yellow product is estimated spectrophotometrically. Ammonia does not interfere with the determination.

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Studies on Sulphatases

14. A PRELIMINARY ACCOUNT OF THE CHONDROSULPHATASE OF *PROTEUS VULGARIS**

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The existence in putrefactive bacteria of an enzyme which liberated sulphuric acid from chondroitin sulphate was first suspected by Neuberg & Rubin (1914). Subsequently, a concentrate of this enzyme, to which the name 'chondrosulphatase' was given, was obtained from an organism resembling *Bacterium fluorescens* 'non-liquifaciens' (syn. *Pseudomonas fluorescens* 'non-liquifaciens') (Neuberg & Hofmann, 1931*a, b*). Other putrefactive bacteria, *Bacterium proteus* (syn. *Proteus vulgaris*) and *Bact. pyocyaneus* (syn. *Pseudomonas aeruginosa*) were also shown to possess chondrosulphatase activity (Neuberg & Hofmann, 1931*a, b*). The ability of micro-organisms, including those isolated from the oral flora (Pincus, 1950), to desulphate mucopolysaccharides isolated from human enamel and dentine (Pincus, 1949; Candelli & Tronieri, 1951) has suggested a relationship between bacterial chondrosulphatase and the production of dental caries. A chondrosulphatase is present in living cultures of *Penicillium spinulosum* (Pincus, 1950) and in the digestive organs of the marine mollusc *Charonia lampas* (syn. *Triton nodiferus*) (Soda & Egami, 1938), but it is not yet clear whether the enzyme is present in mammalian tissues. Thus although no activity could be detected in a number of different rat tissues by sensitive chemical or histochemical methods (Dohlman & Friedenwald,

1955), it was possible to detect appreciable amounts of $^{35}\text{SO}_4^{2-}$ in rat urine after the administration of ^{35}S -containing chondroitin sulphate (Dr C. H. Dohlman, personal communication). There is also a certain amount of somewhat confused evidence which suggests that the enzyme elastase contains more than one component, one of which may be a chondrosulphatase type of enzyme (Pepler & Brandt, 1954; Hall & Gardiner, 1955).

In the studies mentioned above and in other investigations on chondrosulphatase activity in bacteria (Beuhler, Katzman & Doisy, 1951; Reggianini, 1950*a, b*; Konetzka, Pelczar & Burnett, 1954) liberation of sulphuric acid from chondroitin sulphate was always accompanied by the appearance of degradation products possessing reducing activity. Although the mucopolysaccharase (chondroitinase) system responsible for the appearance of reducing activity has in no case been completely separated from the associated chondrosulphatase, in the mollusc preparations a partial separation of the two enzymes has been achieved and there seems to be no doubt as to their separate identities (Soda & Egami, 1938).

In spite of the possible value of a chondrosulphatase as a tool in establishing the structure of chondroitin and related sulphates little is known of the properties of the enzyme. The present report presents the results of a preliminary study of the chondrosulphatase of a strain of *Proteus vulgaris*.

* Part 13: Dodgson, Spencer & Williams, (1956).

MATERIALS AND METHODS

Preparation of potassium chondroitin sulphate. Recently three different types of chondroitin sulphate have been recognized (Meyer & Rapport, 1951; Orr, 1954), and it seems probable that most preparations obtained by simple extraction procedures are unlikely to be homogeneous. However, for these preliminary studies complete homogeneity of substrate is not essential and the product prepared by a modification of the method of Einbinder & Schubert (1950) has been used.

