³⁵S]sulphate by rabbits at two dose levels. Dose 40 mg./kg., determinations made by radioactivity (▲—▲) and by metachromatic reaction (●—●). Dose 20 mg./kg., determinations made by radioactivity (△—△) and by metachromatic reaction (○—○). The logarithm of the corresponding weight of dextran sulphate is plotted against time after injection.

Table 1. Analysis of rabbit urine samples for dextran sulphate
Figures in brackets are percentages of dose.

Sample no.	Volume (ml.)	Dextran sulphate (mg.)	
		Metachromatic estimation	Radioactivity estimation
Rabbit weighing 3.2 kg. given 64 mg. of dextran [³⁵ S]sulphate, i.e. 20 mg./kg.			
1	6.0	4.50	5.70
2	4.8	3.49	4.19
3	5.9	2.01	2.97
4	5.2	1.05	1.79
5	4.7	0.79	1.37
6	5.9	0.35	1.17
Total at 1 hr.	—	12.19 (19.0%)	17.39 (27.1%)
Subsequent 48 hr.	162	7.68	5.05
Total at 49 hr.	—	19.87 (31.0%)	22.44 (35.0%)
Calc. total	—	12.50 (19.5%)	19.50 (30.3%)
Rabbit weighing 3.4 kg. given 136 mg. of dextran [³⁵ S]sulphate, i.e. 40 mg./kg.			
1	7.2	26.0	29.52
2	5.0	10.95	11.03
3	9.0	8.65	10.75
4	8.6	4.73	6.27
5	8.4	2.78	4.76
6	6.0	1.08	3.03
Total at 1 hr.	—	54.19 (39.9%)	65.36 (48.0%)
Subsequent 96 hr.	—	12.10	7.20
Total at 97 hr.	—	66.29 (48.7%)	72.56 (53.3%)
Calc. total	—	58.50 (43.0%)	65.5 (48.2%)

Fig. 2. Paper chromatogram comparing dextran sulphate in urine samples at 10, 20, 50 and 60 min. after injection with a dextran sulphate (*S*) identical in molecular composition with the injected material. Ascending chromatography with 25% (v/v) *n*-propanol in *m*/15 sodium phosphate buffer (pH 6) and Whatman no. 1 paper.

Fig. 3. Paper chromatogram comparing dextran sulphate in urine samples at 10 and 20 min. and also dialysate of the 20 min. sample (*D*) with the dextran sulphate (*S*) and a reference preparation (*OC*) of short chain length. Ascending chromatography with 25% (v/v) *n*-propanol in *m*/15 sodium phosphate buffer (pH 6) and Whatman no. 1 paper.

DISCUSSION

In considering the results of the tracer experiments, shown in Table 1, the significance of the two methods of estimating dextran sulphate should be borne in mind. The metachromatic estimation measures the number of sulphuric acid ester groups in the urine sample. The radioactivity estimation measures the number of sulphur atoms and may include metabolic products of dextran sulphate.

Since the blank urine samples were free from metachromatic material it is probably justifiable to regard the metachromatic estimates on samples numbered 1 to 6 in Table 1 as measuring only dextran sulphate. The estimate by radioactivity is always the higher of the two.

Fig. 1 shows that whatever may be the true basis of the estimates or the causes of error the excretion follows an exponential decay curve, since the logarithm of the amount excreted in each 10 min. period is linearly related to time after injection.

By regarding the ordinates at 10 min. intervals as a series in geometrical progression an estimate of the total excretion may be obtained as the sum to infinity of the series. This provides the calculated totals of Table 1. It will be seen that the amount found by analysis is slightly greater than the total calculated on the assumption that excretion continues at the uniformly declining rate. (The only discrepancy is the metachromatic estimate at 49 hr. for the rabbit given 20 mg./kg., which is too high. This could have been caused by naturally occurring metachromatic substances.)

