None of the equilibrium concentrations discussed here have been corrected for variation of the activity coefficients from unity, but the absence of any apparent dependence of the constants on reactant concentrations suggests that the apparent free energies of hydrolysis calculated here differ little from the true free energies of hydrolysis.

SUMMARY

1. Specific enzymic methods were used to estimate the equilibrium concentrations of $(\alpha + \beta)$ -D-glucose 6-phosphate and of α -D-glucose 1-phosphate in the products of the reaction catalysed by muscle phosphoglucomutase at pH 7.0 and 25° with 25 mM-Mg²⁺. The apparent equilibrium constant, $[(\alpha + \beta)\text{-D-glucose 6-phosphate}]/[\alpha\text{-D-glucose 1-phosphate}]$, was 17 ± 2 and the apparent free energy of phosphoryl transfer ($\Delta G'$) was -1.7 ± 0.1 kcal./mole.

2. Specific enzymic methods were also used to estimate the equilibrium concentrations of $(\alpha + \beta)$ -D-glucose and of $(\alpha + \beta)$ -D-glucose 6-phosphate in the products of hydrolysis of glucose 6-phosphate catalysed by intestinal alkaline phosphatase. Orthophosphate was estimated as phosphomolybdate. At pH 7.0 with 5 mM-Mg²⁺ the apparent equilibrium constant $[(\alpha + \beta)\text{-D-glucose}]/[\text{orthophosphate}]/[(\alpha + \beta)\text{-D-glucose 6-phosphate}]$ was 260 ± 50 , and the apparent free energy of hydrolysis was -3.3 ± 0.1 kcal./mole. There was little change in the apparent equilibrium constant with increasing concentration of Mg²⁺ ions between 0 and 5 mM at pH 7.0.

3. From these results, the apparent free energy of

hydrolysis of α -D-glucose 1-phosphate is -5.0 kcal./mole at pH 7.0 in the presence of Mg²⁺ ions.

We wish to thank Professor S. Angyal, University of New South Wales, for valuable advice on the effect of configuration on thermodynamic properties in hexose phosphates.

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The Chemistry of Connective Tissues

6. THE CONSTITUTION OF THE CHONDROITIN SULPHATE-PROTEIN COMPLEX IN CARTILAGE†

By S. M. PARTRIDGE, H. F. DAVIS AND G. S. ADAIR
 Low Temperature Research Station, Downing Street, Cambridge

(Received 29 July 1960)

It has been known for some time that a large part of the chondroitin sulphate of cartilage is combined with a protein which is not derived from collagen (Shatton & Schubert, 1954; Partridge & Davis, 1958a; Muir, 1958). It appears to be this entity which enters into loose combination with

collagen fibres and soluble collagen to give the tissue its characteristic physical properties and insolubility. Several different procedures have been used for the extraction from cartilage of material containing chondroitin sulphate and protein. The most effective of these have included the use of 10% calcium chloride solution (Meyer & Smyth, 1937; Mathews & Dorfman, 1953; Mathews, 1956;

† Part 5: Thomas & Partridge (1960).

Muir, 1958), extraction with warm aqueous 50 % phenol (Westphal, 1952; Partridge & Davis, 1958*b*), the use of a preliminary heat treatment to effect the release of the complex by thermal shrinkage of the collagen fibres (Partridge, 1948) or extraction with 30 % potassium chloride solution made mildly alkaline by the addition of potassium carbonate (Einbinder & Schubert, 1950; Partridge & Davis, 1958*a*).

Since the physical properties of the products differ widely according to the method of extraction it is probable that these extraction conditions, and the subsequent purification procedures which are necessary to remove collagen, together result in some degree of denaturation or chain rupture. However, quite recently Malawista & Schubert (1958) found that if either fresh or dried cartilage powder is homogenized vigorously with a very large volume of water at temperatures below 15°, a large part of the material passes into solution. The solution is very viscous and about half the dry matter consists of collagen in a soluble and apparently undenatured form, which is readily precipitated by the addition of 2 vol. of ethanol. The chondroitin sulphate-protein complex remains in solution but is precipitated on addition of potassium acetate to the ethanolic solution.

In the present work two sources of the chondroitin sulphate-protein complex were used: material extracted from dried powdered cartilage by homogenizing in a large volume of water and material extracted in the same way from fresh cartilage slices. The complex prepared from dried cartilage proved to be slightly degraded, but because of the very mild conditions used it is thought that the product from fresh cartilage was substantially in the native state.

Evidence has already been given (Partridge & Davis, 1958*a*; Muir, 1958) that the protein component of the complex is firmly bound, and probably chemically combined with the polysaccharide residue, and other evidence is given in the paper which follows (Partridge & Elsdon, 1961). In order to obtain further information about the structure of the complex it was decided to compare the polysaccharide chain weight (as measured by estimating the reducing power due to the terminal residue) with molecular weights determined osmotically, and to study both the native complex and products formed from it by controlled degradation with enzymes.

METHODS

Preparation of cartilage powder. Fresh cartilage from nasal septa of cattle was freed from adhering connective tissue and finely chopped with a scalpel. The pieces were washed twice with 1% (w/v) NaCl solution by gently

shaking and were then rinsed in water and put into acetone. After standing for some time in two or three changes of solvent they were dried in air at 70° and ground in a hammer mill through a sieve with circular apertures 0.56 mm. in diam.

Preparation of chondroitin sulphate-protein complex from fresh cartilage slices. The extraction procedure was based on the method described by Malawista & Schubert (1958). Fresh cartilage was taken as soon as possible after slaughter from cattle 2-3 years old. After removal of adhering connective tissue the cartilage was cut into strips, rapidly washed with 1% (w/v) NaCl and stored at -20°. Batches (10 g.) were taken from storage and cut into slices about 2 mm. thick, the slices being dropped at once into 300 ml. of ice-cold water. The suspension was then vigorously homogenized in an Ato-Mix homogenizer (Measuring and Scientific Equipment Ltd., London) at 12 000 rev./min. for 5 min. or until the temperature rose to 15°. The glass container with the suspension was then cooled in an ice bath to 2° and the homogenization was repeated for a further 5 min. This was repeated twice more, the total time of homogenization being 20 min. at 2-15°. The suspension was centrifuged and 2 vol. of ethanol was added to the supernatant. The mixture was allowed to stand for 2 hr., when Celite 545 (Johns-Manville, London) (10 g.) was stirred in and the suspension centrifuged for 20 min. at 2500 rev./min. The supernatant was filtered, first through a glass-wool plug and then through a fluted filter paper (Whatman no. 54). Potassium acetate (10 g.) was then added to the solution and the precipitate collected by centrifuging. It was washed three times with 75% (v/v) ethanol to remove potassium acetate and then taken up in 40 ml. of water. The solution was colourless, clear and very viscous. It was dialysed in cellophane overnight at 2° against a large volume of distilled water and stored in the frozen state at -20°.