The rings of cartilage from fresh bovine trachea were immersed for 10 min. in acetone, when the hardened non-cartilaginous material could readily be removed. The cartilage was washed, coarsely minced, and 150 g. of the mince was macerated in 200 ml. of water containing 0.25 g. of crude trypsin (British Drug Houses Ltd.) and 0.1 g. of thymol, the whole being adjusted to pH 7.8. After incubating at 37° for 72 hr., 1 l. of a 30% (w/v) solution of KCl was added and the mixture shaken mechanically for 72 hr. at room temperature. The cloudy extract was decanted and stored at 0° while the residue was shaken with a further 1 l. of KCl solution for 72 hr. The two extracts were combined and dialysed for 40 hr. against tap water in order to reduce the salt concentration of the solution and thereby increase the efficiency of the subsequent deproteinization procedure (Strandberg, 1951). Acetic acid (20 ml.) and 20 g. of potassium acetate were added and the solution was stirred for 30 min. with 20 g. of kaolin. The bulk of the kaolin was removed by centrifuging, the solution clarified by filtering through Hyflo Super-Cel (Johns Manville Inc.) and the filtrate dialysed for 24 hr. against tap water. The treatment with acetic acid, potassium acetate and kaolin was repeated, the solution clarified as before and then concentrated to about 300 ml. *in vacuo* at 40°. The solution was poured, with stirring, into 1 l. of ethanol and the whole allowed to stand overnight at 0°. The white flocculent precipitate of potassium chondroitin sulphate was separated, washed well with ethanol and ether and dried *in vacuo*. The product was dissolved in water to give a 3% (w/v) solution, acidified with acetic acid (2 ml./100 ml.) and stirred with kaolin (4 g./100 ml.) for 30 min. The solution was clarified as described above, stirred with a further quantity of kaolin (4 g./100 ml.), clarified again and the filtrate dialysed against several changes (100 vol. in all) of distilled water. Potassium acetate (10 g.) was added to each 100 ml. of diffusate and the potassium chondroitin sulphate precipitated by the addition, with stirring, of 3 vol. of ethanol. The precipitate was separated, washed with ethanol followed by ether, and dried *in vacuo*. Yield, 5–7 g. Analysis (average of three preparations): N, 2.23% (determined by the method of Markham, 1942); SO_4^{2-} ions, 14.19% (determined by the gravimetric procedure of Lugg, 1938, after hydrolysis by refluxing with 4N-HCl for 20 hr.); hexosamine, 26.0% (determined according to Belcher, Nutten & Sambrook, 1954); N:S ratio, 1.08.

Viscosity measurements. The viscosity of solutions of potassium chondroitin sulphate were determined with an Ostwald viscometer having a flow time of 50 sec. for 2 ml. of water. The temperature was maintained at 37° ($\pm 0.05^\circ$) and the time of flow was measured to the nearest 0.1 sec.

Choice of buffer concentration for enzyme experiments. Mathews (1953) has shown that the viscosity behaviour of solutions of chondroitin sulphate is typical of that of linear

polyanions and postulated that the shape of the chondroitin sulphate molecule varies from that of a loose flexible coil at low cation concentration to that of a tightly coiled sphere or near-sphere at high cation concentration. At pH 7.0 the viscosity of solutions of potassium chondroitin sulphate (0.095 or 0.64%, w/v) in sodium acetate–acetic acid solution was independent of the concentration of acetate over the range 0.2–0.5M. Between these limits it can therefore be assumed that there is no gross change in the shape of the chondroitin sulphate molecule and consequently in the spatial distribution of the ester sulphate groupings. A concentration of acetate of 0.2M was chosen for use in the enzyme determinations in order that cations which may be present in the enzyme preparations should not influence the molecular shape. In many of the enzyme experiments acetate solutions were used at pH greater than 7.0. At pH values greater than 6.0 acetate mixtures have little buffering power and in these cases the pH of the incubation mixture was checked at the beginning and the end of the incubation period. No detectable changes in pH were observed.

