Studies on Suramin (Antrypol: Bayer 205)

5. THE COMBINATION OF THE DRUG WITH THE PLASMA AND OTHER PROTEINS

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(*Received* 12 *November* 1945)

In previous investigations (Boursnell, Dangerfield & Wormall, 1939; Boursnell & Wormall, 1939) support was obtained for the view that the long retention of injected suramin in the animal body is largely due to its combination with the proteins of plasma and the body tissues. Little is known about the nature of this combination or the factors governing the dissociation or the 'metabolism' of protein-suramin complexes in the body, and it was thought desirable, therefore, that a further study of these problems should be made. In the investigations described here the amounts of the drug combining with proteins under varying conditions have been determined, and the suramin-protein complexes have been exhaustively extracted with various solvents to find out whether this extraction will remove all or most of the combined or adsorbed drug.

With regard to the retention of suramin in the body for several weeks after the injection, there is no unequivocal proof that it is the unchanged drug which is retained, though the fact that protection against trypanosomiasis is given for many weeks after the injection suggests that no marked chemical change in the drug has taken place during this period. The method of suramin determination which we have devised and used in our investigations, and indeed any chemical method of a similar nature, cannot differentiate between the unchanged drug and other substances which produce aromatic amines when hydrolyzed by acid; thus the chemical determinations on plasma and the tissues merely give a measure of the amount of suramin *plus* any amine-containing 'metabolites' of suramin. Although it is unlikely that any appreciable part of the injected suramin is hydrolyzed to simple aromatic amines and that the latter are retained in the body in combination with proteins, it was decided that it would be of interest to determine (i) whether acid-hydrolyzed suramin can combine with plasma and other proteins, and (ii) how long injected, hydrolyzed suramin is retained in the plasma and tissues of the injected animal. The results of these investigations are described in this paper.

METHODS

Suramin determinations. These were carried out as described previously (Dangerfield, Gaunt & Wormall, 1938), the method involving the heating of the suramin-containing material with HCl, diazotization of the resulting amines and coupling with methyl-α-naphthylamine to give a red colour. All determinations were made with the aid of Lovibond tintometer disks (Boursnell *et al.* 1939; Boursnell & Wormall, 1939), and the use of a control 'backing' tube made it possible to compensate for amines or amine-precursors in normal sera or in the untreated proteins or for any dark colour or other pigment in the filtrates after treatment with kaolin. Duplicate determinations were made in practically every case, and all the figures in this paper, except those in Table 1, have been corrected for the small blank values for normal serum, serum proteins and other proteins. Total N determinations were made on the dried suramin-protein complexes, but it was usually found most satisfactory to record the result in terms of mg. suramin/g. of the dried product.

RESULTS

The combination of suramin with serum proteins

Earlier experiments (Boursnell & Wormall, 1939) have shown that when suramin is added to serum a considerable amount of the drug becomes attached to the proteins and can be found in the ethanol-precipitated proteins. A study of the plasma of rabbits which had previously received intravenous injections of the drug showed a similar fixation of suramin by the proteins, and here also ethanol precipitation gave products containing considerable amounts of the drug. No attempt was made to determine the relative amounts of suramin combined with different fractions of the plasma proteins, but it was found that the precipitated and washed globulin fraction contained an appreciable part, but not the whole, of the suramin present in the original plasma.

In view of these earlier results it was decided to study the following: (i) the amounts of suramin combining with the serum or plasma proteins with varying concentrations of the drug, and (ii) the stability of the protein-suramin complexes to prolonged extraction with various solvents. Preliminary experiments showed that the combination of suramin with serum proteins takes place rapidly, within a few minutes of mixing the solutions. In order to give the mixtures time to reach equilibrium, however, they were usually allowed to stand at room temperature (16–20°) for 1 hr., with occasional shaking. Further experiments showed that it is not necessary to allow the mixture to stand for more

than a few minutes, after precipitating the suramin-protein complexes with ethanol, though a longer period of time (about 1 hr.) was usually allowed. In the early experiments the ethanol-precipitated proteins were exhaustively extracted with a mixture of ethanol and water, but in later experiments this long extraction was made with methanol, in which suramin is very soluble. It was found that all the loosely attached suramin which could be Soxhlet-extracted with methanol was removed from the precipitated proteins by 3 hr. extraction and this was adopted as the standard procedure in most of the later experiments.

