Table 3. *Determination of hydroxypyruvate by enzymic oxidation of diphosphopyridine nucleotide with hydroxypyruvate reductase*

Amounts are μ moles of hydroxypyruvate, weighed as the lithium salt monohydrate, contained in 3.02 ml. in 1 cm. spectrophotometer cells. For details see text.

Amount taken (μ mole)	Found (μ mole)	Recovery (%)
0.10	0.095	95.5
0.20	0.195	98
0.30	0.308	102.5
0.40	0.385	97
	Mean	98

Notes on the enzymic method

Specificity. Pyruvate is reduced at less than 2 % of the rate of an equimolar amount of hydroxypyruvate by this preparation of the enzyme. Since both pyruvate and hydroxypyruvate rapidly oxidize DPNH in the presence of lactic dehydrogenase of muscle, which is obtainable commercially (Boehringer und Soehne, Mannheim), a differential analysis of pyruvate and hydroxypyruvate in mixtures of the two substances is readily performed by a comparison of the density decrease obtained with these two enzymes either separately or consecutively in the same solution.

Recovery of hydroxypyruvate. When recrystallized lithium hydroxypyruvate monohydrate (Dickens & Williamson, 1958) was used as a standard, the recovery was quantitative within the experimental limits of ± 5 % (Table 3).

SUMMARY

1. A colorimetric method for the determination of hydroxypyruvate and glycolaldehyde is de-

scribed. The intensity of the green colour obtained on heating the solution with naphtharesorcinol in 23N-H₂SO₄, measured at 660 m μ , is proportional to the amount of these substances present under the defined conditions of the test.

2. When both substances are present together the estimation is repeated after adsorption of hydroxypyruvic acid on Bio-Deminrolit G.

3. The specificity of the test has been studied, and certain interfering substances are noted. Modifications permitting the estimation of glyceric acid and of glyoxylic acid are indicated.

4. The use of hydroxypyruvate reductase from parsley leaves and lactic dehydrogenase of muscle for enzymic assay of hydroxypyruvate is described. Whereas the former enzyme reacts only with hydroxypyruvate, the latter reduces pyruvate also and the two enzymes may therefore be used in conjunction for quantitative analysis of mixtures of these two keto acids.

The above work was in part supported by a block grant to this Medical School from the British Empire Cancer Campaign, to whom the authors' thanks are due.

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The Degradation of Cartilage Chondroitin Sulphate by the Chondroitinase of *Proteus vulgaris*

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(Received 6 June 1957)

Dodgson, Lloyd & Spencer (1957) have shown a strain of *Proteus vulgaris* (National Collection of Type Cultures, no. 4636) to be a particularly potent source of two enzymes, chondroitinase and chondrosulphatase, which are collectively capable of degrading cartilage chondroitin sulphate with release of reducing substances and sulphuric acid respectively. Subsequent work (Dodgson & Lloyd,

1957a, b) showed that whereas chondroitinase action could proceed independently of the associated chondrosulphatase, the latter enzyme was inactive towards polymer chondroitin sulphate. However, the enzyme was capable of liberating sulphuric acid from the sulphated oligosaccharide fragments which were obtained by degrading cartilage chondroitin sulphate with testicular

hyaluronidase. The present communication describes the isolation and characterization of the sulphated disaccharide resulting from the exhaustive degradation of cartilage chondroitin sulphate by *Proteus* chondroitinase in the absence of chondrosulphatase action. A preliminary report of this work has already been made (Lloyd & Dodgson, 1957).

MATERIALS AND METHODS

Potassium chondroitin sulphate. Three different types of chondroitin sulphate are known to occur in mammalian tissues (Meyer & Rapport, 1951; Orr, 1954; Meyer, Davidson, Linker & Hoffman, 1956). Two of these types, chondroitin sulphates A and C, are present in cartilage, the former predominating. The third type, chondroitin sulphate B, occurs mainly in skin, tendon and heart-valves but is not present in cartilage (Meyer *et al.* 1956). Further comment regarding the differences between the three types is made in a later section. The chondroitin sulphate used in the present work was prepared from fresh bovine tracheal cartilage by the method described by Dodgson *et al.* (1957) and may be presumed to consist mainly of chondroitin sulphate A together with small amounts of the C form.