Measurement of chondrosulphatase activity. Chondrosulphatase activity was measured by estimating the enzymically liberated sulphate by the benzidine micro-method of Dodgson & Spencer (1953). The concentration of the various enzyme preparations was adjusted so that between 20 and 40 μg . of SO_4^{2-} ions was liberated in the incubation mixture. In the original account it was reported that the method gave anomalous results when concentrations of potassium chondroitin sulphate greater than 0.5% (w/v) were present in the incubation mixture. It is now clear that an impure preparation of chondroitin sulphate was used in these early studies, and more recent work has shown that with freshly prepared potassium chondroitin sulphate, quantitative recoveries of added SO_4^{2-} ions could only be obtained when the concentration of chondroitin sulphate in the incubation mixture was less than 0.17% (w/v). When it was necessary to use higher concentrations the original method had to be modified, as described for the assay of glycosulphatase by Dodgson & Spencer (1954). In such cases instead of incubating 0.6 ml. of acetate containing potassium chondroitin sulphate solution with 0.6 ml. of acetate containing enzyme solution, smaller volumes of substrate (0.1 ml. or 0.2 ml.) were incubated with an equal volume of a proportionately more concentrated enzyme solution and the modified procedure then followed (see Dodgson & Spencer, 1954).

Measurement of mucopolysaccharase activity. A variety of methods, both physical and chemical, have been developed for the assay of mucopolysaccharase systems (see Meyer & Rapport, 1952). In the present case the estimation of liberated reducing substances was considered to be the most convenient. The method used was as follows: the enzyme solution (0.6 ml. in 0.2M acetate solution, pH 7.0), contained in a 15 ml. centrifuge tube, was pre-incubated for 3 min. at 37° before the addition of 0.6 ml. of the substrate in 0.2M acetate solution, previously warmed to 37°. After incubation at 37° for the required period, the reaction was stopped by the addition of 2.4 ml. of 0.3N-NaOH, followed immediately by 2.4 ml. of a 5% (w/v) aqueous solution of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, these reagents having previously been standardized against each other as recommended by Somogyi (1945). Suitable control determinations were made in which enzyme and substrate were incubated separately and mixed only immediately before the addition of the

NaOH. The precipitate was removed by centrifuging at 2500 *g* for 20 min., and a portion (2.5 ml.) of the clear supernatant was mixed with 2.5 ml. of Somogyi (1952) copper carbonate reagent in tubes chosen for uniformity of diameter and wall thickness and graduated to 7 ml. The mixture was heated for 10 min. in a boiling-water bath, the mouth of the tube being closed with a glass ball to prevent excessive loss by evaporation. The tube was cooled in running water and 2 ml. of arsenomolybdate reagent (British Drug Houses Ltd., see Nelson, 1944) added. After standing for at least 12 hr. at 2°, the volume was adjusted to 7 ml. with water and the colour densities of both test and control determinations were measured against a reagent blank in 0.5 cm. cells at 750 m μ ., with the Unicam SP. 600 spectrophotometer. Anhydrous D-glucose (Thomas Kerfoot and Co. Ltd.) standards, in the range 10–150 μ g., were treated in the manner described above and a calibration curve was constructed. The results of the enzyme assays were then expressed as μ g. of reducing substance, with glucose as the reference standard. Added glucose could be quantitatively recovered from enzyme solutions in acetate over a wide range of substrate concentrations.

RESULTS

Estimation of the chondrosulphatase activity of growing cultures

A number of micro-organisms obtained from the National Collection of Type Cultures were tested for chondrosulphatase activity in the following way. Sterile nutrient broth (1.0% of Oxoid peptone, 0.3% of Oxoid Lab Lemco and 0.1% of NaCl, all w/v in water) was adjusted to pH 7.0 with N-NaOH or N-HCl, inoculated with the test organism and incubated at 25° for 48 hr. A Roux flask was sterilized by dry heat and sterile nutrient agar added to form a thin layer on one side. The broth culture (5 ml.) was added to the flask, spread evenly over the agar surface and incubated at 25° for 24 hr. The cells were washed from the agar with four 5 ml. portions of sterile water, and the suspension of cells so obtained was centrifuged at 4500 *g* and 0° for 45 min. The precipitated cells were washed twice

with sterile water (5 ml.) and then suspended, in a sterile glass homogenizer, in 10 ml. of sterile 0.2M acetate solution, pH 7.0. To a sterile 15 ml. centrifuge tube containing 0.2 ml. of a 0.75% (w/v) solution of potassium chondroitin sulphate in 0.2M acetate solution, pH 7.0, which had been previously sterilized by Seitz filtration, was added 0.2 ml. of the cell suspension. The tube was incubated for 12 hr. at 37° and the enzymically liberated sulphate estimated by the benzidine micro-method modified for this high substrate concentration as described above. Determinations were carried out in duplicate with suitable controls.