Preparation of chondroitin sulphate-protein complex from dried cartilage powder. The powder (2.5 g.), which had been stored at room temperature for several weeks, was rehydrated by allowing it to stand with 300 ml. of water for 1 hr. The suspension was cooled and extracted with the Ato-Mix homogenizer in the same way as with fresh cartilage slices. As judged from the hydroxyproline content, the chondroitin sulphate-protein complex isolated by precipitation with potassium acetate contained 10-15% of collagen in different preparations and further purification was effected by treatment with a carboxylic acid ion-exchange resin (Partridge & Davis, 1958*a*). The resin (Amberlite CG 50, 100-200 mesh) was prepared by treating it with 2*N*-NaOH and washing by decantation. This was followed by treatment with 2*N*-HCl and further washing. It was then equilibrated with 0.1*M*-potassium acetate of pH 5.0 by stirring with several successive volumes of buffer. The treated resin (dry wt. 1 g.) was added to 180 ml. of an approx. 1% (w/v) solution of the chondroitin sulphate-protein complex and 20 ml. of *M*-potassium acetate buffer (pH 5) was added. The mixture was gently agitated in a mechanical shaker for 1 hr. and the resin was then separated by centrifuging. The process was repeated once more with a fresh batch (1 g.) of resin. After dialysis to remove buffer the complex recovered by drying in the frozen state had a hydroxyproline (colorimetric) content of 0.5%, showing that contamination with collagen was not more than about 3.5%.

Treatment of the chondroitin sulphate-protein complex with papain. Solutions of the chondroitin sulphate-protein complex were found to be too viscous for accurate pipetting and were therefore weighed out. A portion (30 g.) of an approx. 1% (w/v) solution, which had been thawed at room temperature after storage at -20° , was mixed with 24 ml. of water and 6 ml. of 0.2M-potassium phosphate buffer, pH 6.5. Cysteine hydrochloride (1.2 m-moles) and two or three crystals of benzoic acid were then dissolved in the solution. Crystalline papain (3 mg.; Kimmel & Smith, 1954) was added and the pH readjusted to 6.5 by the addition of N-NaOH. The solution was incubated for 24 hr. at 37° and dialysed for 24 hr. against repeated changes of distilled water. A light flocculent precipitate formed during dialysis and was removed by filtration. A portion (40 ml.) of the clear colourless solution was stored at -20° for osmotic-pressure experiments, and the remainder (46 ml.) was reserved for dry-weight determination (by freeze-drying) and for estimation of reducing power.

Treatment of the chondroitin sulphate-protein complex with hyaluronidase. The action of hyaluronidase after varying periods of incubation from 6 hr. to 3 days was investigated as follows. The solution of chondroitin sulphate-protein complex which had been stored at -20° was thawed, thoroughly mixed and its concentration was determined by freeze-drying a sample. The viscous solution contained about 1% of solids and 24 ml. was measured out by weighing. This was mixed with 6 ml. of buffer solution (0.5M-potassium acetate, pH 4.42, containing 0.75M-NaCl). Part of this solution (10 ml.) was used as an untreated control and the remainder (20 ml.) was incubated with ovine hyaluronidase (Benger Laboratories Ltd., 4 mg.) at 37° under toluene. Control solutions containing only hyaluronidase and buffer were also set up. In the experiments in which the incubation time was longer than 24 hr. a further quantity of hyaluronidase (2 mg.) was added after each 24 hr. period.

The control solutions were dialysed against distilled water and the volumes were measured. Part of the untreated control solution was then used for determination of reducing power (glucose equivalent) and the remainder dried from the frozen state for determination of dry matter, ash and nitrogen. The reducing power of the solution after treatment with hyaluronidase was estimated and the glucose equivalent of the incubated enzyme plus buffer control solution was subtracted. The remainder of the solution was adjusted to pH 8 and ultrafiltered through the highly permeable membrane described. Ultrafiltration was allowed to proceed until the volume in the sac was 10–15% of the original volume. Water was then added to make up the original volume and the solution refiltered. This was repeated four times. To test the efficiency of the ultrafiltration process the distribution of reducing power in the successive ultrafiltrates was determined. In one experiment (incubation time 24 hr.) the total glucose equivalent of the solution after treating with hyaluronidase was 7.50 mg. The first, second, third and fourth ultrafiltrates contained respectively 4.74, 1.16, 0.30 and 0.12 mg. of glucose equivalent (total 6.32 mg.), and the glucose equivalent remaining in the sac was 1.44 mg. The small quantity of diffusible reducing substance remaining in the sac was removed by dialysis against a large volume of water. The ultrafiltrates were combined and the volume

was measured; the nitrogen content and glucose equivalent were determined on the solution. The solution was then dialysed through cellophane and a sample from the cellophane sac was dried from the frozen state for determination of nitrogen and ash. The polysaccharide content of the total ultrafiltrate was then calculated from the nitrogen content of the solution and its volume.

The solution remaining in the ultrafiltration sac was free from buffer salts. The reducing power was determined on the solution and a portion was dried from the frozen state for determination of dry weight, nitrogen and ash. The remainder was used for determination of molecular weight by measurement of osmotic pressure.

Purified potassium chondroitin sulphate. The sample was prepared by alkaline degradation and purified by the use of ion-exchange resin as described by Partridge & Davis (1958a) [Found: N, 2.83; hexosamine (colorimetric), 24.7; uronic acid as galacturonic acid (carbazole), 27.1; ash (as K_2SO_4), 30.2%].

Treatment of purified potassium chondroitin sulphate with hyaluronidase. Potassium chondroitin sulphate (2 g.) prepared as described above was dissolved in 200 ml. of 0.1M-potassium acetate containing 0.15M-NaCl and the pH was adjusted to 4.55 by the addition of acetic acid. Ovine hyaluronidase (40 mg.) was then added and the mixture incubated for 120 hr. at 37° under toluene. The solution was then dialysed for 72 hr. in Visking tubes against repeated changes of distilled water. Part of the solution of non-diffusible substances was dried from the frozen state for dry-weight determination and analysis. The total yield was 1.28 g. (anhydrous) (Found: N, 3.24; glucose equivalent, 8.36%). The increased nitrogen content compared with the starting material (N, 2.83%) could be accounted for by the enzyme added.