Chondrosin and N-acetylchondrosin. Chondrosin, β -D-(β -D-glucopyranosyluronic acid)-2-deoxy-2-amino-D-galactose, was prepared by the method of Davidson & Meyer (1954) and was subsequently converted into the corresponding N-acetyl derivative by treatment with keten, the procedure described by Weissmann & Meyer (1954) for the preparation of N-acetylhyalobiuronic acid being used. Davidson & Meyer (1954) have shown that in chondrosin the uronic acid is linked to the galactosamine residue through the reducing group of the former. However, the actual position of linkage has not yet been established with certainty. Similar considerations therefore apply to N-acetylchondrosin and to the N-acetylchondrosin sulphate described in a later section. In addition, the position of the sulphate group in the latter compound is not known.

Enzyme preparations and methods of assay. Bovine testicular hyaluronidase was prepared by the method of Dorfman (1955). The preparation of concentrates of chondroitinase and chondrosulphatase from extracts of *Proteus vulgaris* has already been described (preparations C2 and D respectively, see Dodgson & Lloyd, 1957b). Methods for the assay of hyaluronidase and chondroitinase (based on determination of release of reducing substances) and for chondrosulphatase (based on determination of liberated SO_4^{2-} ions) have already been described (Dodgson *et al.* 1957).

Analytical methods. Total nitrogen was determined by the method of Markham (1942), uronic acid by the carbazole method of Dische (1947) and amino nitrogen by the method of Moore & Stein (1948). The hexosamine and ester sulphate contents of various compounds were determined by the methods of Belcher, Nutten & Sambrook (1954) and Dodgson & Spencer (1953) respectively, after hydrolysis of the compounds with 4N-HCl for 15 hr. at 100°.

Descending paper chromatography. This was carried out on Whatman no. 1 paper. The solvent system used was butan-1-ol-acetic acid-water, either in the proportions 50:12:25 or 50:15:35. Hexosamine-containing oligo-

saccharides were detected on chromatograms with Elson-Morgan reagents (see Partridge, 1948), compounds containing free amino groups with a solution of ninhydrin in butan-1-ol (Payne & Kieber, 1954) and materials possessing general reducing properties by using a solution of aniline hydrogen phthalate in *n*-butanol-saturated water (Partridge, 1949).

Charcoal-column chromatography. Enzyme digests of chondroitin sulphate were freed from enzyme and electrolyte by chromatography on columns consisting of a mixture (1:1) of Darco G-60 charcoal (Atlas Powder Co., New York, U.S.A.) and Celite no. 535 (Johns Manville Co., New York, U.S.A.). The dimensions of the columns were adjusted according to the concentration of chondroitin sulphate originally present in the enzyme digest, 3 g. of the adsorbent mixture being used for each 100 mg. of chondroitin sulphate. Before use, columns were washed for several hours with water, followed by 50% (v/v) ethanol and finally with water again, flow rates being adjusted to approx. 2 ml./min. by application of air under pressure.

