PARTIAL PURIFICATION OF BOVINE SULFATION FACTOR

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Summary

A method for the partial purification of sulfation factor (S. F.) from bovine serum utilizing the techniques of ultrafiltration and molecular sieve chromatography is presented. The method is simple and approximately 50% recoveries of the initial S. F. activity have been consistantly obtained. A 400-fold purification is obtained when the potency is based on biuret reactive material; on a dry weight basis, a 125-fold purification. From the data presented herein, it appears, that serum S. F. is similar in size to the muscle S. F. of Hall but considerably smaller than that reported by others. It is suggested that freezing and thawing of the samples prior to and during the purification procedures may dissociate S. F. from a large protein or macromolecule which would lead to artificially high molecular weight assignments.

Sulfation factor (S. F.) is a constituent of blood which stimulates the conversion of chondroitin to chondroitin sulfate by cartilage. Sulfation activity is related to pituitary homeostasis since it is elevated in acromegalics and depressed in hypopituitary dwarfs (1). Although growth hormone is the pituitary agent which stimulates sulfation *in vivo*, it apparently is not S. F. since growth hormone is ineffective in modulating sulfation activity *in vitro* (2). Although the nature of S. F. is unknown, it has been postulated that it may be a catabolite ("active core") of growth hormone (3).

In a recent communication a scheme yielding a 65-fold purification

of S. F. from acromegalic plasma was presented (4).

We wish to report on our efforts to purify S. F. from bovine serum. A 400-fold purification has been obtained utilizing the techniques of ultrafiltration and molecular sieve chromatography.

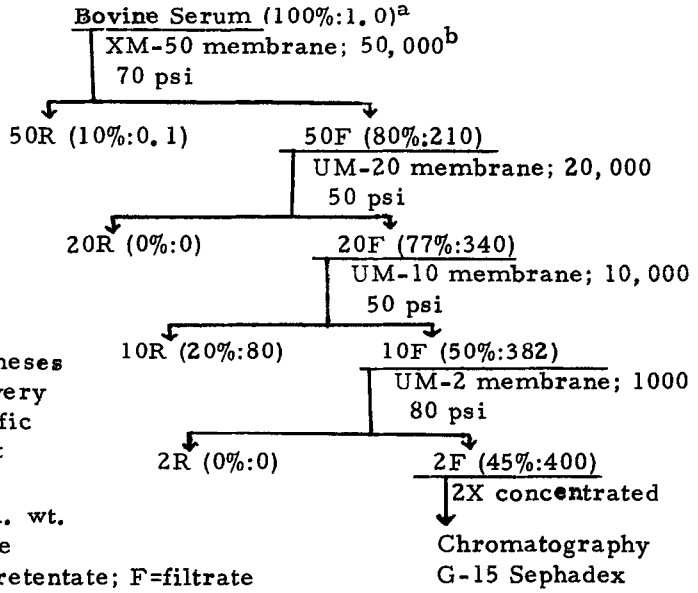
Methods

Bovine blood was collected in vessels which were kept in ice. Serum was prepared by centrifugation on the same day and stored at -10° in volumes of 200 mls. The serum was thawed slowly and batches of 200 to 400 mls were ultrafiltered through a series of membranes in a Model 401 ultrafiltration cell (Amicon Corp., Lexington, Mass.) as depicted in Table 1. The ultrafiltration cell was kept in ice during the filtration and the filtrate collected at 4-6°. The BS-2F fraction was concentrated 2-fold by lyophilization and then subjected to molecular sieve chromatography employing Sephadex G-15 equilibrated and eluted with 0.001 M ammonium formate. All filtrates were stored at -10°. The S. F. activity (c/m/mg cartilage) was assayed using the in vitro incorporation of ^{35}S into chondroitin sulfate by costal cartilage obtained from hypophysectomized rats (5). The sulfation activity of test samples was corrected for endogenous activity. Protein was determined by the biuret reaction.

Results

A flow diagram of the purification scheme employed and the sulfation activities obtained are shown in Table 1. Fractionation through the XM-50 membrane represents the most effective single step purification in the overall procedure representing a recovery of 80% of the initial activity and about a 200-fold purification. The majority of serum components having a molecular weight $> 50,000$ are retained by the

Table I - Summary of Purification Procedure of Bovine Sulfation Factor

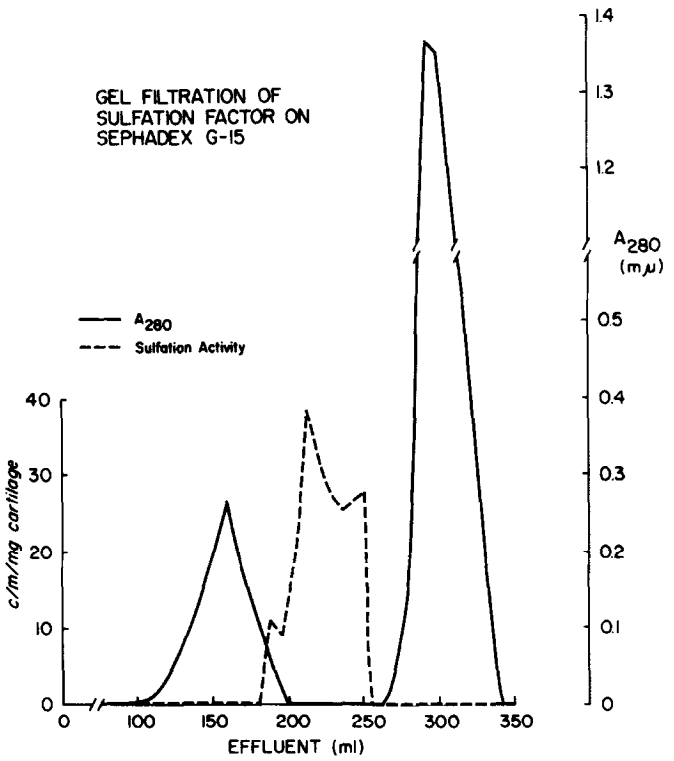


[a] Values in parentheses represent % Recovery and Relative Specific Activity/mg biuret positive material