Estimation of hexosamines. Total hexosamine was estimated colorimetrically by the method of Elson & Morgan (1933) as modified by Rondle & Morgan (1955) after hydrolysis with 4N-HCl for 16 hr. in a sealed tube at 100° . Glucosamine and galactosamine were estimated separately by the chromatographic method of Gardell (1953) after hydrolysis under the same conditions as were used for the colorimetric method. In some experiments glucosamine and galactosamine were determined by the use of a 9 mm. $\times$ 15 cm. Moore, Spackman & Stein (1958) column and by elution with 0.35M-sodium citrate buffer, pH 5.26, at 50° (cf. Eastoe, 1954). The peaks due to the hexosamines were estimated by the use of the ninhydrin reagent of Yemm & Cocking (1955).

Estimation of hydroxyproline. As a rapid method for use where the content of collagen was high, the colorimetric method of Newman & Logan (1950) as modified by Miyada & Tappel (1956) was employed. This method gave high results when used to estimate the hydroxyproline content of samples containing less than about 5% of collagen. This was probably due to interference from tyrosine or tryptophan in the non-collagenous protein (see Miyada & Tappel, 1956). For final analysis of samples with a low hydroxyproline content the material was refluxed with 6N-HCl for 24 hr. and the hydroxyproline peak separated chromatographically with a 15 cm. Moore & Stein (1951) column. The column was equilibrated and eluted at 37° with 0.1M-sodium citrate buffer, pH 3.0, and concentrations in the peaks were measured by use of the ninhydrin reagent of Yemm & Cocking (1955).

Estimation of reducing power due to the terminal residue in chondroitin sulphate chains. The measurement of reducing power by standard methods proved to be subject to a number of errors when applied to chondroitin sulphate. As a model substance the oxidation of the purified preparation of potassium chondroitin sulphate described above was studied, and three methods of determining the reducing power were investigated: (1) The Somogyi (1937) copper-reduction method as modified by Nelson (1944). (2) The Hagedorn & Jensen (1923) alkaline-ferricyanide method as modified by Hanes (1929). (3) The alkaline-iodine method as described by Weissmann, Meyer, Sampson & Linker (1954).

Results from the iodine method were consistently about 50% higher than those from copper reduction, but since Weissmann *et al.* (1954) have shown that *N*-acetylglucosaminic acid required 3.2 equiv. of I_2 /mol. instead of 2 equiv./mol. this result is understandable. The alkaline-ferricyanide method gave results which were high and erratic and accordingly all further determinations were carried out by copper reduction. The essential validity of the method as applied to pure preparations of chondroitin sulphate was shown by analysis of a sample which had been dialysed exhaustively in cellophane. The chain weight by copper reduction was 16 600, and the molecular weight, determined osmotically, was 16 100. The procedure finally adopted was as follows.

Samples of the solution to be estimated, containing up to 0.3 mg. of glucose equivalent, were placed in 17 mm. test tubes provided with calibration marks at 2 and 25 ml. If the solution was very viscous, as with the native chondroitin sulphate-protein complex, the solution was weighed in the tubes. The volume was then made up to the 2 ml. mark with water. Usually three pairs of duplicates, containing solutions of different concentration, were taken for each estimation. The Somogyi copper reagent A (2 ml.; Nelson, 1944) was then added and the tubes were heated in a boiling-water bath for 10 min. The tubes were not disturbed whilst in the bath. After cooling, 2 ml. of the Nelson (1944) arsenomolybdate reagent was added and the tubes were well shaken. They were then made up to the 25 ml. mark and read with the Hilger Spekker absorptiometer with 1 cm. cells and an Ilford no. 604 green filter. A calibration curve was constructed for glucose with quantities in the range 0.03–0.3 mg. and the reducing power of the test solution was expressed in terms of mg. of glucose equivalent/ml. When solutions which contained buffer or added enzyme were examined, the glucose equivalent of the buffer or the enzyme was determined separately and subtracted.

Preparation of membranes. The membrane of high permeability used for ultrafiltration was made from a solution composed of 4 g. of pyroxylin (Imperial Chemical Industries Ltd.; reference no. HL 120/170), 50 ml. of absolute ethanol, 50 ml. of anhydrous ether and 18 ml. of ethylene glycol. Five coats of this solution were poured over a glass tube rotated at 15 rev./min. The tube was dried for 20 hr. at 18° in a room with a humidity of 58%. The membrane was 13.5 cm. long, 2.22 cm. in diam. and 0.032 cm. thick. At a pressure of 340 mm. Hg the rate of ultrafiltration of water was 0.77 ml./min. at 16°. This rate is comparable with the rate calculated for a membrane with cylindrical pores of diameter 212 Å. The membranes for the osmotic experiments were prepared as described by Adair (1949). All calcula-

tions of ultrafiltration rates given later, represented by the symbol q^* , refer to a temperature of 16°, a pressure of 441 mm. Hg and an area of 28.4 cm.².

RESULTS

Properties of the complex extracted from dried cartilage powder and from fresh cartilage

In fourteen experiments the yield of crude chondroitin sulphate-protein complex obtained from 2.5 g. of dried cartilage powder varied from 0.39 to 0.56 g. The hydroxyproline content of the product varied from 1.46 to 2.34%, indicating contamination with collagen to the extent of 10–15%. After a single treatment with the resin IRC-50 the hydroxyproline content (colorimetric) was decreased to 0.5–0.9%, but further resin treatment was ineffective in decreasing the hydroxyproline content of any sample below 0.3–0.5%. This corresponded to a maximum of 3.5% of collagen. [Analysis of a bulked sample of the product from 10 preparations after vacuum-ice drying showed: N, 4.83; hydroxyproline (colorimetric), 0.29; glucosamine, 3.3; galactosamine, 19.4; ash (as K_2SO_4), 25.5; uronic acid (carbazole), 22.4%.]