EXPERIMENTAL AND RESULTS

Time-course of the degradation of chondroitin sulphate by Proteus chondroitinase

It had previously been established (Dodgson & Lloyd, 1957b) that *Proteus* chondroitinase could be studied independently of the associated chondrosulphatase by taking advantage of the fact that the latter enzyme could be completely inhibited by 0.1M- Na_3PO_4 without affecting chondroitinase activity. In practice the required amount of the enzyme preparation containing both chondroitinase and chondrosulphatase (preparation C2, see Dodgson & Lloyd, 1957b) was dissolved in a known volume of 0.2M-sodium acetate-acetic acid mixture, pH 7.5, before adding an equal volume of a 0.2M-solution of Na_3PO_4 . The pH of the mixture was re-adjusted to 7.5 with acetic acid and the whole incubated at 37.5° for 30 min. The treated enzyme preparation exhibited no sulphatase activity towards chondroitin sulphate or the sulphated oligosaccharides arising from the exhaustive degradation of chondroitin sulphate by testicular hyaluronidase (cf. Dodgson & Lloyd, 1957b). The inhibition could not be reversed by dialysis of the treated preparation. In all experiments involving chondroitinase preparation C2 which had been treated in this way was used.

When examining the degradation of chondroitin sulphate by testicular hyaluronidase a similar mixture of sodium acetate, acetic acid and Na_3PO_4 was used as buffer at pH 4.5. This ensured that both chondroitinase and hyaluronidase experiments were performed in a similar electrolyte environment. When treated as described above chondroitinase showed maximum activity towards chondroitin sulphate (1%, w/v solution, final concentration) at pH 7.8 when assayed over a period of 1 hr. at 37.5°. Under similar conditions testicular

hyaluronidase showed maximum activity at pH 4.5.

Fig. 1 shows the time-courses of the degradation of chondroitin sulphate by chondroitinase and testicular hyaluronidase respectively. In each case 10 ml. of a 1% (w/v) solution of enzyme in the acetate-phosphate buffer described above and at the pH appropriate to the enzyme was incubated at 37° with an equal volume of a 2% (w/v) solution of chondroitin sulphate in the same buffer. Samples were withdrawn at suitable time intervals and assayed for release of reducing substance. It can be seen from Fig. 1 that the release of reducing substance proceeded more rapidly with chondroitinase than with testicular hyaluronidase, the reaction being complete in 24 hr. with the former enzyme, since no further increase in reducing activity occurred on addition of a further 3 ml. of freshly prepared enzyme solution at the twenty-eighth hour. Paper chromatography of the material incubated with chondroitinase, with the solvent systems described earlier, showed the presence of one carbohydrate component only ($R_f = 0.17$ and 0.22 in the solvent systems 50:12:25 and 50:15:35 respectively). The component gave positive reactions for reducing groups and hexosamine but a negative ninhydrin reaction, indicating the absence of free amino groupings. Paper chro-

matography of the solutions incubated with hyaluronidase under similar conditions showed the presence of three carbohydrate components containing reducing groups and hexosamine but no free amino groups. In addition, some material remained at the starting point. None of these components was as mobile as the chondroitinase product. However, if the hyaluronidase incubation period was prolonged to 48 hr. a faint spot corresponding to this product was obtained (see Fig. 2). Further comment on these points will be made later. In all cases control experiments were made in which enzyme preparation and substrate were incubated separately and chromatographed under the conditions outlined above. No mobile components were observed.

Isolation of the product of chondroitinase action

Preliminary experiments were conducted in order to establish the conditions necessary for the separation of the product of chondroitinase action from the other components of the incubation mixture. In a typical experiment 15 ml. of a 1% (w/v) solution of chondroitinase in the acetate-phosphate mixture described earlier was incubated for 24 hr. at 37° and pH 7.8 with an equal volume of a 2%

Fig. 1. Time-course of the enzymic degradation of cartilage potassium chondroitin sulphate. Experimental conditions are described in the text. ●, Chondroitinase of *Proteus vulgaris*; ○, testicular hyaluronidase.

Fig. 2. Paper chromatography of the products of the enzymic degradation of cartilage potassium chondroitin sulphate. I, Degradation for 24 hr. with *Proteus* chondroitinase; II and III, degradation for 24 and 48 hr. respectively with testicular hyaluronidase. R_f values in the solvent system butan-1-ol-acetic acid-water (50:12:25) were: A, 0.17 (0.22); B, 0.079 (0.19); C, 0.037 (0.12); D, 0.01 (0.08). Figures in parentheses are the values obtained with the same solvent system in the proportions 50:15:35.

