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DETECTION AND ESTIMATION OF CHONDROITIN SULPHATE IN SUBMICROGRAM QUANTITIES AS A COMPLEX ON ACRYLAMIDE GEL

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SUMMARY

A complex between chondroitin sulphate and the histone fraction F1 was formed on acrylamide gel and separated from excess of histone by an electrophoretic method. The stability of the complex was demonstrated by staining reactions and also by radioactive determinations, for which the histone was highly labelled with [^{14}C]-dimethyl sulphate before formation of the complex. The complex was resistant to electrophoresis for long periods compared with the time required to separate the complex from free histone. The radioactivity was proportional to the 0.7th power of the amount of chondroitin sulphate on the gel. 10^{-8} g of chondroitin sulphate could be detected by the amount of radioactivity bound.

INTRODUCTION

Precipitation of a chondroitin sulphate with histone was observed by AGRELL AND HEBY[1] using a double diffusion technique in agarose. The binding of histone by agar gel prevents the migration of the protein in an electrical field. In the present experiments a sample of chondroitin sulphate was applied to acrylamide gel by electrophoresis. After a short time interval, the current was switched off and a sample of the histone fraction F1 was applied to the same gel with the voltage gradient reversed. The histone and chondroitin sulphate bands crossed in the gel. If histone was in excess, a histone–chondroitin complex was left at the crossing point and excess histone migrated off the gel. The complex was immobile and stable to electrophoresis for a period many times that required to resolve the complex from free histone. In order to quantitate the interaction, the histone F1 was methylated with [^{14}C]-dimethyl sulphate. The application of the interaction to the estimation of submicrogram quantities of chondroitin sulphate is discussed.

MATERIALS AND METHODS

Chondroitin sulphate

This was prepared as the barium salt from bovine nasal cartilage by the method of MALAWISTA AND SCHUBERT[2]. The sulphur content (measured by Dr. G. WEILER

and Dr. F. B. STRAUSS, Oxford) was 3.1 %. The material was dissolved in 40 % w/v sucrose to give a series of dilutions from 0.01 to 3.0 mg/ml. These were stored frozen at −8°. The histone fraction F1 was that used in a previous investigation[3]. The protein was methylated by EDLBACHER's[4] method as follows: 30 mg of fraction F1 which had been prepared by precipitation as the hydrochloride from aqueous acetone was dissolved in 1.4 ml water. 0.6 ml 0.1 *N* NaOH was added and also 0.1 mC [^{14}C]-dimethyl sulphate (22.7 mC/mmole from the Radiochemical Centre, Amersham) dissolved in 0.1 ml ethanol. The system was mixed well and left stoppered at room temperature (22°) for 2 h. One drop of aqueous 0.5 % w/v bromophenol blue solution was added and 2 drops of 6 *N* HCl to render the solution acid. The methylated histone was precipitated by addition of 10 ml acetone and washed by decantation with ten washes of 10 ml acetone. The acetone was drained off, the protein allowed to become air dry and redissolved in 10 ml 40 % w/v sucrose. The solution was divided into several parts and stored frozen at −20°. The methylation was almost quantitative as judged by the radioactivity of the protein solution (10^7 disintegrations/min/ml). It is believed that methylation occurs at the ε-amino group of lysine. Some previous experiments (with Dr. G. WILTSHIRE) had shown a considerable (80 %) reduction in N-amino after methylation of bovine plasma albumin with excess dimethyl sulphate under alkaline conditions.

Acrylamide gel electrophoresis

The electrophoresis was done in capillary tubes (50 × 1 mm) obtained by cutting disposable 100 μl micropipettes (Dispo, Scientific Products, Evanston, Ill., U.S.A.) crosswise in half. The tubes were not cleaned prior to use. The tubes were filled by capillary action with the gel system described by REISFELD *et al.*[5]; the acrylamide concentration being 28 % w/v in the stock solution and 7 % in the final gel. The tubes were sealed at the lower end by standing them in a plastic clay (Seal-Ease, Clay-Adams Inc., New York). The gels were water-layered in the usual way. Before setting the top of the gel was sharpened by withdrawing some of the boundary into a fine syringe needle attached to a micrometer syringe (Kontes Glass Co., Vineland, N.J., U.S.A.) by narrow flexible tubing.