The course of extraction from fresh cartilage was rather different. The yield of complex from eighteen batches, each of 10 g. of cartilage slices (about 2.5 g. dry wt.), varied from 0.326 to 0.420 g. Separation of soluble collagen by precipitation with ethanol was more nearly quantitative than with the extracts of dried cartilage, and the hydroxyproline content (colorimetric) of the samples was in the range 0.31–0.55%. Treatment with ion-exchange resin (IRC-50) did not decrease this figure and no further purification was attempted. A representative sample of the chondroitin sulphate-protein complex, drawn from twelve batches, had: N, 5.72; glucosamine, 3.4; galactosamine, 20.2; ash (as K_2SO_4), 22.68; uronic acid (carbazole), 20.0%.

It is known that, in addition to chondroitin sulphate A, bovine hyaline cartilage contains smaller quantities of other polysaccharides: in particular chondroitin sulphate C and keratosulphate (Meyer, Linker, Davidson & Weissmann, 1953). It is commonly accepted that these two polysaccharides are also combined in some way with protein but it is not known if they exist as independent polysaccharide-protein complexes or if two or more polysaccharide chains of different types may be attached to the same protein. At present no means of separating the macromolecular complexes are known to the authors and, in view of the difficulty of avoiding partial degradation even with very mild treatments, no extensive fractionation of the extracted complex was

Table 1. *Number-average chain weight (Cn) and number-average molecular weight (Mn) of preparations from dried cartilage powder*

	N (%)	Cn	Mn
(A) Water extract	5.13	18 000	140 000 ± 20 000
(B) (A) treated with papain	2.86	18 100	14 000 ± 3 000
(C) Purified chondroitin sulphate (K ⁺ salt) prepared by alkaline degradation	2.83	16 600	16 100 ± 1 000
(D) (C) treated with hyaluronidase followed by dialysis			
Preparation 1	3.24	2 150	3 800
Preparation 2	3.28	2 860	4 900

Table 2. *Chain weight (Cn), molecular weight (Mn) and number of reducing residues per molecule (R) of preparations from fresh cartilage*

Values (*) are calculated for the potassium salt.

Preparation	Source	N (%)	Cn	Mn	R
E1	Water extract	5.25	43 000	1.5 × 10 ⁶ *	23–115
E2	Water extract	5.72	53 000	1.5 × 10 ⁶ *	19–95
F1	Preparation E1 treated with papain	2.87	51 000	28 500 ± 2 000*	0.56
F2	Preparation E1 treated with papain	2.87	50 000	27 500 ± 1 000*	0.55
G	Preparation E2 treated with hyaluronidase followed by ultrafiltration	9.85	7 730	170 000 ± 2 000*	23 ± 2

attempted. It has not been considered necessary to distinguish between chondroitin sulphate A and chondroitin sulphate C in this work since the two polysaccharides are closely related isomers. In the paper that follows (Partridge & Elsdon, 1961) some evidence is presented in favour of the suggestion that keratosulphate forms an integral part of the complex containing chondroitin sulphate. The content of glucosamine in the ethanol-fractionated water extract is less than 15% of the total hexosamine, showing that the keratosulphate content of the total polysaccharide could not be greater than about 15%.

Determination of chain weight (Cn) by measurement of reducing power due to the terminal residue

In applying the method of chain-weight determination described above to the degradation products of the chondroitin sulphate-protein complex, there were several sources of error which appeared to have little importance with the pure polysaccharide:

(1) Losses in reducing power due to Maillard (1912) reactions. This error was reduced by avoiding the storage of dried products. Samples for analysis were stored as solutions in the frozen state at -20°.

(2) Loss of reducing power due to the use of ethylenediaminetetra-acetate (EDTA) in the experiments with papain. Ogston & Sherman (1959) have shown that EDTA in the presence of trace amounts of heavy metals probably acts as an oxidation catalyst. The use of EDTA proved to be unnecessary and was avoided in further experiments.

(3) Loss of reducing power due to the use of ether or other peroxide-containing solvents.

(4) Errors due to the reducing power of the protein moiety, which appeared to be small.

The procedures for the preparation of samples for analysis described under Methods were designed to avoid these sources of error so far as was possible. Replicate determinations of reducing power on the same sample agreed to within ± 2% but determinations on different samples of chondroitin sulphate-protein complex showed ± 6% variation in one series.

The number-average chain weight (Cn) of a preparation was calculated from the expression

$$Cn = \frac{180 \times 1000}{\text{glucose equivalent}},$$

where 180 is taken as the molecular weight of glucose and 'glucose equivalent' is defined as the reducing power of 1 g. of sample expressed as mg. of glucose. The results obtained with extracts of dried cartilage powder are summarized in Table 1 and those with extracts from fresh cartilage in Table 2. The values for Cn in the Tables are not corrected for the varying amounts of protein in the different preparations.

Determination of molecular weight (Mn) from osmotic-pressure experiments

The majority of the measurements of osmotic pressure were made with a high concentration of diffusible electrolyte and very low concentrations, usually less than 0.2 g. of chondroitin sulphate/100 ml., in order to decrease the partial pressure,

π_i , due to the net excess of diffusible ions inside the membrane. Formula (1), used for calculating π_i for ideal solutions, was derived from more general formulae published by Adair & Robinson (1930):

$$\pi_i = RT (C_e \alpha)^2 / 4I. \quad (1)$$

(R , Gas constant; T , absolute temperature.) Since all pressures are given in mm. Hg at 0° , the product RT is equal to 17 034. The symbol C_e denotes the equivalent concentration of the potassium or sodium salt of chondroitin sulphate, discussed below, and α denotes the degree of ionization of this salt. The symbol I denotes the ionic strength of the diffusible electrolytes in the external solution or dialysate. Most of the colloidal solutions were dialysed with large volumes of an external solution made up with 0.015M- Na_2HPO_4 , 0.005M- NaH_2PO_4 and 0.5M- NaCl . This external solution has pH 6.96 at 1° and I 0.55.