For the determination of dry weight 0.2 ml. of the cell suspension and 0.2 ml. of the substrate solution were incubated as above in a weighed sterile tube. Substrate was included in case appreciable bacterial growth occurred under these conditions during the incubation period. The cells were collected by centrifuging, washed several times with water and the tubes dried overnight at 105° before weighing. Results are recorded in Table 1.

Purification and properties of the chondrosulphatase of Proteus vulgaris (N.C.T.C. 4636)

Table 1 shows that *P. vulgaris* 4636 was particularly rich in chondrosulphatase activity, and this organism was selected as a suitable source of the enzyme. The organism was cultured as follows: large flasks, each containing 16 l. of nutrient broth (see previous section), were sterilized. To each flask was added 20 ml. of a 24 hr. broth culture of the organism and the flasks were incubated at 25°, aeration and stirring being provided by a sterile air stream. Maximum growth was obtained after 5 days, when the cells were harvested with the aid of a Sharples centrifuge, suspended in water and again separated by centrifuging. The cells were macerated in acetone at 0°, filtered at the pump, washed well with acetone at 0° and dried *in vacuo*. Yield, 4–5 g./16 l. of broth (preparation A). During later stages of the work acetone-dried cells from large-scale fermentations (320 l.) of the organism were prepared in analogous fashion at the M.R.C. Antibiotic Research Station, Clevedon.

Properties of preparation A. Maximum chondrosulphatase activity was obtained at pH 7.0 (Fig. 1) when measured over a period of 1 hr. at 37° with a final substrate concentration of 0.045% (w/v) in the presence of 0.2M acetate solution. Although the pH curve did not vary when the concentration of acetate was increased to 0.5M the enzyme activity was a little lower. At pH 7.0 maximum enzyme activity was obtained at a substrate concentration of 0.045% (w/v) (Fig. 2). Under these optimum conditions the chondrosulphatase activity of a number of different samples of preparation A was approximately 2 μ g. of sulphate liberated/hr./mg.

Table 1. *Chondrosulphatase activity of certain bacteria*

National Collection of Type Cultures no.	Organism	Sulphate liberated in 12 hr. (μ g./mg. dry wt. of cells)
4175	<i>Proteus vulgaris</i> I	19.4
4636	<i>Proteus vulgaris</i> I	102.5
8311	<i>Proteus vulgaris</i> II	29.0
8313	<i>Proteus vulgaris</i> II	51.2
5940	<i>Pseudomonas fluorescens</i>	0
6751	<i>Pseudomonas fluorescens</i>	0
912	<i>Pseudomonas ovalis</i>	0
7914	<i>Pseudomonas ovalis</i>	0
5731*	<i>Alcaligenes metalcaligenes</i>	0

* National Collection of Industrial Bacteria no.

The optimum conditions did not vary from preparation to preparation.

The optimum pH for mucopolysaccharase activity with a substrate concentration of 0.045% was greater than 7.5. However, no attempt was made to establish the actual optimum pH and all determinations of mucopolysaccharase activity were made with the optimum conditions for chondrosulphatase activity. Under these conditions preparation *A* liberated 5.8 $\mu\text{g.}$ of reducing substance/hr./mg.

Fig. 1. pH-Activity curve of the chondrosulphatase of *P. vulgaris* (preparation *A*) acting on 0.045% (w/v) potassium chondroitin sulphate in 0.2M acetate solution over 1 hr. at 37°.

Fig. 2. Substrate concentration-activity curves for the chondrosulphatase of *P. vulgaris* (preparation *A*, ●; *B*, ■) acting on potassium chondroitin sulphate in 0.2M acetate, pH 7.0. Incubation was for 1 hr. at 37°.

