oxidation of DOPA proceeded at a rate of 432 μl O_2/h, while that of 3,4-dihydroxyphenylacetaldehyde progressed sluggishly at 37 μl O_2/h under these conditions. We conclude that 3,4-dihydroxyphenylacetaldehyde is indeed a substrate for this enzyme, but that it is oxidized at an extremely slow rate.

These studies *in vitro* indicate that 3,4-dihydroxyphenylethylamine is metabolized to 3,4-dihydroxyphenylacetaldehyde which may be oxidized by DPN-dependent aldehyde dehydrogenase to 3,4-dihydroxyphenylacetic acid.

The author wishes to thank Mrs. L. STRANDHOLM, Miss B. ISHIDA and Mr. C. DE BRUIN for their participation in this work. This work was supported by U.S. Public Health Grant no. B 1572.

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Received January 26th, 1959

Action of the chondroitinase of *Proteus vulgaris* on hyaluronic acid

Enzymes capable of degrading hyaluronic acid have been classified as follows[1]: endoglucuronidases, yielding a tetrasaccharide as reaction product (*e.g.* leech hyaluronidase); endohexosaminidases, yielding a different tetrasaccharide as the main reaction product and acting by a process of transglycosylation (*e.g.* testicular hyaluronidase), this type of enzyme will also degrade chondroitin sulphates A and C; endohexosaminidases, yielding the Δ 4,5-unsaturated disaccharide, 3-O-(β-D-Δ 4,5-glucoseenpyranosyluronic acid)-N-acetyl-2-deoxy-2-amino-D-glucose (*e.g.* staphylococcal hyaluronidase). All bacterial hyaluronidases which have previously been studied belong to the last group.

Recently DODGSON AND LLOYD[2] showed that the chondroitinase of *Proteus vulgaris* (National Collection of Type Cultures No. 4636) degraded cartilage chondroitin sulphate in a manner analogous to that of testicular hyaluronidase. It was therefore of interest to examine the activity of the Proteus enzyme towards hyaluronic acid.

Several different preparations of a chondroitinase concentrate (the C2 concentrate described by DODGSON AND LLOYD[3]) were used during the course of the work. The activities of these and other enzyme preparations towards hyaluronic acid were followed by determination of liberated reducing material according to the method of SOMOGYI[4], using glucose as the reference standard (see DODGSON, LLOYD AND SPENCER[5]). The optimum pH for chondroitinase activity towards sodium hyaluronate (0.75 mg/ml), in the presence of a mixture (1:1) of 0.2 *M* sodium acetate–acetic acid

and 0.2 *M* Na_2HPO–NaH_2PO_4 buffers, was in the region of 4.7–5.0. Enzyme activity under these conditions varied somewhat from preparation to preparation but was usually such that at 38°, 150–250 μg reducing material was liberated/h/mg enzyme.

In the degradation experiments, sodium (Wyeth, Philadelphia) or potassium (Sehering A.G., Berlin) hyaluronate (at concentrations ranging from 10–50 mg/ml) was exhaustively digested at 38°, in the presence of toluene but in the absence of buffer, with preparations (5–20 mg/ml) of testicular (at pH 4.5) or staphylococcal (pH 6.1) hyaluronidases or with chondroitinase (pH 4.85). Initially the pH was adjusted to the appropriate value with a trace of glacial acetic acid; the pH changes during the incubation period were negligible. Occasionally, acetate and phosphate buffers were incorporated into the incubation mixtures but superior chromatograms were subsequently obtained when buffers were omitted. At the end of the incubation period (usually 48 h) protein was removed from the incubation mixtures by treatment[4] with $Ba(OH)_2$–$ZnSO_4$. On other occasions the incubation mixtures were dialysed in narrow-bore (0.6 cm diameter) Visking dialysis tubes for 18 h against two changes of distilled water (40 vol. in all) at 38°. The dialysates were then concentrated by lyophilization. This was a particularly successful way of recovering the degradation products from the digests; oligosaccharides containing up to at least eight units readily passing through the membrane.

Determination of the u.v.-absorption spectrum of the concentrated, protein-free digests showed that, irrespective of the particular experimental conditions used for the degradation and recovery of the products, only the staphylococcal digests gave the absorption maximum at 230–232 mμ which is indicative[6,7] of the presence of the Δ 4,5-unsaturated disaccharide.