Many experiments have been carried out with varying amounts of suramin added to human serum, and the ethanol-precipitated proteins have been extracted with various solvents. It is not necessary to give a detailed account of the individual experiments and it will perhaps be sufficient to record that the suramin-serum protein preparations still contained very appreciable amounts of suramin after each of the following Soxhlet extractions: (i) with aqueous ethanol [a mixture of 2 vol. of ethanol to 1 vol. of H_2O, giving a 6 : 1 ethanol-water ratio in the vapour phase (*Handbook of Chemistry and Physics*, 1936, p. 1294)] for 6 hr., with a change of the extracting fluid after 3 hr. extraction, and (ii) with methanol for 3–12 hr. The amount of suramin left in the preparations after these extractions varied considerably, but it depended largely, of course, on the relative amounts of suramin and serum originally used; another factor influencing the final result may have been the physical state of the powder during the extraction, and the agreement between the values for duplicate preparations (separately extracted) from a suramin-serum mixture was often much less satisfactory than in the experiments with the other proteins; there is also the possibility that the combining power for suramin might vary somewhat from one sample of serum to another. No attempt has so far been made to determine the maximum amount of suramin which will combine with a given weight of serum proteins, but in one experiment a fully extracted product contained 5% of suramin, equivalent to about 3·5 mol. of suramin/mol. of protein, assuming a mean mol. wt. of 100,000 for the serum proteins.

The following experiment may be regarded as typical of a large number carried out with the objects mentioned above, and it will perhaps serve to indicate the general methods used in attempts to obtain the maximum solvent extraction of loosely attached suramin from the suramin-protein complexes. To five 20 ml. samples of human serum were added 1 or 2 ml. of suramin solutions of concentration ranging from 0·2 to 9·6% (w/v), the mixtures being shaken and allowed to stand for 1 hr. at room temperature. Three 4 ml. samples from each mixture were each added to 12 ml. of absolute ethanol and the mixtures shaken well and left for some time. The precipitated proteins were stirred well with a glass rod to break up any large particles, centrifuged, washed with methanol, and Soxhlet-extracted for 6 hr. with methanol. The products were dried *in vacuo* over $CaCl_2$ and then 'air-dried' to constant weight (in most other experiments, however, the hygroscopic material was weighed out as rapidly as possible after removal from the desiccator). Suramin determinations gave the following results:

mg. suramin added to 20 ml. serum	2·0	19·9	60	119	192
mg. suramin/g. in the extracted product	0·0	7·7	16	29	50

The combination of suramin with casein and gelatin

Casein

Exp. 1. The suramin (20 mg.) was dissolved in 2 ml. of water and added to 20 ml. of a filtered casein solution (approx. 5%) prepared as described by Cole (1933, p. 127). The mixture was shaken and samples were taken (*a*) at once, (*b*) after 1 hr., and (*c*) after 2 hr. at 17°. Each sample (2 ml.) was run into 6 ml. of ethanol, and a few drops of a conc. $MgCl_2$ solution were added to assist precipitation; the centrifuged proteins were washed twice with about 8 ml. of methanol each time, then Soxhlet-extracted with methanol for 6 hr. and dried *in vacuo* over $CaCl_2$.

The mean values obtained for the suramin contents of the samples were (*a*) 18 mg., (*b*) 16 mg. and (*c*) 17 mg./g. of dried protein complex.

Exp. 2.

	Approx. 5% casein solution (ml.)	mg. suramin (dissolved in 1 ml. H_2O)
Mixture A	10	154
Mixture B	10	54
Mixture C	10	20

Samples (2 ml.) of each mixture, and of a control mixture (with no suramin), were run into ethanol (containing $MgCl_2$) and the precipitates washed and Soxhlet-extracted (3 hr.) with methanol as in Exp. 1.

The dried preparations contained 182 mg. (A), 104 mg. (B) and 39 mg. (C) of suramin/g. It is of interest to note that with the two lower concentrations of suramin (B and C), between 80 and 90% of the suramin added to the casein solution became firmly attached to the protein. With the highest concentration of suramin (mixture A) only a little over 60% of the added suramin was found in the extracted protein precipitate.