Solubilization of the chondrosulphatase. Homogenization of water suspensions of the acetone-dried cells in a glass homogenizer, followed by standing at 2° for periods of 2–8 hr., did not liberate the chondrosulphatase into solution. Grinding in a bacterial mill (Booth & Green, 1938) was equally unsuccessful. Alternate freezing and thawing followed by standing at 2° for 5 hr. released 15% of the enzyme activity into solution. Incubation in water at 25° or 37° did not solubilize the enzyme but incubation at 37° in 0.2M acetate solutions at pH 7.0–8.0 liberated considerable amounts of the enzyme into solution. The behaviour of the mucopolysaccharase activity during these various treatments closely paralleled that of the sulphatase.

Preparation *A* was homogenized in 0.2M acetate solution, pH 7.0 (1 g. of *A*/20 ml. of solution), the suspension was re-adjusted to pH 7.0 and the whole incubated for 2 hr. at 37°. The cellular debris was removed by centrifuging at 18 000 *g* and 2° for 20 min., and the supernatant solution was dialysed against several changes of distilled water over a period of 16 hr. in the refrigerator. The diffusate was clarified by centrifuging at 18 000 *g* and 2° to give a cell-free extract which was used as the starting material for further purifications. The white powder obtained by freeze-drying this extract (preparation *B*, yield about 0.1 g./g. of *A*) was completely soluble in water.

Properties of preparation B. At a substrate concentration of 0.045% (w/v) preparation *B* gave a pH curve which was identical with that obtained with preparation *A* (Fig. 1). However, the substrate concentration-activity curve at pH 7.0 was quite different from that obtained for preparation *A* (see Fig. 2) and maximum enzyme activity was now obtained at a substrate concentration of 0.125% (w/v). With this concentration of substrate the optimum pH remained unchanged at 7.0. Several different preparations of *B* gave the same optimum pH and substrate concentration and under these conditions possessed an average activity of 19 $\mu\text{g.}$ of sulphate liberated/hr./mg. The mucopolysaccharase activity, when measured under the same conditions, was 32 $\mu\text{g.}$ of reducing substance/hr./mg.

pH Inactivation and precipitation. The extent to which chondrosulphatase and mucopolysaccharase were inactivated over 1 hr. at 37° was identical, within the limits of experimental error, over the whole of the pH range tested, both enzymes being completely inactivated at pH lower than 5 or greater than 11. However, at 0° the enzymes were less sensitive to pH inactivation and at pH below 5.8 material containing both chondrosulphatase and mucopolysaccharase was precipitated from a 0.5% (w/v) water solution of preparation *B*. The precipitation of both enzyme activities was closely parallel throughout the pH range tested, maximum pre-

precipitation occurring at pH 4.0. At this pH the precipitated material contained 75 % of the total sulphatase activity and 70 % of the total mucopolysaccharase activity. The extent to which the enzymes were precipitated at pH 4.0 was decreased in the presence of 0.2M acetate solution. The material precipitated at pH 4.0 contained appreciable amounts of nucleic acid as shown by the ultraviolet-light absorption spectrum. After removal of this nucleic acid from solutions of the precipitated material by the addition of protamine sulphate, the enzyme activities could no longer be precipitated by adjusting the pH to 4.0. This suggested that the initial precipitation of the enzymes at this pH might be dependent on the presence of nucleic acid and that more complete precipitation might be achieved by increasing the concentration of nucleic acid. Addition of yeast nucleic acid (L. Light and Co. Ltd.) before adjusting to pH 4.0 increased the precipitation of the sulphatase to 93 % and the mucopolysaccharase to 87 %. On the basis of these results preparation B was further purified in the following manner.