[b] Approximate mol. wt. cutoff of membrane

Abbreviations: R=retentate; F=filtrate

membrane (BS-50R) and represent approximately 95% of the biuret positive material of serum. The resultant filtrate, BS-50F, although more viscous than water, is clear and colorless. The BS-50F was passed through a UM-20 membrane. The retentate (BS-20R) contained no measurable S. F. activity and accounted for most of the remaining biuret positive material. Essentially all the S. F. activity was found in the filtrate (BS-20F). Subsequent ultrafiltration through a UM-10 membrane resulted in retention of 1/5 of the total S. F. activity having a specific activity of 80. The filtrate (BS-10F) was finally passed through a UM-2 membrane and a white, tacky substance was retained. The majority of the S. F. activity resided in the filtrate (BS-2F), and based on biuret positive material represented a 400-fold purification of S. F. (85-fold purification based on dry weight) and contained 45% of the initial activity. The 280 mμ absorbance and the S. F. activity obtained upon chromatography are shown in Fig. 1. The fractions con-



Legend to Fig. 1

Molecular sieve chromatography of S. F. 95 mls of twice concentrated BS-2F was applied to a Sephadex G-15 column (2.0 x 40 cm) and eluted with 0.001 M ammonium formate. The flow rate was 10 ml per hr and 5 ml fractions were collected. All operations were done at 4°. Each fraction was lyophilized, dissolved in H_2O and aliquots were assayed for S. F. activity.

taining S. F. activity were confined to a single peak which was eluted after the void volume. The eluates which possessed S. F. activity gave positive tests to ninhydrin. No increase in the relative specific activity of S. F. on the basis of biuret reaction was noted in these fractions although small molecular weight contaminants were removed. A 125-fold purification was obtained on a dry weight basis however.

Discussion

The technique of ultrafiltration offers a method for the relatively

crude separation of serum which has definite advantages over other methods of fractionation. Large volumes can be handled with relative ease which is of extreme importance when isolating material such as S. F. which in all likelihood is present in minute amounts. In addition no extremes of pH and temperature are encountered and the deleterious effects that can arise with the use of organic solvents and salts are circumvented. One definite drawback we encountered in ultrafiltration is the slow rate of filtration in the initial fractionation, i.e. using membrane XM-50. The initial flow rate of 2 ml/min decreased to 0.3 ml/min as the viscosity of the retentate increased. However, very little leakage of large molecular weight proteins was observed even after continuous ultrafiltration for 72 hours. The subsequent ultrafiltration steps posed no similar problem since rapid flow rates were obtained (>5 ml/min.).

Approximately one-half of the initial S. F. activity was recovered in the BS-2F fraction and represents a 400-fold purification. This latter value is an approximation based on biuret positive material since it has been reported that S. F. is proteinaceous (6). Van Wyk et al. (4) based their 65-fold purification of S. F. on the dry weight of the isolated material relative to the dry weight of the starting material. Calculated on a dry weight basis, our procedure yields a 125-fold purification.

It is of interest to note that the substance retained on the UM-2 membrane completely inhibited S. F. activity. In mixing experiments, the S. F. activity of BS-2F and of unfractionated serum was inhibited 100% by this material. It is known that addition of serum to a final concentration of $>30\%$ in the assay system results in a reduction of S. F. activity. It would be of great physiologic importance if the in-

hibition by BS-2R is specific for S. F.

The serum employed in this purification scheme has been frozen and thawed at least once prior to ultrafiltration. Ultrafiltration of serum which is not so treated results in a different and unpredictable fractionation. In several studies utilizing unfrozen sera, the S. F. activity distributed 60%-40% and 80%-15% in the 50R and 50F fractions, respectively which would indicate a molecular size in the range of 30,000 to 50,000. The ultrafiltration separation patterns of S. F. from frozen and thawed serum and its behavior on G-15 Sephadex indicate that the molecule is quite small ($\leq$ 5000 molecular weight). It is known that several low molecular weight hormones such as thyroxine and cortisone occur in blood as protein conjugates. An explanation for these discrepancies may be that S. F. exists in blood bound to a transport macromolecule or as an aggregate which can be dissociated by repeated freezing and thawing. This explanation could account for the conflicting data (3, 7) which have indicated S. F. as being a small molecule ($<$20,000 molecular weight), a large molecule, a dissociable molecule and a molecule which may be protein bound. In this connection it is interesting to note that Van Wyk et al. (4) tentatively assigned a molecular weight of $\geq$ 20,000 to plasma S. F. although the procedures employed by this group subsequent to Sephadex chromatography were similar to those of Hall et al. (unpublished results cited in reference 4) who have isolated a skeletal muscle sulfation substance which is a 'peptide'. In both those studies, the sulfation activity resided in the identical zone of the high-voltage electrophoretogram.

We are presently processing larger quantities of serum in order to characterize S. F. and to study further the sulfation inhibitory substance.

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