The clay seal was removed by cutting off the end of the tube with an ampoule file. The tubes were supported in a conventional two-tier apparatus made from Perspex (Imperial Chemical Industries). The water seal in which the tubes were supported was provided by a sheet of 1/8 in. thick (3 mm) non-intercellular neoprene (Expanding Rubber Company, Croydon, Surrey), sandwiched between the bottom of the upper buffer compartment and another layer of Perspex. The sandwich was held together with nylon nuts and bolts (Nyloy Screws, London) and butyl rubber washers. The buffer compartments were filled with the buffer system of NAGAI *et al.*[6].

Application of the samples was by means of a calibrated microsyringe (Hamilton, Whittier, Calif., U.S.A.). This was supported in a frame constructed from a desk lamp (Terry, Redditch, Worcs.) with an aluminium bar, 7.5 × 1.5 × 1.5 cm, with a spring clip in place of the lamp reflector and socket. The chondroitin sulphate was applied to the top of the gel and migrated downwards towards the anode. When the histone was added to the top of the gel the field was reversed so that the bottom compartment contained the cathode and the chondroitin sulphate already on the gel began to migrate upwards to meet the labelled histone. In either case, the magnitude

of the voltage across the electrodes was 100 V. After electrophoresis the gels were extracted by breaking the tubes by hammering with the "sharp" end of a glass pestle. The gels were protected during this procedure by placing the tubes in a groove in a Perspex block from which they projected only slightly. The broken glass was washed off by brief immersion in running tap water.

Staining procedures

Protein was located on the gel with 1 % w/v amidoschwarz (G. T. Gurr, London) in 7 % w/v aqueous acetic acid. Chondroitin sulphate was located with 0.1 % Alcian blue (G. T. Gurr, London) in 0.6 *N* HCl. After staining the background was reduced by washing in 7 % acetic acid and 0.6 *N* HCl respectively.

Radioactive counting

The gels were counted in a Packard TriCarb Scintillation Counter with phosphor as described by LAURENCE AND BUTLER[7]. The position of the complex was determined by staining some of a series of electropherograms for protein. The staining and washing necessary to determine the position of a band did not affect the radioactivity subsequently obtained and these stained gels could be included in the radioactive sample. As the position of a band did not depend upon the amount of chondroitin sulphate used, it was only necessary to stain some of the more dense complexes. A thin slice of the gel was removed from the origin and discarded. Two consecutive 0.5 cm segments of the gel were then cut out, the first of which contained the complex, the second was used as a blank. Radioactivity was eluted into the counting vials by leaving the gel segments in 0.4 ml 25 % w/v tetraethylammonium hydroxide for 2 days before adding 10 ml of the phosphor.

Radiological precautions

It should perhaps be emphasized that the methylated histone is highly radioactive and strongly bound. Care must therefore be taken to avoid personal contact with this reagent, and spent buffer and the capillary tubes after the electrophoretic run.

RESULTS

The formation of a histone–chondroitin sulphate complex on acrylamide gel and its subsequent stability and immobility are illustrated in Fig. 1. In these experiments the chondroitin sulphate (3 μg in 1 μl of solution) was allowed to run for 5 min before adding the unlabelled histone solution (2 μl $\equiv$ 6 μg protein) in excess and reversing the electrical field. Fig. 1 shows the results obtained at various times after the start of the histone migration. After 15 min the excess histone has already passed the chondroitin sulphate. The complex that resists electrophoresis for at least 90 min may be stained with either protein or mucopolysaccharide stains.

The stability of the complex is also indicated by radioactivity measurements on the extracted gels. Table I gives data of the counts/min obtained in an experiment similar to that shown in Fig. 1. Table I also contains the results of allowing the chondroitin sulphate to run for 30 min before addition of methylated histone and field reversal. The counts/min obtained for the shorter time of pre-running were higher