Referring again to Fig. 1, the difference in slope of both pairs of lines is significant (lower pair: $t = 4.37$, $P < 0.01$; upper pair: $t = 2.58$, $P < 0.05$). This implies that the *S* atoms from dextran sulphate are leaving the rabbit more rapidly than they can be accounted for as sulphuric ester groups. The excess sulphur may be in the form of fragments of the dextran sulphate molecule with less than four glucose units or inorganic sulphate so remaining undetected by toluidine blue.

During the first hour after injection the dextran sulphate excreted by the kidney appears to be a low-molecular-weight fraction of that injected. Probably it is excreted so rapidly because it is insufficiently firmly bound to proteins of the clotting system. Dextran sulphate of such short chain length (*OC*) has been shown (Ricketts, 1954) to have relatively little effect upon blood clotting time when injected. Since as much as 48% may ultimately be excreted it is very probable that larger dextran sulphate molecules appear in the urine later on, but their concentration is so low that it is difficult to investigate their molecular weight.

It should be noted that these experiments were carried out with doses larger than a single

therapeutic dose and involving an amount of dextran sulphate corresponding to two or three days of therapy.

SUMMARY

1. Dextran [^{35}S]sulphate with molecular-weight distribution and biological properties similar to that used as a clinical anticoagulant has been prepared.

2. Intravenous injection of dextran sulphate is followed by renal excretion in exponentially decreasing amount. About 30% is eliminated in 2 days rising to 50% at 4 days.

3. Initially the excreted dextran sulphate is a low-molecular-weight component of the injected material.

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Inhibitors and Activators of Brain Hexokinase

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Inhibitions and activations of hexokinase by specific proteins are of great interest because of their possible connexion with the mechanism of hormonal action. Colowick, Cori & Slein (1947) found that a protein fraction of the anterior pituitary inhibited the hexokinase activity of brain and muscle extracts and that the inhibition was enhanced by adrenocortical extract and counteracted by insulin. Attempts to confirm this result have not always been successful. Abood & Gerard (1951) have reported that a protein in nerve extracts specifically inhibits brain hexokinase; inhibitors of hexokinase have also been found in extracts of spleen (Broh-Kahn & Mirsky, 1947), pancreas (Zakharyn, 1951) and placenta (Gerrard, private communication). Weil-Malherbe & Bone (1951a-d) made a detailed study of brain hexokinase and discovered activators of the enzyme in erythrocytes and muscle, and inhibitors and activators in human plasma. Zacco & Sevag (1952) have published a preliminary report on an inhibitor in human plasma. Further experiments with inhibitors and activators of hexokinase are described in this paper.

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EXPERIMENTAL

The experimental arrangements, analytical methods and composition of the medium for the assay of hexokinase activity were as used by Weil-Malherbe & Bone (1951a). Hexokinase activity was determined by measuring glucose disappearance (Nelson, 1944). Because of the high adenosinetriphosphatase (ATPase) activity of many of the preparations used, it was usually found convenient to make up the test medium in the following order and final concentrations (M): water; glucose, 0.00185–0.002; MgCl_2 , 0.0067; NaHCO_3 or KHCO_3 , 0.02; activators or inhibitors; NaF , 0.024. The reaction was started by the addition of adenosine triphosphate (ATP) to give a final concentration of 0.0033–0.005 M. Incubations were usually carried out at 30° for 15–20 min. in an atmosphere of N_2 containing 5% (by vol.) CO_2 . Any deviations from these conditions are mentioned in the text.

Brain extracts. Most of the experiments were carried out with an extract prepared by double extraction of rat brain at 0° with glass-distilled water and centrifuging in an angle centrifuge at 3500 rev./min. for 5 min. (Weil-Malherbe & Bone, 1951a). This preparation will be referred to as 'standard brain extract'. Some brain extracts were fractionated by centrifuging at 20 000 g for 20 min. in a refrigerated centrifuge, a procedure which practically eliminates particulate constituents other than microsomes. The supernatants will be referred to as high-speed extracts.