The dialysed cell-free extract (preparation B before freeze-drying) was cooled to 2° and the

sodium salt of yeast nucleic acid [0.2 ml. of a 5 % (w/v) aqueous solution at pH 7.4/20 ml. of extract] was added. The pH of the mixture was adjusted to 4.0 with acetic acid and the whole allowed to stand for 30 min. at 2°. The precipitated material was removed by centrifuging at 4 500 g and 2° for 30 min., suspended in one-fifth of the original volume of water, and dissolved by adjusting the pH to 7.4 with N-NaOH. The solution was adjusted to pH 6.7 with N-HCl and an aqueous solution of protamine sulphate (1 %, w/v), adjusted to the same pH, was added until a portion of the clear supernatant obtained by centrifuging at 18 000 g for 5 min. at 2° no longer gave a precipitate on adding further protamine sulphate solution. The pH of the nucleic acid-free supernatant was adjusted to 8.0 with N-NaOH, and the whole cooled to 2°. Any excess of protamine sulphate which was precipitated was removed by centrifuging at 18 000 g at 2° for 20 min., and the supernatant was dialysed against 20 vol. of distilled water at 2° for 12 hr. A small amount of insoluble material separated out during dialysis and was removed by centrifuging. The diffusate was freeze-dried to give a white powder (preparation C) which was completely soluble in water. The average yield was 100 mg./10 g. of preparation A.

Properties of preparation C. The optimum conditions for the chondrosulphatase were identical with those for preparation B, and under these conditions the activity corresponded to a liberation of 65 µg. of sulphate/hr./mg. The mucopolysaccharase activity corresponded to the release of 107 µg. of reducing substance/hr./mg. The ability of preparation C to liberate sulphate from the sodium or potassium salts of a number of synthetic or naturally occurring sulphate esters was measured at 37° over a period of 24 hr. No sulphatase activity towards any of the compounds tested (Table 2) was observed.

Time course of the enzymic degradation of chondroitin sulphate

During the concentration procedures no appreciable separation of chondrosulphatase and mucopolysaccharase activities was achieved and it was not possible therefore to gauge the extent to which the activities of the two enzymes were interdependent. With this point in mind the time-activity curves of the enzymes were examined (Figs. 3, 4). The curves for preparation A (Fig. 3, curves 1, 2 and 3) show that, whereas liberation of material possessing reducing activity begins immediately, there is a distinct lag in the appearance of inorganic sulphate. This lag is prolonged by increasing the concentration of chondroitin sulphate in the incubation mixture. These findings suggested that sulphatase activity was dependent on preliminary mucopolysaccharase attack. However, when the

Table 2. *Sulphate esters not attacked by the chondrosulphatase of Proteus vulgaris 4636*

Preparation C (0.6 mg. in 0.6 ml. of 0.2M acetate, pH 7.0) was incubated with 0.6 ml. of a solution of the sulphate ester in the same solution. Except where otherwise stated the concentration of the sulphate ester in the incubation mixture was 0.1 % (w/v), and under such conditions added sulphate could be quantitatively recovered. In no case was enzymic liberation of sulphate observed, even after incubation for 24 hr. at 37°.

Polysaccharide sulphates:

Heparin
Carrageenin
Agar
Fucoidin
Chondrus ocellatus mucilage (Mori, 1953)

Other carbohydrate sulphates:

Glucose 6-sulphate (0.01M)
Uridine diphosphate acetylgalactosamine sulphate (0.8 mM)
Acetylgalactosamine sulphate (0.8 mM)

Aryl sulphates:

2-Hydroxy-5-nitrophenyl sulphate (0.02M)
p-Nitrophenyl sulphate (0.0025M)
p-Acetylphenyl sulphate (0.007M)
Tyrosine *O*-sulphate (0.02M)

Steroid sulphate:

Dehydroisoepiandrosterone sulphate (0.2 mM)

Mustard-oil glycoside sulphate:

Sinigrin (potassium myronate)

Alkyl sulphates:

Ethyl sulphate
Choline sulphate

experiments were repeated with preparation *B* (Fig. 4), no such time-lag was observed. The essential difference between preparations *A* and *B* is that, in the latter, sulphatase and mucopolysaccharase have been extracted from the acetone-dried cells by incubation with acetate solution and subsequently separated from the cell debris. Preliminary incubation of preparation *A* for 2 hr. with 0.2M acetate solution, pH 7.0, before addition of substrate, eliminated the lag phase completely (Fig. 3, curve 4) irrespective of the concentration of chondroitin sulphate subsequently used in the assay procedure. Removal of the lag phase could

not be achieved by carrying out a similar incubation in water, and it is known that this treatment does not extract the sulphatase and mucopolysaccharase from the acetone-dried cells.