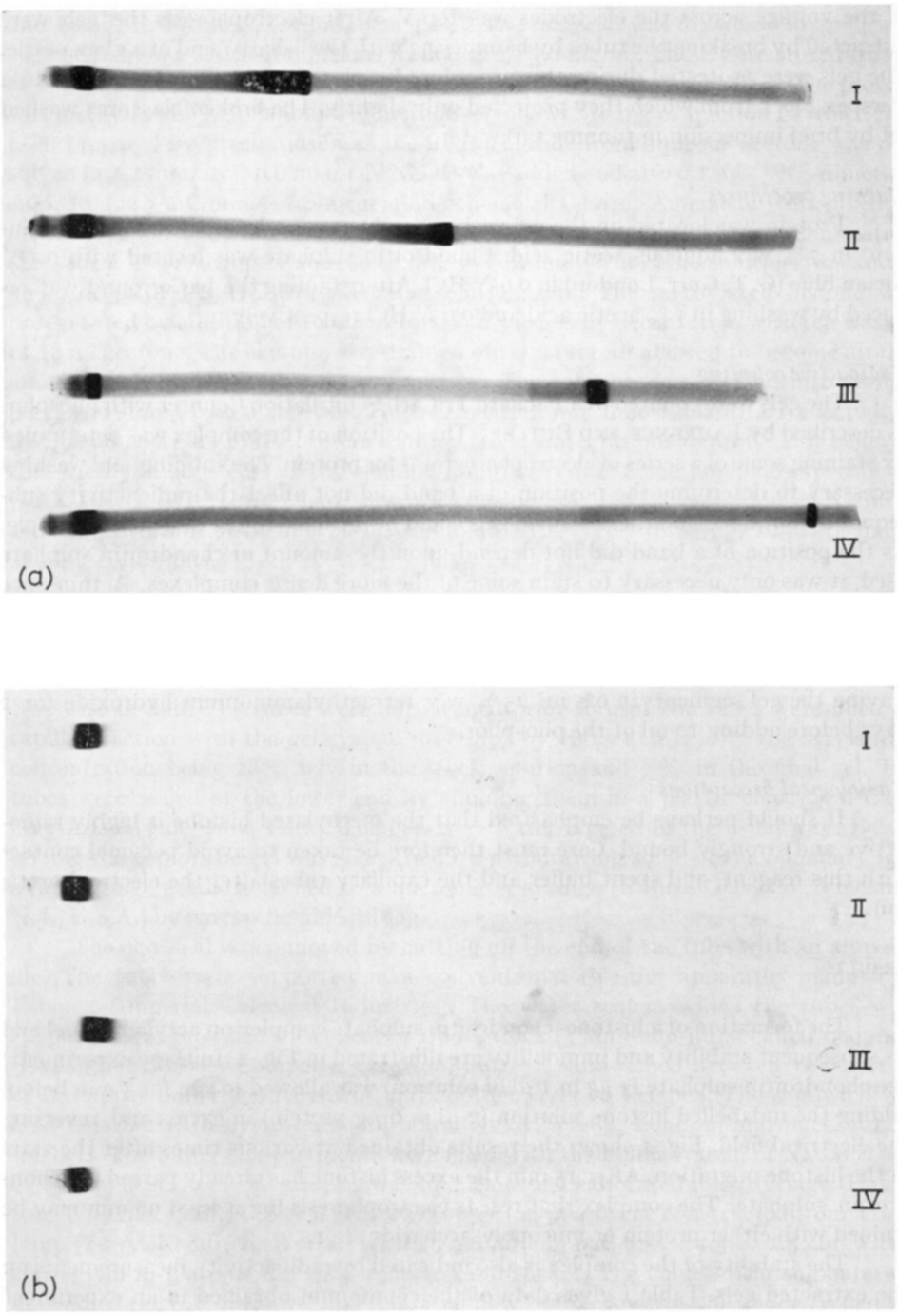

Fig. 1. Separation of excess histone and stability of the chondroitin sulphate–histone complex. (a) Protein staining; (b) alcian blue staining. Duration of the electrophoresis of histone: (i) 5 min, (ii) 30 min; (iii) 60 min; (iv) 90 min.

TABLE I

DETERMINATION OF THE STABILITY OF THE HISTONE–CHONDROITIN SULPHATE COMPLEX TO ELECTROPHORESIS

All table entries are in counts/min. 3 μg of chondroitin sulphate were allowed to react with an excess of histone. T_1 = time for which chondroitin sulphate was pre-run, T_2 = time of subsequent electrophoresis of labelled histone.

T_2 (*min*)	T_1 (*min*)	
	5	*30*
15	7250	6800
30	6600	5650
60	6950	5750
90	6200	5600
120	6100	5600

than those obtained for the longer time. When chondroitin sulphate is run for 30 min the complex is more diffuse, and it was necessary to take 2 cm pieces of gel in order to include the entire complex. After a fairly rapid fall in radioactivity within the first 30 min period, the values obtained between 30 min and 120 min showed a slower decrease which was very slight in the case of the 30-min pre-run.