Gelatin

Leaf gelatin (1·2 g.) was dissolved in 37·5 ml. of warm water, 12·5 ml. of 0·75 M-$NaHCO_3$-H_2CO_3 buffer of pH 7·5 were added and the temperature adjusted to 37°. The suramin (50 mg. dissolved in 2 ml. of water) was then added, the mixture shaken, and samples taken (*a*) immediately,

(*b*) after 1 hr., and (*c*) after 5 hr. at 37°, the mixture being shaken at intervals throughout the experiment. Each 5 ml. sample was run into 30 ml. of ice-cold ethanol and the solution centrifuged; the precipitates were washed twice with about 8 ml. of methanol each time and were then Soxhlet-extracted with methanol for 6 hr., and finally dried *in vacuo* over $CaCl_2$.

Suramin determinations gave the following results: (*a*) 37 mg., (*b*) 39 mg. and (*c*) 37 mg. of suramin/g. dried protein complex. Calculations from the N and suramin values showed that about 90 % of the added drug had become firmly attached to the gelatin.

The combination of hydrolyzed suramin with proteins

Experiments have been carried out to determine whether the products obtained by acid hydrolysis of suramin combine with serum and other proteins in the same way as does the unhydrolyzed substance. To enable a proper comparison to be made, amounts of hydrolyzed suramin were used equivalent to the amounts of unhydrolyzed drug used in some of the experiments described above.

Experimental. Suramin (0·50 g.) was heated with 3 ml. of water and 3 ml. of 10N-HCl in a stoppered tube in a boiling water-bath for 7 hr. About 1 ml. of water and 1 drop of phenol red solution were then added and the mixture treated with 40 % (w/v) NaOH to give pH about 8·0. The heavy white precipitate which had formed during the acid hydrolysis almost entirely dissolved on the addition of the NaOH. Distilled water was added to make the final volume 10 ml., and the solution (*X*) was warmed slightly to dissolve the small amount of solid matter. Another hydrolyzed suramin solution (*Y*) was prepared in a similar manner by 8 hr. hydrolysis of 0·51 g. of suramin. Amine determinations on solutions *X* and *Y*, which were prepared for the injection experiments described later in this paper and which therefore had to be very concentrated, showed that the suramin had apparently not been completely hydrolyzed to simple amines, possibly because of the high concentration of the drug and the separation of solid matter during the hydrolysis; the amine contents of *X* and *Y* showed that the solutions were equivalent to completely hydrolyzed 4·1 and 4·2 % suramin respectively. It was, of course, not essential for these experiments that the suramin should be completely converted into simple amines, and it was thought desirable that the same solutions (*X* and *Y*) should be used for both sets of experiments.

Portions of solutions *X* and *Y* were allowed to react with serum and a casein solution as follows:

A. 20 ml. of human serum + 1·33 ml. of hydrolyzed suramin solution (*X*).

B. 21 ml. of approx. 5 % casein solution + 0·5 ml. of hydrolyzed suramin solution (*Y*).

The mixtures A and B were shaken and allowed to stand at 16° for 1 hr. Two 10 ml. samples of each mixture were run into 30 ml. of ethanol, a few drops of a conc. $MgCl_2$ solution being added to the samples of B to aid precipitation, and the precipitated proteins were removed by centrifuging and washed twice with about 8 ml. of methanol. Half of each preparation was Soxhlet-extracted with methanol for 6 hr., and all the products were dried *in vacuo* over $CaCl_2$. Suramin determinations gave the following results:

Complex of hydrolyzed suramin with	Total amine (as mg. of suramin/g. dry product)	
	Not Soxhlet-extracted	Soxhlet-extracted
Serum proteins	6·7	0·2
Casein	3·8	3·3

The above figures for the total amine *plus* amine-precursor in the products show that although a small amount of hydrolyzed suramin becomes associated with serum proteins, the attachment is unlike that occurring with the unhydrolyzed drug, for in the former case the amount attached is relatively small and almost all of it is readily extracted by methanol. With casein, the amount of hydrolyzed suramin combining with the protein is also relatively low, being approximately one-fifth of the amount of the unhydrolyzed drug combining under comparable conditions; in this case, however, extraction with methanol does not remove the attached 'amines' possibly because they are firmly linked up with the acid groups in the casein.