(w/v) solution of chondroitin sulphate in the same acetate-phosphate mixture. The incubation mixture was subsequently transferred to a charcoal column (prepared as described above) which was then washed with water until PO_4^{3-} ions could no longer be detected in the eluate. Elution of oligosaccharide material was then attempted by successive washings with 5 and 15% ethanol followed by 0.1, 0.5, 1, 1.5, 2 and 5% pyridine (all percentages being v/v). Eluates were collected automatically in 10 ml. fractions, which were screened for uronic acid by the method of Dische (1947), a new solvent being introduced when the eluates no longer contained uronic acid. Most of the uronic acid-containing material was present in the ethanol eluates but a small amount was present in the 0.1% pyridine eluate. These eluates were concentrated separately by distillation *in vacuo* at 40° and examined by paper chromatography. Although the elution pattern obtained suggested that separation of three distinct components had been achieved, in actual fact each eluate contained the same single component, the chromatographic mobility of which was identical with that of the component originally demonstrated by chromatography of the whole incubation mixture. A similar phenomenon was observed by Linker, Meyer & Hoffman (1956) during the chromatographic separation (charcoal-Celite column) of the unsaturated uronide resulting from the degradation of hyaluronic acid by bacterial hyaluronidase.

Isolation of the degradation product was carried out on a larger scale as follows. The incubation mixture consisted of 1 g. of chondroitinase and 2 g. of chondroitin sulphate in a total volume of 200 ml. of the usual acetate-phosphate buffer at pH 7.8. After incubation for 24 hr. at 37° the mixture was transferred to a charcoal column which was subsequently washed with water in order to remove electrolyte. The column was then eluted in the manner already described, the eluates being collected automatically in 25 ml. fractions which were screened for uronic acid. Fig. 3 shows the elution pattern obtained. Peak materials were concentrated to low bulk separately, by distillation *in vacuo* at 40° , and examined by paper chromatography. Peaks 1-4 (see Fig. 3) corresponded to the single component already described. The remaining peaks (5-7) represented three further, poorly resolved, components of lower chromatographic mobilities. Mention will be made of these components later.

The concentrates corresponding to peaks 1-4 were combined, reduced to low bulk by distillation *in vacuo* at 40° and passed through a Dowex 50 ion-exchange column (17 cm. $\times$ 2 cm., K^+ form, 50-100 mesh; Dow Chemical Co., Michigan, U.S.A.). The eluate and water-washings from the column

were combined, concentrated to low bulk by distillation *in vacuo* at 40° and finally freeze-dried. The product was a pale-yellow powder; yield, 520 mg.

Characterization of the product of chondroitinase action. The product was chromatographically homogeneous and gave positive tests for hexosamine and reducing groups but no ninhydrin test for free amino groups. The compound had $[\alpha]_D^{20} -14 \pm 2^\circ$ in water ($c=2$) (Found: N, 2.5; amino N, 2.5; ester SO_4^{2-} ions, 17.8; uronic acid 35.8; hexosamine, 33.2. The dipotassium salt of *N-acetylchondrosin sulphate* (new compound), $\text{C}_{14}\text{H}_{21}\text{O}_{15}\text{NSK}_2$, requires N, 2.5; amino N, 2.5; ester SO_4^{2-} ions, 17.4; uronic acid, 35.0; hexosamine, 32.4%). There was no evidence of the presence of an unsaturated linkage in the uronic acid moiety (cf. the unsaturated uronide resulting from the action of bacterial hyaluronidases on hyaluronic acid; Linker *et al.* 1956) since the compound failed to take up bromine and had no characteristic ultraviolet-absorption peak at $232\text{ m}\mu$. Hydrolysis of the compound and subsequent treatment of the hydrolysate under the conditions outlined by Davidson & Meyer (1954) for the preparation of chondrosin from chondroitin sulphate, yielded material which moved as a single spot on paper chromatography with a mobility identical with that of chondrosin ($R_F=0.13$ in the solvent system 50:12:25).