Determination of α . It is difficult to make direct measurements of α for solutions with high concentrations of diffusible salts, and therefore measurements were made with solutions in water. One solution, with a final concentration of 0.889 g. of the potassium salt of chondroitin sulphate/100 ml., gave an osmotic pressure of 288 mm. Hg at 0° and an 'apparent molecular weight' of 526. A more dilute solution, with 0.49 g./100 ml., gave a pressure of 164.1 mm. and an apparent molecular weight of 509. The term apparent molecular weight denotes the ratio M/ϕ , where M is the average molecular weight and ϕ is a type of osmotic coefficient, which represents the ion-pressure difference π_i and deviations from the ideal solution laws. The ratio M/ϕ is calculated from formula (2):

$$M/\phi = 10RT(C/\pi) = 170\,342 (C/\pi). \quad (2)$$

(π , Osmotic pressure, mm. Hg at 0° ; C , g. of chondroitin sulphate/100 ml. of solution.) Values of M/ϕ for the solutions of the potassium salts of chondroitin sulphate in water, 526 and 509, are small compared with the mean molecular weight (M 14 300) estimated for solutions with I 0.55; thus the factor ϕ is large and the greater part of the observed osmotic pressures of the solutions in water is due to K^+ ions. It may be noted that the observed values of M/ϕ are approximately twice the equivalent weight, 268, calculated on the assumptions that the carboxyl groups and the sulphate groups are fully ionized. If the degree of ionization (α) be 0.5, and the equivalent weight be 268, the product $C_e \alpha$ in equiv./l. is 0.0187C.

If it is assumed that $C_e \alpha$ is 0.0187C when I is 0.55 it follows that $\pi_i = 2.6C^2$. For a dilute solution the pressure π_i is very small (0.026 mm. Hg when C is 0.1 g./100 ml.) and for the purpose of calculating molecular weight by the modified

form of van't Hoff's law (equation 3) measurements were made on solutions where C was usually less than 0.2:

$$\pi = \pi_p + \pi_i = (10RT/M) C + KC^2. \quad (3)$$

(π_p , Pressure due to colloidal ions; KC^2 , a small term, proportional to C^2 , which represents the term π_i and the effects of deviations from the ideal solution laws.) The value 2.6 is not exact and the range of application of equation 3 should be limited to solutions where C is less than 0.2 and M is less than 100 000 and π_p is much greater than π_i . Preliminary experiments on solutions with higher concentrations (0.3–0.8 g./100 ml.) gave variations in M greater than the experimental errors.

With the samples of chondroitin sulphate prepared by dissociation with alkalis or by papain treatment the values of π_i were much smaller than the observed osmotic pressures, when I was 0.55, and it was thus possible to estimate their molecular weights within the limits given (Tables 1 and 2). In other preparations the molecular weights were very high or very low. Details of the treatment of these samples are given below.

Complex prepared from fresh cartilage. From viscosity and light-scattering data, Mathews & Lozaityte (1958) report, for the chondroitin sulphate-protein complex, weight-average molecular weights of 4×10^6 or more. A solution of the complex prepared from fresh cartilage with a final concentration of 0.17 g./100 ml. gave an osmotic pressure of 1.03 mm. of toluene at 0.68° , equivalent to 0.067 mm. Hg at 0° . The apparent molecular weight M/ϕ is 434 000. The observed osmotic pressure, 0.067 mm., is only slightly greater than the partial pressure, π_i 0.051 mm., estimated by assuming that the equivalent weight is 268, the equivalent concentration is 0.0052 and α is 0.5. The result is consistent with that of Mathews & Lozaityte since M/ϕ is much less than M when the greater part of the observed osmotic pressure is due to inorganic ions.

Complex prepared from dried cartilage. A sample of the complex prepared from dried cartilage gave a pressure of 4.0 mm. toluene at 0.7° , equivalent to 0.26 mm. Hg at 0° , for a solution containing 0.155 g./100 ml. This pressure is larger than the pressure of 0.06 mm. estimated for the diffusible ions in a solution containing the same concentration of chondroitin sulphate. The ratio M/ϕ is 102 000, and the mean molecular weight M is approx. $140\,000 \pm 20\,000$.

Purified sodium chondroitin sulphate prepared by alkaline degradation. Samples of chondroitin sulphate, degraded by alkalis, gave much higher pressures and smaller molecular weights. One solution (0.1 g./100 ml.) gave a pressure of 16.7 mm.

toluene at 0.7°, or 1.08 mm. Hg at 0°. The ratio M/ϕ was 15 700 and the mean molecular weight M was 16 100. A solution with 0.369 g./100 ml. gave a pressure of 4.31 mm. Hg and a molecular weight of 15 900. A membrane of permeability q'' 0.0116 was used for these measurements.

Another sample prepared by dialysis with a more permeable membrane (q'' 0.0805) gave an average molecular weight of 19 000. Comparisons of initial and final concentrations showed that about 30% of the chondroitin sulphate had diffused across the membrane or had been adsorbed by the membrane in the course of 7 days. These losses of chondroitin sulphate were observed with solutions of high ionic strength (I 0.55). A membrane of high permeability (q'' 0.164) retained chondroitin sulphate for months, when water was used as solvent.

Purified chondroitin sulphate treated with hyaluronidase. Samples of chondroitin sulphate which had been degraded by alkalis and then digested with hyaluronidase contained substances which diffused across most collodion membranes. The apparent osmotic pressure π^* given by these membranes is the pressure at which gains in volume due to osmosis are balanced by losses due to ultrafiltration of solvent and leakage of the solute. This pressure π^* may be less than the pressure π given by an ideal membrane impermeable by the solute. Losses of solute and apparent osmotic pressures were influenced by the properties of the membranes.

An increase in the permeability for water, symbolized q'' , was correlated with a reduction in the pressure π^* and an increase in the uncorrected molecular weight M^* calculated from π^* . The smallest value of M^* , 3052, was obtained with the least-permeable membrane with an ultrafiltration rate of 0.0041 ml./min. A value of 3587 was obtained with the second membrane, with an ultrafiltration rate of 0.011 ml./min. The maximum value for M^* , 4875, was given by the third membrane with an ultrafiltration rate of 0.0258 ml./min. These figures are useful as an upper limit. The corrected values must be smaller than M^* .

Comparison of chain weight and osmotic molecular weight

Preparations from dried cartilage. The results of the osmotic-pressure experiments carried out with preparations from dried cartilage powder are compared with the determinations of chain weight in Table 1. The initial chondroitin sulphate-protein complex had Mn about 140 000 and the polysaccharide chain weight was only 18 000. After papain treatment there was a decrease in nitrogen content due to removal of the protein moiety; the polysaccharide chain weight remained about the same but the osmotic molecular weight was decreased to a figure closely approximating to the

chain weight. This suggests that the chondroitin sulphate-protein complex is composed of a series of chains of chondroitin sulphate, linked together by the protein to form a larger molecule. The chain weight of the purified chondroitin sulphate agreed well with its molecular weight and the two values lie fairly close to those for the complex after papain treatment. This might suggest that there is little chain rupture during the purification of chondroitin sulphate by treatment with alkali, but in fact the preparation was extensively dialysed through Visking tubing and considerable losses of material were observed owing to diffusion of chain fragments through the membrane.