It has also been shown that during the extraction of the two enzyme systems from the acetone-dried cells by pre-incubation with acetate, polysaccharide material, which is present in preparation *A*, is degraded to give material possessing reducing activity. There is no detectable increase in reducing activity when the cells are incubated in water alone.

Fig. 3. Time-activity curves for the release of sulphate and reducing activity from solutions of potassium chondroitin sulphate (KCSA) in 0.2M acetate solution, pH 7.0, by the same concentration of preparation *A*. Incubations and pre-incubations were carried out at 37°. Curve 1. ▲, 0.045% KCSA. Curve 2. ■, 0.09% KCSA. Curve 3. ●, 0.125% KCSA. Curve 4. □, Enzyme incubated for 2 hr. before incubation with 0.125% KCSA. Curve 5. ○, Release of reducing substance from the enzyme preparation incubated in the absence of KCSA.

Fig. 4. Time-activity curves for the release of sulphate and reducing activity from solutions of potassium chondroitin sulphate (▲, 0.045%; ■, 0.09%; ●, 0.125%) in 0.2M acetate solution, pH 7.0, by the same concentration of preparation *B*. Incubation was carried out at 37°.

The presence in *P. vulgaris* of polysaccharide material which can be degraded by testicular hyaluronidase has already been noted by Warren & Gray (1954). Fig. 3, curve 5, shows part of the time-activity curve for the appearance of reducing activity when preparation *A* is incubated in 0.2M acetate solution in the absence of potassium chondroitin sulphate. Release of reducing activity is complete in 2 hr. This time course is substantially the same as that obtained when testicular hyaluronidase is also present in the incubation mixture. The degradation of endogenous polysaccharide during the incubation of preparation *A* emphasizes the need for strict control determinations when assaying the mucopolysaccharase activity of such preparations.

To summarize: during the initial stages of the incubation of preparation *A* with chondroitin sulphate in the presence of acetate solution, mucopolysaccharase activity proceeds normally but sulphatase activity progressively increases, as some change, dependent on the presence of acetate, occurs. It is possible that salt solutions other than acetate might be capable of inducing this change but this has not been tested. The rate at which the change proceeds is progressively delayed by increasing the concentration of chondroitin sulphate. The nature of the change is not clear, but increasing 'solubilization' of the enzyme and degradation of the polysaccharide material present in the acetone-dried cells of the bacterium may be involved. In preparation *B* the preliminary change has already occurred during the preparative procedure, and sulphatase activity proceeds normally. The time-activity curves obtained with *B* gave no indication that sulphatase activity is dependent on preliminary degradation of the substrate by the mucopolysaccharase system.

The anomalous time-activity curves given by preparation *A* help to explain why the apparent optimum substrate concentration obtained for this preparation differs from that obtained for *B* (Fig. 2). With *A*, increasing substrate concentration lengthens the period of lag before the appearance of full sulphatase activity, and consequently, when measured over a period of 1 hr., there is an apparently greater activity at a substrate concentration of 0.045% than at 0.125%. When measured over periods longer than 2 hr. greatest activity is obtained with a substrate concentration of 0.125%, the optimum substrate concentration for preparations *B* and *C*. The anomalous time curves also explain why the substrate concentration-activity curve for preparation *A* when measured over 1 hr. shows inhibition by excess of substrate (Fig. 2). Inhibition by excess of substrate has been noted for the chondrosulphatase of viable cultures of *P. vulgaris* (Konetzka *et al.* 1954).