Further evidence as to the nature of the oligosaccharide was obtained as follows. The oligosaccharide (75 mg. in 2 ml. of 0.2M-sodium acetate-acetic acid mixture, pH 7) was incubated with

Fig. 3. Elution pattern resulting from the charcoal-column chromatography of the products of the degradation of chondroitin sulphate by the chondroitinase of *Proteus vulgaris*. Experimental points were obtained for each fraction (25 ml.) but have been omitted for the sake of clarity. Elution solvents were: fractions 1-25, 5% ethanol; 26-40, 15% ethanol; 41-56, 0.1% pyridine; 57-68, 0.5% pyridine; 69-76, 1% pyridine; 77-90, 1.5% pyridine; 91-102, 2% pyridine; 103-120, 5% pyridine.

3 ml. of a 1.2% solution of chondrosulphatase in the same acetate mixture for 3 hr. at 37°. No increase in reducing activity occurred during this treatment. The incubation mixture was transferred to a charcoal column (12 cm. $\times$ 1 cm.) which was subsequently washed with water (200 ml.) followed by 5% (v/v) pyridine (200 ml.). The pyridine eluate was concentrated to dryness *in vacuo* at 35°, the residue dissolved in water and examined by paper chromatography together with samples of the original starting material, chondrosin and *N*-acetylchondrosin and with the solvent system 50:12:25. The component resulting from the action of chondrosulphatase on the unknown oligosaccharide had a chromatographic mobility identical with that of *N*-acetylchondrosin ($R_F = 0.32$). A small amount of the starting material ($R_F = 0.17$) remained unchanged. It seems clear from the collective results that the ultimate product of the degradation of chondroitin sulphate by *Proteus* chondroitinase is *N*-acetylchondrosin sulphate.

*Incomplete degradation of chondroitin sulphate
by Proteus chondroitinase*

Examination of the charcoal-column eluates after the large-scale degradation of chondroitin sulphate by chondroitinase showed that, in addition to *N*-acetylchondrosin sulphate, other oligosaccharides were also produced and appeared in the 1.5, 2 and 5% pyridine eluates (see Fig. 3, peaks 5-7). It was possible that these materials represented intermediate products in the degradation of chondroitin sulphate. Further experiments were therefore designed in order to establish the nature of the products of the incomplete degradation of chondroitin sulphate.

Chondroitinase (60 mg.) was incubated, for 15 hr. only, with chondroitin sulphate (300 mg.) in a total volume of 30 ml. under the conditions described earlier. The incubation mixture was transferred to a charcoal column which was subsequently washed with water until no further PO_4^{3-} ions could be detected in the eluate and then eluted with 5% (v/v) pyridine. Fractions containing uronic acid were combined, concentrated to low bulk *in vacuo* at 40° and finally freeze-dried. Paper chromatography under the conditions described previously showed the presence of four components in the freeze-dried material. All the components gave positive tests for hexosamine and general reducing groups but no ninhydrin test for free amino groups. The principal component had a chromatographic mobility which was identical with that of *N*-acetylchondrosin sulphate, whereas the remaining components exhibited a pattern of decreasing mobility. The chromatographic pattern as a whole was identical with that obtained when

chondroitin sulphate was degraded with testicular hyaluronidase for a period of 48 hr. (see Fig. 2), and it must therefore be assumed that the same oligosaccharide series is obtained in each case.