When the purified chondroitin sulphate was treated with hyaluronidase and dialysed through cellophan about 36% of its weight was lost by diffusion through the membrane. The remainder (preparation 1) consisted of material of Cn 2150. The osmotic-pressure determinations on this material gave a rather higher figure for Mn and since it was observed that there were losses of chondroitin sulphate through the osmotic membrane it was thought that the higher value was due to loss of material of lower molecular weight during osmotic equilibration. Accordingly the experiment was repeated with a new preparation (preparation 2 in Table 1), but this time Cn was determined on the material remaining in the sac after conclusion of the osmotic experiment. Cn so determined was rather higher, 2860, but the agreement with Mn on the same preparation was still not as close as would be expected by taking into account the known errors.

Preparations from fresh cartilage slices. The values obtained for the osmotic molecular weight of samples of chondroitin sulphate-protein complex from dried cartilage powder were considerably lower than several estimates given in the literature for the weight- and number-average molecular weights of material extracted from fresh cartilage. The possibility that the process of drying, milling or storing powdered cartilage had resulted in some degradation was therefore considered. Water extracts were made by the use of the homogenizer from sliced fresh cartilage obtained from selected young animals. The extracts were found to be more viscous than those from dried material, and it was also found that, provided that the extraction was carried out below 15°, the collagen separated very sharply on the addition of ethanol.

A similar set of values for Cn and Mn , obtained with material from fresh cartilage, is given in Table 2. The osmotic molecular weight for these preparations ($1-5 \times 10^6$) was much higher than for those obtained by extraction of dried cartilage. The values for Cn in Table 2 were determined on the solution remaining in the sac after the osmotic

experiments, in order to avoid the possibility of error due to the loss of low-molecular-weight material by adsorption on the membrane or by diffusion through it. After treatment with crystalline papain Mn fell to 28 000 but Cn remained substantially the same at 50 000. Since the molecular weight cannot be less than the chain weight, and both are number averages, it was thought that the value for the apparent chain weight (Cn) may be high due to the presence of chains in which the terminal reducing residue was masked.

The masking of terminal groups by oxidation during the course of treatment with papain readily occurs if EDTA is present and may be catalysed by traces of heavy metals. Thus when the reaction was carried out in the presence of cysteine but not EDTA the results of a number of determinations of Cn on different preparations were in the range $45-55 \times 10^3$, but with EDTA also present the results were erratic and values up to 85×10^3 were found. It is thought possible that cysteine also promotes heavy-metal catalysis to a lesser extent and that the true value of Cn may be rather lower than 50 000.

Experiments with hyaluronidase

After treatment of the native complex with hyaluronidase, followed by ultrafiltration, the chondroitin sulphate content of the complex fell to approx. 30 %. The chain weight (uncorrected for protein content) fell to 7730 but the osmotic molecular weight (170 000) remained at more than 20 times this value. In the last column of Table 2, calculated values for the number of reducing residues (R) in the molecule for each preparation

Fig. 1. Action of hyaluronidase on a model molecule in which the chondroitin sulphate chains are combined at several points to a protein core. The protein core is depicted by a heavy line and the arrow heads indicate the reducing terminals of the chondroitin sulphate chains.

are given. For the native complex the range for R is 19–115; for the papain-treated preparations the values for R are less than unity owing to the apparent masking of the reducing terminals of some of the chains; for the hyaluronidase-treated preparation, which contains a very large proportion of protein, R is 23 ± 2 . It thus appears that the effect of hyaluronidase is to decrease the length of the chondroitin sulphate chains to points near the protein core, forming a new unit of Mn 170 000 which bears 23 short polysaccharide chains.

Observation of the course of reaction with hyaluronidase may yield information of another sort. Thus if each polysaccharide chain were combined at several points to a protein core, then prolonged action of hyaluronidase would be expected to break down the loops so formed and the product would have a higher reducing power than the starting material. In Fig. 1 this situation is depicted; the heavy line represents the protein core and the arrowheads represent the terminal reducing residues of the polysaccharide chains. It is clear, however, that an experiment of this sort will be ambiguous unless there is assurance that fragments of polysaccharide chains not attached to protein have been removed from the system. To this end a membrane of very high permeability was employed for ultrafiltration (for details of the membrane see the Methods section), and comparative experiments were carried out in which the degree of polysaccharide-chain breakage was varied by varying the time of digestion with hyaluronidase and the amount of enzyme. A series of experiments of this kind should result in end products which approach constant reducing power when no free chains remain of a size sufficient to retain them in the sac used for ultrafiltration. An analysis of the final experiments of the series appears in Table 3. It will be observed that with preparation 1 (incubation time 24 hr.) the mixture in solution after treatment with hyaluronidase had an average polysaccharide chain length of 4500 whereas with preparation 2 (incubation time 72 hr.) this average was 2450. In each ultrafiltration experiment the total glucose equivalent of the solution to be ultrafiltered was recovered nearly quantitatively in the ultrafiltrate and in the solution remaining in the sac. In the first experiment the chondroitin sulphate-protein complex taken had 25 ± 2 reducing terminals/ 10^6 g. of complex, whereas the 'core material' remaining in the sac had 41 ± 2 terminals/ 10^6 g. of starting material. With preparation 2 the chondroitin sulphate-protein complex (a different extract) had 19 ± 2 reducing residues/ 10^6 g. and the core substance remaining in the sac had 23 ± 2 reducing residues, calculated on the same weight of initial material. The results of the series showed that the values

Table 3. *Ultrafiltration of hyaluronidase-treated complex*

	Preparation 1 (incubated for 24 hr.)	Preparation 2 (incubated for 72 hr.)
(A) Original chondroitin sulphate-protein complex		
Dry weight (mg.)	195	251
Ash (% as Na ₂ SO ₄)	—	22.7
Nitrogen (%)	5.25	5.72
Total glucose equivalent (mg.)	0.86	0.86
C _n	40 600	53 000
Number of reducing terminal residues in 10 ⁶ g. of complex	25 ± 2	19 ± 2
(B) Chondroitin sulphate-protein complex after treatment with hyaluronidase		
Dry weight (mg.)	195	251
Total glucose equivalent (mg.)	7.84	18.1
C _n	4 500	2 450
(C) In ultrafiltrate		
Dry weight (mg.)	100.2	208
Ash (%)	—	28.0
Nitrogen (%)	2.78	2.93
Total glucose equivalent (mg.)	6.32	17.44
C _n	4 530	2 200
(D) In sac		
Dry weight (mg.)	107.7	44.6
Ash (%)	11.19	8.35
Nitrogen (%)	8.85	9.85
Total glucose equivalent (mg.)	1.44	1.04
C _n	13 500	7 730
Number of terminal residues in 10 ⁶ g. original wt. of complex	41 ± 2	23 ± 2

approached a 1:1 ratio as the degree of degradation increased. It is therefore concluded that each polysaccharide chain is attached to the protein core by a single linkage.