The injection of hydrolyzed suramin into rabbits

Rabbit no. 495 (wt. 1·9 kg.): 1st injection. 2·7 ml. of a hydrolyzed suramin solution prepared by heating 0·50 g. of suramin with 2 ml. of 10N-HCl for 6 hr. and subsequent neutralization with 40 % (w/v) NaOH to pH 8, were injected into the marginal vein of one ear; amine determinations on the hydrolyzed solution showed that it contained the equivalent of 7 % of suramin and thus the injection was comparable with an injection of 0·1 g. suramin/kg. of body weight. A sample of blood (6 ml.) was taken from the other ear of the rabbit before the injection and further samples (4 ml. in the early stages and later rising to 14 ml.) were taken 5 min., 1 hr., 6 hr., 24 hr. and 96 hr. after the injection. The oxalated blood samples were centrifuged and duplicate suramin determinations were made on each plasma.

2nd injection. The same rabbit received 6 days later a similar injection of hydrolyzed suramin equivalent to 0·1 g. suramin/kg. (4·5 ml. of solution *Y* described earlier in this paper).

Rabbit no. 497 (wt. 1·95 kg.). This rabbit received one intravenous injection of 4·7 ml. of hydrolyzed

suramin solution *X* (equivalent to 0·1 g. suramin/kg.).

The results of these 'total amine' determinations show that a very small fraction only of the hydrolyzed suramin is retained in the blood plasma a few hours after the injection, and 48 hr. later merely a trace of the injected material is left in the plasma. The results of the above experiments are compared, in Fig. 1, with those obtained in an earlier experiment (Dangerfield *et al.* 1938) in which an equivalent amount of unhydrolyzed suramin was injected. It can also be seen, from a comparison with the earlier results, that on the average, there was more 'total amine' in the plasma 90 days after the injection of unhydrolyzed suramin than there was 6 hr. after the injection of an equivalent amount of the hydrolyzed drug.

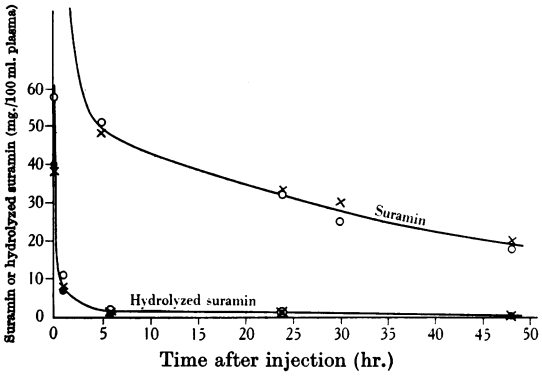

Fig. 1. Comparison of concentrations in plasma of suramin and hydrolyzed suramin after the injection of equivalent quantities (0·1 g. suramin/kg. body wt.) into rabbits. (Values for suramin obtained from Dangerfield *et al.* 1938.) The values have all been corrected for the corresponding 'blank' value (0·9 or 1·0 mg./100 ml.) for the pre-injection plasma samples.
Suramin: × rabbit no. 468; o rabbit no. 449.
Hydrolyzed suramin: × rabbit no. 497, 1st injection.
o rabbit no. 495, 1st injection.
• rabbit no. 495, 2nd injection.

Table 1. *Amine plus amine-precursor content (in terms of mg. suramin per g. of tissue or per ml. of plasma) of the tissues of normal rabbits and of rabbits injected with hydrolyzed or unhydrolyzed suramin*

Tissue	Normal rabbits*	Rabbit injected with hydrolyzed suramin	Rabbits injected with suramin*
Kidney	0·043	0·044	0·158
Liver	0·040	0·043	0·056
Spleen	0·060	0·060	0·108
Lung	0·039	0·039	0·056
Muscle (abdominal wall)	0·026	0·012	0·043
Plasma	0·009	0·009	0·036

* Average values obtained in earlier experiments (Boursnell & Wormall, 1939).

In earlier experiments (Boursnell & Wormall, 1939) it was found that the injection of suramin into rabbits leads to the retention of significant amounts of suramin or some related substance in the tissues for many weeks after the injection. It was therefore thought that it would be of interest to determine whether the rabbits used in the above experiment showed any similar storage of amine or amine-precursor. Rabbit no. 495 was killed 9 days after the second injection, and suramin determinations were made on the tissues as described previously (loc. cit.). The results obtained (cf. Table 1) did not differ significantly from the 'blank' values for the corresponding tissues of normal rabbits, and they were certainly very considerably lower than the values previously obtained for the tissues of rabbits 21 days after an injection of a much smaller amount of unhydrolyzed suramin (0·03 g. suramin/kg. of body weight, i.e. only 15 % of the equivalent of the hydrolyzed suramin injected into rabbit no. 495).