French & Knapp (1950) have observed empirically that the log of the partition function

$$[\log (1/R_F) - 1]$$

is an additive property of the various structural features of a given oligosaccharide molecule. Moreover, it is related to the degree of polymerization of that molecule and, to some extent, to the type of linkage involved in the formation of the molecule. These workers showed that a plot of the log of the partition function against the degree of polymerization for a homologous series of oligosaccharides yielded a straight line. Since these early observations this principle has been successfully applied to a number of different known homologous series of glucose and fructose oligosaccharides (e.g. French, 1951; French & Wild, 1952, 1953; White & Secor, 1953; Feingold, Avigad & Hestrin, 1956) and to the oligosaccharides

Fig. 4. Relationship between assumed degree of polymerization and paper-chromatographic mobilities of the oligosaccharide series resulting from the incomplete degradation of chondroitin sulphate by *Proteus* chondroitinase or the 48 hr. degradation by testicular hyaluronidase. The polymer repeating unit is assumed to be *N*-acetylchondrosin sulphate. ●, Solvent system butan-1-ol-acetic acid-water in the proportions 50:12:25; ○, the same solvent system in the proportions 50:15:35.

resulting from the degradation of hyaluronic acid by testicular and bacterial enzymes (Weissmann, Meyer, Sampson & Linker, 1954; Schütte & Greiling, 1955).

Examination of the chromatographic behaviour of the oligosaccharide series resulting from the incomplete degradation of chondroitin sulphate by *Proteus* chondroitinase or from the 48 hr. degradation by testicular hyaluronidase showed that this series also obeys this principle. If it is assumed that *N*-acetylchondrosin sulphate is the repeating unit of each oligosaccharide and that the degree of polymerization has whole-number values, then a linear relationship exists between the degree of polymerization and the log of the partition function (Fig. 4). It would therefore appear that these oligosaccharides constitute a homologous series, namely di-, tetra-, hexa- and octa-saccharides, and that the action of testicular hyaluronidase towards chondroitin sulphate is essentially similar to that of chondroitinase, although with regard to the preparations used in the present work the latter enzyme is the more efficient of the two.

DISCUSSION

On the basis of their action towards hyaluronic acid and chondroitin sulphate two types of aminopolysaccharase systems have previously been differentiated. That of mammalian origin, testicular hyaluronidase, degrades both aminopolysaccharides to give mainly tetrasaccharide products, although disaccharides and higher oligosaccharides may also be formed in small amounts (Weissmann *et al.* 1954; Schütte & Greiling, 1955; Hoffman, Meyer & Linker, 1956). Evidence indicates that the products of testicular hyaluronidase action have the same repeating units as the original polymers. In contrast, the hyaluronidases of bacterial origin degraded hyaluronic acid to yield mainly a disaccharide, although higher oligosaccharides may also be produced (Schütte & Greiling, 1955; Linker *et al.* 1956). This disaccharide is not identical with the repeating unit of the original polymer and has been identified as the unsaturated uronide 3-*O*-(β -D- Δ -3:4-glucoseenpyranosyluronic acid)-*N*-acetyl-2-deoxy-2-amino-D-glucose (Linker *et al.* 1956). Chondroitin sulphate A is not attacked by these bacterial hyaluronidases (Meyer & Rapport, 1951) unless ester sulphate groupings are first removed by mild acid hydrolysis (Linker *et al.* 1956). The action of bacterial hyaluronidases on the desulphated, partially degraded product yields an unsaturated disaccharide similar to that obtained from hyaluronic acid, although its structure does not appear to have been established with certainty (Linker *et al.* 1956).

Little is known of the effects of testicular and

bacterial hyaluronidases on chondroitin sulphates B and C. Preliminary reports (Meyer & Rapport, 1951) indicate that neither compound is attacked by the bacterial enzymes. Testicular hyaluronidase is able to degrade chondroitin sulphate C but is without action on the B form (Meyer *et al.* 1956).