The concentrated digests were chromatographed on Whatman 3 MM paper with the solvent system butan-1-ol–acetic acid–water in the proportions 50:12:25, 50:15:35 or 44:16:40. Subsequently the chromatograms were sprayed with aniline hydrogen phthalate (for compounds containing reducing groups) or with acetylacetone-dimethylaminobenzaldehyde reagent[8] (for N-acetylated hexosamine derivatives).

The staphylococcal digests contained one component only, the chromatographic mobility of which was identical with that of an authentic specimen of the Δ 4,5-unsaturated disaccharide. The testicular hyaluronidase digests showed the expected[9] series of oligosaccharides, the fastest-moving component having a mobility identical with that of authentic N-acetylhyalobiuronic acid. This oligosaccharide series, the predominant member of which was the tetrasaccharide, gave the expected straight-line relationship when log $[(1/R_F) - 1]$ was plotted against the degree of polymerization (*cf.* ref. 2). The chondroitinase digests showed an oligosaccharide series identical with that obtained with the testicular enzyme, with the tetrasaccharide again predominating. There was no spot corresponding in mobility to that of the Δ 4,5-unsaturated disaccharide.

With some chondroitinase preparations two additional reducing spots appeared on the chromatograms. One of these (R_F, 0.22 in the 50:12:25 solvent system) gave no colour with the acetylacetone-dimethylaminobenzaldehyde reagent and had a mobility identical with that of glucuronic acid. The mobility of the other (R_F, 0.37) was identical with that of N-acetylglucosamine. Similar observations were made by LINKER, MEYER AND WEISSMANN[9] during a study of the degradation of hyaluronic acid with crude preparations of testicular hyaluronidase. These workers showed that such

preparations also contained a β-glucuronidase and a β-glucosaminidase, the former being capable of hydrolysing the terminal glucuronosidic bonds of the even-numbered oligosaccharides (tetra-, hexa-, etc.) which resulted from the action of the hyaluronidase. The terminal glucosaminidic bonds of the odd-numbered oligosaccharides which were formed as a result of the intervention of the β-glucuronidase were then hydrolysed by β-glucosaminidase.

It has been possible to show that *Proteus vulgaris* also possesses a β-glucuronidase and a β-glucosaminidase, both of which appear to variable extents in the C2 concentrates. As in the case of crude testicular hyaluronidase preparations[9] it seems probable that the action of the Proteus β-glucosaminidase is dependent on the preliminary activity of the β-glucuronidase since both the glucuronic acid and N-acetylglucosamine spots no longer appeared on chromatograms when 0.02 *M* ammonium 1:4 saccharolactone (which inhibits only the β-glucuronidase) was incorporated into the original incubation mixtures. N-Acetylhyalobiuronic acid was not hydrolysed by the Proteus C2 concentrate. The appearance of glucuronic acid in the chondroitinase digests is further evidence that the enzyme does not produce oligosaccharides containing terminal Δ 4,5-unsaturated uronic acid residues since such compounds are not substrates for β-glucuronidases[10].

It is clear from the collective results that Proteus chondroitinase is quite different from other bacterial hyaluronidases which have previously been described. On the other hand, the enzyme shows a striking similarity to testicular hyaluronidase. This is further emphasized by the fact that Dr. K. MEYER (personal communication), using C2 concentrates supplied by this laboratory, has recently observed transglycosylation reactions towards individual hyaluronate oligosaccharides similar to those shown by testicular hyaluronidase.

I wish to thank the staff of the M.R.C. Antibiotic Research Station, Clevedon, for the mass-culture of *Proteus vulgaris*, and Drs. A. ALBURN, H. J. ROGERS, H. GIBIAN and J. LENS for gifts of hyaluronic acid and various hyaluronidase preparations. Dr. K. MEYER kindly provided samples of N-acetylhyalobiuronic acid and the Δ 4,5-unsaturated disaccharide. This investigation was supported by a research grant, A. 1982, from the Arthritis and Metabolic Diseases Division of the U.S. Public Health Service.

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Received January 7th, 1959