DISCUSSION

A considerable amount of evidence is available that suramin reacts with plasma and other proteins, and a reaction of this type may be significant in connexion with the anticomplementary and anticoagulant action of this drug. Klopstock (1932), for example, concludes that suramin changes the isoelectric point of certain of the plasma proteins, and that with higher concentrations of the drug there is a reaction with specific groups in the protein molecule. Other investigators have reported that suramin, although not a general enzyme poison, has an inhibitory or toxic action on certain enzymes, e.g. trypsin (Beilinsohn, 1929), fumarase (Quastel, 1931) and the enzymes giving post-mortem production of acid in muscle and liver (Fürth, Scholl & Herrmann, 1932). Evidence of combination of suramin with proteins was obtained in our earlier investigations (Boursnell & Wormall, 1939) by the chemical determination of suramin in the precipitated proteins of (*a*) mixtures of suramin with serum or plasma, and (*b*) the plasma of rabbits which had received intravenous injections of the drug, and there appears to be little doubt that the long retention of the drug in the body is due largely to its combination with proteins. It is interesting in this connexion to note that as long ago as 1921 Mayer & Zeiss suggested that the injected drug is bound to the plasma proteins.

The results of the investigation described in the present paper indicate that the combination between suramin and plasma proteins is of a firm nature, and that the compounds or complexes produced retain very considerable amounts of suramin

even after exhaustive extraction with solvents in which suramin is very soluble. The amount of suramin combining with a given weight of the protein depends largely on the concentration of the drug in the plasma or serum. With high concentrations (e.g. 1 g. of suramin/100 ml. of serum) as much as 5% of firmly attached suramin has been found in the solvent-extracted protein preparation, but, of course, plasma concentrations of this order would not be reached in individuals receiving intravenous injections of the drug in the usual amounts (1 or 2 g. per injection). The exact nature of the combination of suramin with proteins is not known, and although the combination is apparently something other than ordinary adsorption, we have no evidence as yet of a reaction with specific groups in the protein molecule. Evidence of a reaction of this type would, of course, be most useful and might offer some explanation for the important pharmacological or therapeutic actions of the drug. It is hoped, therefore, that further investigation will throw some light on this problem. It would also be of interest to know the trypanocidal activities of the suramin-protein complexes compared with those of equivalent amounts of the uncombined drug, and also whether the rate of *in vivo* liberation or dissociation of the drug from the complex is determined solely by the normal breakdown or 'turnover' of the plasma proteins.

Combination with suramin is not confined to the plasma and similar proteins, for a reaction occurs with gelatin and casein; indeed, the experiments described in this paper suggest that the amount of suramin firmly attached to casein or gelatin may be appreciably greater than that attached to the serum proteins under comparable conditions. It seems probable, therefore, that there is a general reaction between suramin and any protein, but the above-mentioned work of other investigators on the specific inactivation of certain enzymes by the drug and also the specific nature of its anticomplementary and anticoagulant actions suggest that there may also be a more specific reaction with specially active groups of certain proteins. The combination is rapid, and under the conditions of our experiments with serum proteins, casein and gelatin, it is completed within a few minutes of mixing the solutions. With some of the lower concentrations of suramin used in these experiments at least 70% and sometimes as much as 90% of the added drug was found to be firmly attached to the separated and solvent-extracted gelatin and casein complexes, but appreciably less was fixed by the serum proteins. Up to the present no attempt has been made to determine the maximum combining power of proteins for suramin, but preparations of the serum protein complexes containing over 5% of suramin, and of the casein complexes containing 18% of suramin have been obtained.

The possibility of some breakdown of the suramin molecule in the animal body cannot be excluded, and the products of this 'metabolism' might conceivably combine with the plasma and tissue proteins and be determined as suramin in our experiments. A study has therefore been made of the capacity of acid-hydrolyzed suramin to combine with proteins, though it is not suggested that any degradation of the drug in the body is likely to be as complete as that effected by HCl. The results of our investigations show that the amines present in acid-hydrolyzed suramin solutions do not become firmly attached to serum proteins and only a relatively small amount becomes attached to casein. Furthermore, hydrolyzed suramin injected intravenously into rabbits is not retained in the plasma for any very appreciable length of time, nor is there any increase in the amine *plus* amine-precursor content of the tissues of the injected animal similar to that which follows the injection of the unhydrolyzed drug. The conclusion can be reached, therefore, that the long retention of 'amine-precursors' in the plasma and tissues of rabbits injected with suramin cannot be due in any way to the retention of relatively simple amines in combination with the body proteins.