The rate of liberation of sulphate from chondroitin sulphate by enzyme preparations *A*, *B* and *C* decreased markedly with time and it was of interest to see whether total liberation of sulphate could be achieved or whether smaller sulphated fragments, resistant to sulphatase attack, would remain. With 2.4 mg. of preparation *B* in 1.2 ml. of acetate solution, pH 7.0, the ester sulphate present in 200 μ g. of potassium chondroitin sulphate was completely liberated after incubation at 37° for 30 hr. When the amount of chondroitin sulphate present was increased to 500 μ g., only 83% of the ester sulphate was hydrolysed by the same amount of enzyme during 30 hr.

DISCUSSION

The preliminary studies on the specificity of the chondrosulphatase show it to be devoid of activity towards the substrates of aryl-, glyco-, steroid- and myro-sulphatases (cf. Buehler *et al.* 1951). Early preparations of bacterial chondrosulphatase were reported to liberate sulphate from glucose 6-sulphate and myrosin (Neuberg & Hofmann, 1931*a*), but it now seems likely that these observations were due to the presence in the preparations of other sulphatases. The fact that chondrosulphatase failed to hydrolyse the polysaccharide sulphates listed in Table 2 is perhaps not surprising, since none of these compounds contain the sulphated acetylgalactosamine residues present in chondroitin sulphate. However, in view of the recent suggestion that the uridine diphosphate acetylgalactosamine sulphate isolated from hens' oviducts might be an intermediate in the synthesis of chondroitin sulphate (Strominger, 1955), it is particularly interesting that the *Proteus* chondrosulphatase failed to liberate sulphate from this compound or from acetylgalactosamine sulphate derived from it.

No significant separation of the chondrosulphatase and mucopolysaccharase activities was achieved during the purification procedure described. The extent to which the two activities are interdependent is still therefore not clear and further attempts are now being made to separate the chondrosulphatase from the mucopolysaccharase before investigating the mechanism and specificity of the sulphatase action in greater detail.

SUMMARY

1. A number of bacteria were examined for the presence of chondrosulphatase activity. *Proteus vulgaris*, N.C.T.C. no. 4636, contained appreciable amounts of the enzyme and was selected for further study.

2. Concentrates of the chondrosulphatase have been prepared and a preliminary study of its properties has been made. Such concentrates possess

mucopolysaccharase activity which it has not so far been possible to remove.

3. The chondrosulphatase did not attack a number of other carbohydrate sulphate esters or esters which are known to be substrates for other types of sulphatases.

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Formation of Ammonia from Glutamine by Rat-brain Particles

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Weil-Malherbe (1953) showed that in washed or dialysed rat-brain homogenates the rate of production of ammonia from glutamine was accelerated in the presence of inosine triphosphate (ITP) or of both inosinic acid and creatine phosphate. He suggested that the amide group of glutamine was transferred to ITP or another inosine phosphate to give the corresponding adenine compound, possibly at the expense of high-energy phosphate, followed by its deamination.

Reinvestigation of the problem with an enzyme preparation from rat brain apparently confirmed Weil-Malherbe's findings. However, further experiments failed to support his interpretation of the results.

It was noted that phosphate and sulphate stimulated production of ammonia under the conditions of the test (cf. Greenstein & Leuthardt,

1948). The apparent stimulation of production of ammonia from glutamine by inosine phosphates can be satisfactorily explained by the presence of phosphate and sulphate. These would be present in Weil-Malherbe's preparations. The effect of various ions on the reaction glutamine $\rightarrow$ glutamic acid + NH_3 was measured.

MATERIALS AND METHODS

Barium creatine phosphate was prepared by the method of Ennor & Stocken (1948), and the barium salt of inosinic acid (IMP) according to Ostern (1932) (see Lehninger, 1951). The barium salt of ITP was prepared according to Kleinzeller (1942). The preparation of the sodium salts from the barium salts was achieved by decomposition with sodium sulphate. Barium creatine phosphate was dissolved in water, and barium inosine mono- and tri-phosphates were dissolved in chilled N-HCl ; the barium was precipitated by the addition of a slight excess of $0.5\text{M-Na}_2\text{SO}_4$, the precipitate centrifuged off and the supernatant neutralized with NaOH (Eggleston

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