DISCUSSION

Chondroitin sulphate is now regarded as a linear polymer of the substituted disaccharide *N*-acetylchondrosine sulphate. Crystalline derivatives of chondrosine were isolated from the acid-hydrolysis products of chondroitin sulphate by Hebling (1914) and by Levene (1941), and structures for the disaccharide, differing in detail, were formulated by other workers (Meyer, Odier & Siegrist, 1948; Wolfrom, Madison & Cron, 1952; Meyer & Baldin, 1953). Davidson & Meyer (1954), in agreement with Masamune, Hiyama & Kobayasi (1942), offered the first conclusive evidence for the constitution of chondrosine as 3-*O*-(β -D-glucopyranosiduronic acid)-2-amino-2-deoxy-D-galactose.

Early methylation studies (Bray, Gregory & Stacey, 1944) suggested that the polysaccharide may be highly branched, but the isolation of chondrosine in high yield (60–70%) from the acid-hydrolysis products of chondroitin sulphate (Wolfrom *et al.* 1952; Davidson & Meyer, 1954) shows that glycosidically-linked branch chains

cannot be present in large amount. The polysaccharide contains alternate hexosaminidic and uronic bonds and both bovine testicular hyaluronidase and dilute acids degrade it by hydrolysing the hexosaminidic links (Rapport, Meyer & Linker, 1951). In chondroitin sulphate A the sulphate ester residue is linked to galactosamine through position C-4, but bovine nasal-septum cartilage also contains a small amount of the isomeric chondroitin sulphate C, which contains C-6 galactosamine-linked sulphate (Mathews, 1958; Hoffman Linker & Meyer, 1958).

Chondroitin sulphate exists in hyaline cartilage as a protein-containing complex of high molecular weight, but even when extracted under mild conditions the product appears to be polydisperse in molecular size. Webber & Bayley (1956) gave values for the molecular weight of the complex, as estimated by sedimentation and viscosity measurements, of about 1.0×10^6 , and Bernadi (1957) reports 1.98×10^6 from viscosity, sedimentation and light-scattering. Webber & Bayley (1956) studied the product obtained from the chondroitin sulphate-protein complex after tryptic digestion of the protein moiety and reported that the number-average molecular weight of the polysaccharide residue was 39 000 (sedimentation and viscosity), but a value of 145 000 was found for the weight-

average molecular weight from light-scattering data. Their product was accordingly regarded as widely polydisperse.

A different conclusion was reached by Mathews (1956), who isolated a chondroitin sulphate-protein complex from an extract of bovine hyaline cartilage and after treating it with proteolytic enzymes found that the product behaved as a monodisperse solution with molecular weight 50 000. Later Mathews & Lozaityte (1958) from light-scattering and viscosity data proposed that the basic molecular unit of the chondroitin sulphate-protein complex is a rod of length 3700 Å and 4.0×10^6 molecular weight. To account for the linear dimensions and distribution of matter in the particle they proposed that the protein moiety forms a core running the length of the rod, along which is distributed about 62 units of chondroitin sulphate, each of 50 000 molecular weight. Evidence was also presented which suggests the possibility that aggregates of much higher molecular weight may be formed by both lateral and end-to-end association of the basic units and that additional protein may be involved in this process.

In the present work the problem has been approached in another way. Since it is known that there are few, if any, glycosidically-linked side chains in the chondroitin sulphate of hyaline cartilage, it was thought that the observed formation of very large molecules was probably through links or branching points which do not involve the reducing group of the terminal galactosamine residue. Provided that the terminal residue is not masked in this or any other way, the number-average weight of linear chondroitin sulphate chains making up a multiple-chain molecule can be determined by measurement of the reducing power. The result can then be compared directly with the number-average molecular weight of the whole molecule, determined osmotically, to give the number of chains in the molecule.

The chain weight (C_n) of the complex extracted by water was $40\text{--}50 \times 10^3$ in different preparations. If this value is corrected for the non-chondroitin sulphate content of the complex (about 25 %), the true polysaccharide chain weight becomes $30\text{--}40 \times 10^3$. Osmotic measurements carried out with the chondroitin sulphate-protein complex give pressures corresponding to a molecular weight in the range $1\text{--}5 \times 10^6$. This estimate could not be obtained with greater precision since with polyions of such high molecular weight the Donnan correction for pressure due to the counter ions is a very high proportion of the total osmotic pressure. The two results together suggest that, as a minimum, the particles of chondroitin sulphate-protein complex in solution contain 20 chondroitin sulphate chains.

A similar result was obtained in another way. After removal of the protein from the complex by treatment with papain, the osmotic molecular weight of the preparation fell to 28 500, with a possible error of ± 1000 , showing that the papain treatment had released at least 30 chondroitin sulphate chains from combination in the molecule. The value for the osmotic molecular weight of the chondroitin sulphate released lies rather below that obtained for the corrected chain weight determined for the original molecule, and suggests that a few of the polysaccharide chains had masked terminal residues.

When the complex was treated with hyaluronidase, sections of the polysaccharide chains were split off, leaving a protein-containing core apparently intact. Short-chain fragments were separated from the core by ultrafiltration through a membrane of high permeability and, in experiments where degradation with the enzymes proceeded far enough, separation of the core from chondroitin sulphate chain fragments could be taken to completion by final dialysis. In a series of experiments on these lines the reducing power of the core material remaining in the membrane sac approached a constant value that was close to the reducing power of the original complex. This indicates that each polysaccharide chain in the chondroitin sulphate-protein complex is attached to the protein core at one point or one small region only. The osmotic molecular weight of the core material, which contained about 30 % of chondroitin sulphate, was 170 000; this leads to the value 120 000 for the non-chondroitin sulphate material of the core. It is shown in the next paper that this material is a mucoprotein containing a considerable amount of neutral sugar and hexosamine.