SUMMARY

1. A further study has been made of the combination of suramin with the serum proteins. This combination is of a firm nature, and after long extraction with various solvents these suramin-protein complexes still contain very appreciable amounts of the drug.

2. Suramin readily combines with other proteins such as casein and gelatin.

3. Suramin hydrolyzed by HCl does not combine to any appreciable extent with serum proteins; with casein there is some combination, but the amount of amine attached is very much less than the amount of unhydrolyzed suramin which combines with this protein under similar conditions.

4. The injection of hydrolyzed suramin into a rabbit does not lead to any increase in the 'total amine' contents of the organs and other tissues of the body. Suramin injections are followed by a marked increase in these values, as was described in earlier reports.

The authors are pleased to express their gratitude to the Medical Research Council for a personal grant to one of them (H. M. D.), and for an expenses grant, and to Prof. D. Keilin, F.R.S., for laboratory facilities in the Molteno Institute, Cambridge. Thanks are also tendered to Dr J. C. Boursnell for help with a few of the determinations.

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Chemistry of Tissues

2. POLYSACCHARIDES SHOWING BLOOD GROUP A-SPECIFICITY AND THE NATURE OF THE CONSTITUENT UNITS OF THE STABLE CARBOHYDRATE RESIDUE OF THE A SUBSTANCE FROM PEPSIN

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(*Received* 15 *September* 1945)

The chemistry of the blood group substances presents problems of lively interest in both the protein and carbohydrate fields and it is apparent that precise structural knowledge of these substances is eminently desirable in order to understand the remarkable differences in specificity which exist between the various groups. Two types of material, differing in immunological behaviour but sharing a common group specificity, are available for examination. One type, which probably contains lipid constituents in addition to carbohydrate, can be extracted by organic solvents from human tissues and erythrocytes, while the other type can be extracted by water from certain human and animal tissues and secretions. Adequate recent reviews on the subject have been presented by Wiener (1943) and Morgan (1944). One of the most striking observations in considering the field as a whole is the absence of any reliable method for preparing the 'group substances proper' from erythrocytes themselves and the consequent failure to establish more than a serological connexion between these group substances proper and the related substances in tissues. Nevertheless, the determination of the chemical nature of the tissue substances does present a relatively easy approach to the problem. The main part of the work described herein was completed some 6 years ago and was largely designed to find a suitable source of the factors and to work out a method for giving the relatively large amounts of carbohydrate material necessary for study by the methylation and other techniques. The purification was controlled by determining the power of each fraction to inhibit the isoagglutination reaction only, for no opportunity was found to carry out the haemolytic inhibition test (cf. Morgan, 1944). The properties of the substances examined are set forth in the experimental section and are summarized in Table 1. Their significance is discussed later. Table 2 shows typical specimens of blood group A factors described in the literature.

EXPERIMENTAL

The following modification of the method of Sevag (1934) was found suitable for the preparation of a soluble polysaccharide-amino-acid complex from a wide variety of sources.

The mucin or minced tissue suspension was heated at 100° for periods up to 30 min. in a steamer and where necessary a little $CaCO_3$ was added to maintain neutrality. The solution was cooled and filtered. To each 100 ml. of filtrate there was added sodium acetate (12–16 g.) and 20% (w/v) acetic acid (10–12 ml.) to adjust the solution to pH 4·8. It was then vigorously shaken with chloroform (or carbon tetrachloride) (20 ml.) and with butanol (2 ml.) for 30 min. The mixture was centrifuged and the upper layer separated and again shaken with Sevag's reagents several times until there was no further coagulation of extraneous protein material. The crude polysaccharide was precipitated from the solution by addition of 3–4 vol. of ethanol. It was then fractionated by precipitation from 5% aqueous solution containing a few drops of 1% calcium chloride, by gradual addition of ethanol. The fractions were dried by being washed with absolute ethanol and ether and then kept in a vacuum at 20°.