The sensitivity of the complex formation was tested by taking a series of dilutions of chondroitin sulphate. 0.5 μl quantities of chondroitin sulphate were used and applied by means of a Hamilton 1 μl syringe with a Chaney adaptor and run for 5 min. 2 μl quantities of the stock methylated fraction F1 solution were then applied and the electrophoresis was continued for 90 min with reversed polarity. A blank sample of 0.5 μl 40 % sucrose was run and the radioactivity of this blank was subtracted from all the values of radioactivity obtained with the samples to correct for histone adsorbed to the gel. The results are shown in Fig. 2, and the relationship between net counts/min (measured over 10 min corrected for blank) and amount of chondroitin sulphate applied was linear when plotted on a logarithmic scale for both axes. The slope of the graph is 0.7 and so the counts/min were proportional to the

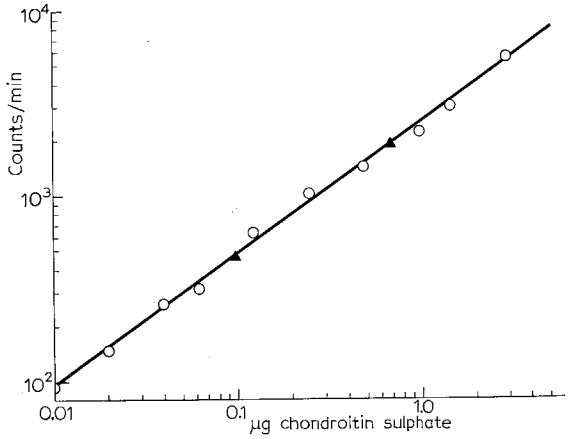

Fig. 2. Relationship between net counts/min extracted from the gel and the amount of chondroitin sulphate applied. ○ = known dilutions of chondroitin sulphate; ▲ = unknown dilutions with concentration subsequently revealed.

0.7th power of the chondroitin sulphate concentration under these conditions. In addition to the known dilutions, two samples were made up with concentration unknown to us until after the determination had been made. These samples are indicated on Fig. 2 by the point indicated by ▲. The lowest amount of chondroitin sulphate used (10^{-8} g) gave a net radioactivity (95.7 counts/min) that was still in excess of the blank value (85.1 counts/min) and so the gross radioactivity of this sample was more than twice "background". All samples were run in pentuplicate to allow for technical failures. Usually at least four samples survived to be counted.

DISCUSSION

The difference in the binding of methylated histone fraction F1 to a blank gel and to a similar gel containing 10^{-8} g chondroitin sulphate emphasises the relatively weak adsorption of the modified protein by the gel. The weight ratio of acrylamide to chondroitin sulphate was about 6×10^4 but the ratio of binding was about unity. Some of the "binding" of the histone to the blank gel may have been due to formation of aggregates that move only slowly in the gel. It was found that a considerable sticking (about 500 counts/min) of the radioactivity occurred to the first millimetre of the gel. This was removed in the experiments with radioactivity by rejecting a thin layer at the top of the gel. The radioactivity at the origin increased slightly over the range of chondroitin sulphate concentrations which suggests that a small part of the mucopolysaccharide sample did not penetrate the gel.

The histone fraction F1 was a cationic polymer readily available to us. This substance has the advantages of a relatively low and controlled molecular weight and the tendency to aggregate is not too pronounced[8]. From the work of AGRELL AND HEBY[1] it is obvious that other cationic polymers and perhaps some polyamines might be tried as a substitute for fraction F1. Methylation is a convenient method of labelling as the basic properties of the protein are conserved or perhaps even enhanced.

The reaction obtained is specific to acidic polysaccharides and other polymers that form aggregates with histone at the pH of the gel (4.6). It is necessary that the polymer should readily penetrate the gel; hyaluronic acid will not penetrate but heparin will do so. The latter forms a complex with similar properties to those described here. The binding for a given weight of heparin was about twice that for the same weight of chondroitin sulphate and this ratio corresponds roughly to the relative amounts of sulphate in the two substances. It is obviously necessary to liberate the chondroitin sulphate from its protein complex by hydrolysis of the protein. When this is done, competition between the histone and proteins in the sample for sites on the chondroitin sulphate should be minimized.

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