Fig. 2. Model proposed for the structure of the chondroitin sulphate-protein complex. The protein core (heavy line) carries at least 23 chondroitin sulphate chains of C_n 28×10^3 . These are connected by a single linkage which does not involve the terminal reducing residue (indicated by arrow heads). The core also carries, at some point along its length, a second polysaccharide material (indicated by a circle) which is rich in galactose and glucosamine.

The model which emerges is depicted in Fig. 2. In this, the particle of minimum molecular weight is conceived as a single protein chain (the heavy line), along which are distributed a large number of chondroitin sulphate chains each attached at a single point, the majority bearing a reducing terminal residue (represented by an arrow head). The protein chain also carries, at some point or points along its length, further polysaccharide material (represented by a circle) which contains neutral sugars and hexosamine.

The work reported here thus essentially confirms and extends the proposal by Mathews & Lozaityte (1958), although the values for the molecular weight of the protein-free chondroitin sulphate chains are considerably lower than those given by these authors and by Mathews (1956) and Mathews & Dorfman (1953). The molecular dimensions of the basic unit of the chondroitin sulphate-protein complex must remain in doubt because of the heterogeneity of its solutions and the possible formation of aggregates.

Determinations of the reducing power of the core material given in this paper showed that the core contained 23 reducing chains, each consisting, as an average value, of about 4–5 disaccharide repeating units. If the mucoprotein core, together with its 23 reducing chains, represents a residue of the smallest molecular unit of the chondroitin sulphate-protein complex, the molecular weight of the whole unit would be about 750 000 and the particles in solution with observed molecular weights of $1\text{--}5 \times 10^6$ must be regarded as aggregates. However, it is possible that labile bonds in the mucoprotein core are split during the course of treatment with hyaluronidase and that the true molecular weight of the complex is accordingly higher.

SUMMARY

1. The polysaccharide chain weight (C_n), estimated by measuring the reducing power of the terminal residue, was compared with the osmotic molecular weight (M_n) for chondroitin sulphate-protein complexes extracted from bovine hyaline cartilage.

2. Water extracts prepared from fresh cartilage slices in a high-speed homogenizer yielded a chondroitin sulphate-protein complex containing approx. 75% of chondroitin sulphate having M_n $1\text{--}5 \times 10^6$ and C_n 50×10^3 . Thus the 'osmotic molecule' contained 20–100 chondroitin sulphate chains terminating in reducing residues.

3. Removal of protein by digestion with papain decreased M_n to $28 \times 10^3 \pm 2000$ and C_n remained unchanged. The result indicates that the chondroitin sulphate chains in the complex are linked together by protein and that the reducing terminals

of a proportion of the chondroitin sulphate chains freed by papain are masked.

4. When the native complex was treated with ovine hyaluronidase and the chondroitin sulphate chain fragments were removed by ultrafiltration, the chondroitin sulphate content of the remaining core was decreased to about 30% and C_n was 7730. The protein-rich core had M_n 170×10^3 , showing the presence of 23 chondroitin sulphate chains of decreased length.

5. The complex had approximately the same reducing power before as after treatment with hyaluronidase and ultrafiltration, thus showing that each chondroitin sulphate chain is attached to protein at one point only.

6. It is concluded that the complex may exist in solution as a macromolecule of M_n $1\text{--}5 \times 10^6$ or an aggregate of smaller molecular units of M_n at least 750×10^3 . The units contain at least 23 chondroitin sulphate chains of C_n 28×10^3 . These are connected to a protein core by a single linkage which does not involve the terminal reducing residue.

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The Chemistry of Connective Tissues

7. DISSOCIATION OF THE CHONDROITIN SULPHATE-PROTEIN COMPLEX OF CARTILAGE WITH ALKALI

BY S. M. PARTRIDGE AND D. F. ELSDEN

Low Temperature Research Station, Downing Street, Cambridge

(Received 29 July 1960)

In the preceding paper a model was proposed for the constitution of the chondroitin sulphate-protein complex of cartilage in which at least 23 chondroitin sulphate chains are connected to a protein core by single linkages thought to be of covalent character. In the work now described, the dissociation of the complex in alkaline solution was studied more closely. Electrophoresis experiments in columns stabilized by a density gradient suggested that alkaline dissociation is irreversible and follows a time course related to the hydroxyl ion concentration. The protein core split off was isolated and analysed and was found to carry a second amino polysaccharide containing glucosamine and galactose.

METHODS

Chondroitin sulphate-protein complex. The complex was prepared from dried cartilage powder as described in the preceding paper. As it was important to decrease contamination with collagen to the lowest possible extent, treatment with ion-exchange resin (Amberlite CG 50) was repeated three or four times until the hydroxyproline content was less than 0.2% as judged by ion-exchange chromatography of the hydrolysed product.

Zone electrophoresis. The experiments were carried out

with an electrophoresis column stabilized by a sucrose density gradient. The method used was generally similar to that described by Svensson, Hagdahl & Lerner (1957), but since the design of the apparatus was different, and has proved to be advantageous in some respects, a drawing of it (Fig. 1) is given here. The apparatus was symmetrical and consisted of two similar glass columns, A and A', provided with ball-joints and arranged over a U tube B. Duplicate sets of columns with different diameters were provided so that the apparatus could be adjusted to suit the scale of the separation. The electrical circuit was completed by the provision of two vessels, C and C' (diameter 7.5 cm.), containing large silver-silver chloride electrodes and connected through large-bore taps, D and D'. A bridge E was provided to secure hydrostatic equilibrium when the taps D and D' were closed.

In the experiment described the columns were 2.7 cm. in diameter and the height between H and J' was 34 cm. The columns were not provided with water jackets and the experiment was carried out at room temperature.

The apparatus was first filled with buffer (0.005N-NaOH containing 0.015N-NaCl) to a point above the level of the side arms D and D'. A reservoir filled with dense buffer (600 ml., 0.01N-NaOH containing 0.025N-NaCl and 50% w/v, sucrose) was connected in series with a mixing chamber (capacity 550 ml.) and a metering pump. The mixing chamber was provided with a magnetic stirrer. The buffer stream of increasing density was pumped from the mixing chamber into the U tube through the tap I. The