It is clear from the present work that *Proteus* chondroitinase degrades cartilage chondroitin sulphate in a manner analogous to that of testicular hyaluronidase, although the disaccharide *N*-acetylchondrosin sulphate, rather than the corresponding tetrasaccharide, is the predominating product. We are thus provided with the first example of a bacterial aminopolysaccharase which is analogous in action to the mammalian enzyme. It has already been pointed out that cartilage chondroitin sulphate consists mainly of chondroitin sulphate A with smaller amounts of the C form. The differences between the two isomers are not great and have been discussed at length by Meyer *et al.* (1956). Both isomers appear to contain the same repeating unit but differ in optical rotation and in their solubility in mixtures of calcium acetate buffer and ethanol. On the other hand, it has recently been established that chondroitin sulphate B differs from A and C with respect to the uronic acid moiety, which is now known to be iduronic acid (Cifonelli, Ludoweig & Dorfman, 1957). Since testicular hyaluronidase is without action on chondroitin sulphate B, it will now be interesting to see if similar considerations apply to chondroitinase.

Dodgson & Lloyd (1957*b*) have already shown that chondroitinase-free chondrosulphatase is inactive towards polymerized cartilage chondroitin sulphate but liberates sulphuric acid from the sulphated products arising from the exhaustive degradation of chondroitin sulphate by testicular hyaluronidase. The present work has shown that *N*-acetylchondrosin sulphate is also a substrate for chondrosulphatase. It is now hoped to isolate various sulphated oligosaccharides after the incomplete degradation of chondroitin sulphate by chondroitinase in order to examine the possible relationships between chondrosulphatase activity and increasing degree of polymerization.

SUMMARY

1. The exhaustive degradation of small amounts of cartilage chondroitin sulphate by the chondroitinase of *Proteus vulgaris*, N.C.T.C. no. 4636, yields a single product containing approximately equimolar amounts of uronic acid, ester sulphate and hexosamine.

2. Acid hydrolysis of this product yields chondrosin, whereas enzymic removal of ester sulphate groupings by the chondrosulphatase of

Proteus vulgaris yields *N*-acetylchondrosin. On the bases of these findings, together with analytical results, the product is considered to be *N*-acetylchondrosin sulphate.

3. When chondroitinase action is not allowed to proceed to completion a series of oligosaccharides is obtained, the principal member being identical with the single product obtained by exhaustive degradation. This series of oligosaccharides is identical with that arising from the prolonged degradation (48 hr.) of chondroitin sulphate with testicular hyaluronidase.

4. Evidence suggests that this oligosaccharide series consists of di-, tetra-, hexa- and octa-saccharide homologues, the smallest member being *N*-acetylchondrosin sulphate.

This work was aided by apparatus grants from the Royal Society, the Medical Research Council, the Wellcome Trust and Imperial Chemical Industries Ltd. One of us (A. G. Lloyd) is grateful to the Medical Research Council for a studentship.

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Electrophoretic Studies on Bacteria

2. THE EFFECT OF ENZYMES ON RESTING SPORES OF *BACILLUS MEGATERIUM*, *B. SUBTILIS* AND *B. CEREUS**

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(Received 27 March 1957)

In a previous paper (Douglas, 1955) the electrophoretic behaviour of resting spores of *Bacillus subtilis* and *Bacillus megaterium* was reported, as a function of pH and ionic strength, together with the effect on the behaviour of addition of surface-active agents. It was concluded that the outermost, electrokinetically effective, surface layers of the *B. subtilis* and *B. megaterium* spores were essentially non-ionogenic, acquiring their charge by

the adsorption of ions, and that they were different with respect to either or both of their chemical and physical structures. Comparison with the corresponding behaviour of bacterial cells and of protein, lipid and other surfaces, as recorded in the literature, suggested that the effective surface of *B. subtilis* spores was essentially polysaccharide; for the *B. megaterium* spores the evidence suggested surface lipid, with possibly a protein component in a complex so as to render the normal ionogenic groups inoperative.

* Part 1: Douglas (1955).