In one experiment, 50 g of Fuller's earth was shown to adsorb 7 g of protein without any significant loss of hyaluronic acid. This treatment caused little loss in intrinsic viscosity. It was shown, too, that an excess of Fuller's earth removed some hyaluronic acid. The cetylpyridinium chloride precipitate from 850 ml of synovial fluid was treated with successive 25-g portions of Fuller's earth without removal of the Fuller's earth from the previous additions. The first treatment removed 3500 mg of protein from the 3850 mg originally present, and 50 mg of hyaluronic acid was lost from the original 440 mg. The 4th treatment decreased the amount of protein from 20 mg to 13 mg while the loss of hyaluronic acid was 85 mg from the 300 mg remaining at this stage.

In subsequent preparations, the treatment was slightly modified. Two treatments with purified Fuller's earth were performed according to the preparation described in Table I. The Fuller's earth was removed by centrifugation before the second treatment was given. As the results in Table I indicate, this procedure was more effective for the removal of protein.

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A carbazole method for the differential analysis of glucuronate, glucosiduronate and hyaluronate

In 1955, FISHMAN AND GREEN[1] devised a method for the separate analysis of free and conjugated glucuronic acid by the naphthoresorcinol color reaction which is applied before and after oxidation of the reducing aldehyde group of free glucuronic acid by alkaline iodine. However, there is a need for an analytical method for the same mixture by DISCHE's carbazole reaction[2] since its reaction conditions are strong enough to hydrolyze the mucopolysaccharides as well as simple *O*-glucosiduronic acids. Accordingly, in the present communication, experimental conditions

are described for analyzing a mixture of free, conjugated and mucopolysaccharide uronic acid based on hypoiodite oxidation[1] and on the DISCHE carbazole reaction. Finally, the analysis of hyaluronate itself became possible after it was discovered that the mucopolysaccharide coprecipitates with HgS from aqueous solution.

Preparation of calibration curve. A mixture of 1 ml of carbonate buffer solution[1], 1 ml of iodine solution[1], 0.1 ml of $NaHSO_3$ solution[1], and 0.2 ml of 10 N acetic acid is shaken well. To this solution are added 3 ml of standard glucuronic acid solution (range, 0–80 μg glucuronic acid) and 0.7 ml of 0.25 M $HgCl_2$ solution. After 10 min standing at room temperature, the mixtures are centrifuged, and a 1.0-ml aliquot of the supernatant is transferred to a test tube and cooled in ice. To this is added 6 ml of conc. H_2SO_4–borate mixture[3] previously cooled in an ice bath. The mixtures are heated in a boiling-water bath for 20 min and 0.2 ml of carbazole reagent[2] is added to each. The tubes are shaken well and heated another 10 min in a boiling water bath and cooled. Color is measured at 540 mμ.

Analysis of total glucuronic acid. Total glucuronic acid (value A) is analyzed by the regular DISCHE carbazole reaction[2] using a H_2SO_4–borate mixture[3].

Analysis of a mixture of free and conjugated glucuronic acid. The specimen (3 ml), 1 ml each of carbonate buffer solution and of I_2 solution are pipetted into semimicro test tubes and allowed to stand for 20 min at room temperature. To this mixture are added 0.1 ml of $NaHSO_3$ solution and 0.2 ml of 10 N acetic acid. Next, the tubes are shaken well to release CO_2. The I^- is precipitated by first the addition of 0.7 ml of 0.25 M $HgCl_2$ and then by allowing the tubes to stand 10 min. When polysaccharides are present, it is sometimes necessary to accelerate and to complete the precipitation of HgI_2, by first heating (100°) for 3 min and then cooling*. This red precipitate is removed by centrifugation and 1.0-ml samples of the supernatant are pipetted into glass-stoppered test tubes. Next, 6 ml of conc. H_2SO_4–boric acid mixture[3] are added to each, and the tubes heated in a boiling-water bath for 20 min. After addition of 0.2 ml of carbazole reagent, the tubes are shaken well and heated for 10 min and after they are cooled the absorbancies are measured at 540 mμ. The corresponding glucuronic acid value B represents glucosiduronic acid and also mucopolysaccharide glucuronic acid when it is present.

This value is subtracted from the total glucuronic acid figure (A–B) to give the free reducing glucuronic acid value.

Analysis of hyaluronate glucuronic acid. Another aliquot (3.0 ml) of the $HgCl_2$ supernatant is pipetted into semimicro test tubes, together with 0.1 ml of 1 N HCl and 0.1 ml of 1.0 % thioacetamide solution. Hg^{2+} is then converted to insoluble HgS by heating at 60° for 30 min. After being cooled by tap water, the mixtures are centrifuged, and the supernatants are analyzed by the carbazole method. The difference between this value C and value B is attributed to polysaccharide glucuronic acid.

Adsorption of hyaluronic acid on mercuric sulfide**. The following substances

* If precipitation of HgI_2 was insufficient, free I_2 would be liberated after addition of H_2SO_4, and during heating, glucuronic acid would be oxidized and consequently the values of glucuronic acid would diminish. In many cases, small particles of HgI_2 or HgS float on the surface of the solution and these cannot be removed even by centrifugation. Addition of 1 drop of 0.1 % Alconox detergent solution along the inner side of the tube wall will precipitate the particles which can then be effectively sedimented by centrifugation.

** 100 μg of polysaccharide in 0.03 ml of distilled water, 1 ml of 0.1 *N* acetic acid, 0.2 ml of 1 % $HgCl_2$, and 0.2 ml of 0.5 % thioacetamide solution were mixed together, warmed at 60° for for 30 min, centrifuged, and then the supernatant was analyzed by the carbazole method.

and their percentage adsorption on HgS were as follows; hyaluronic acid, 100; chondroitin sulfate C, 96.5; oxidized starch, 76; di- and tetrasaccharide of heparin, 72; Type-III capsular polysaccharide, 48.5; tetrasaccharide of chondroitin sulfate C, 30; chondroitin sulfate B, 26; and heparitin sulfate, 0.

TABLE I

ANALYSES OF MIXTURES OF FREE, CONJUGATED, AND POLYSACCHARIDE GLUCURONIC ACID

Glucuronic acid in prepared sample (μg)			*Found (μg)*		
Free	*Conjugated*[1]	*Polysaccharide***	*Free*	*Conjugated*	*Polysaccharide*
10.0	10.0	—	9.7	8.3	—
10.0	70.0	—	9.8	70.5	—
15.4	—	13.35	14.5	—	12.35
40.0	40.0	—	42.6	38.4	—
70.0	10.0	—	68.5	13.0	—
10.0	10.1	8.8	9.0	10.7	8.1
20.0	20.2	17.6	17.6	21.7	16.7

* Diphenylglucosiduronic acid.
** Potassium hyaluronate, (product from Chugai Pharmaceutical Co., Ltd., Tokyo (Japan). Glucuronic acid, 37.0%; glucosamine, 41.7%).

The performance of the method has been tested by analyzing mixtures of known composition (Table I) and is considered satisfactory.

It should also be noted that the present conditions utilizing carbazole give all the desired values in a relatively short time. A mixture of free and conjugated glucuronic acid can be analyzed within 1 h, and a mixture of free, simple conjugated, and polysaccharide glucuronic acid within 2 h.

Terminal reducing glucuronic acid and endo-glucuronic acid of oligosaccharides will act as free and conjugated glucuronic acid, respectively, in this analytical method. Therefore, this method is expected to be useful in the analyses of oligosaccharides which have glucuronic acid as the terminal reducing sugar and should yield information about sugar units of unknown oligosaccharides.

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