The degradation of hyaluronate, the chondroitin sulfates and heparin by bacterial enzymes (flavobacterium)

The structures of chondroitin sulfate B (ChS-B), of heparin and of heparitin sulfate[1] are still unknown. A study of structure based on degradation of these mucopolysaccharides by adaptive bacterial enzymes appeared feasible when such enzymes became available. In this paper preliminary data on the breakdown of various acid mucopolysaccharides by cell free extracts of the flavobacterium[2], both unadapted and adapted to heparin or ChS-B, will be presented.

All extracts were prepared by ammonium sulfate precipitation of dilute K_2HPO_4 extracts of the acetone-dried bacteria. The precipitates were dialysed and lyophilized.

A. *Unadapted bacteria.* It was found previously that, with heparin as carbon source, extracts of a strain of flavobacterium hydrolysed heparin, hyaluronate and chondroitin sulfate of cartilage. The hydrolysis of the last two was apparently due to a constitutive enzyme. Accordingly, extracts of the unadapted strain were prepared grown on a trypticase–phytine–glucose medium[2]. With hyaluronate as a substrate, the unadapted enzyme produced a disaccharide unsaturated in the Δ 4–5 position of the uronidic moiety and identical with that produced by other bacterial hyaluronidases[3] as evidenced by paper chromatography and by adsorption at 230 mμ.

In contrast to other bacterial hyaluronidases which do not hydrolyse chondroitin sulfates A and C, extracts of the unadapted flavobacterium hydrolysed these two polysaccharides at a high rate with the production of unsaturated di- and oligosaccharides, which proved to be only partly sulfated. Apparently in a primary reaction, sulfated, unsaturated oligosaccharides are produced which are desulfated in a secondary reaction by a sulfatase similar to the chondrosulfatase recently found in *Proteus vulgaris*[4]. With the normal sulfated tetrasaccharide of ChS-A as substrate (obtained by digestion with testicular hyaluronidase), the unadapted enzyme yields two disaccharides, one normal, the other unsaturated, indicating that, as with other bacterial hyaluronidases, unsaturation occurs only during the opening of a hexosaminidic group.

B. *Heparin-adapted bacteria.* Extracts of this adapted organism contain a "bacterial" hyaluronidase. They further hydrolyse heparin and heparitin sulfate at a rapid rate with the production of oligosaccharides possessing glucosamine or N-acetylglucosamine, respectively, as reducing endgroups. The color value of the uronic acid drops during the hydrolysis concomitantly with the increase in reducing sugar while the hexosamine values remain unaffected. Chemically desulfated heparin and heparitin sulfate are resistant to the enzyme action, indicating that a sulfate group is essential for the glycosidic cleavage. One of the products appears to be an unsaturated disaccharide not identical with that derived from hyaluronic acid. This finding again emphasizes the marked difference in the structure of heparin and hyaluronate.

C. *ChS-B adapted bacteria.* The carbon source for this strain was a sample of β-heparin generously supplied by Dr. A. WINTERSTEIN of Basle. (β-heparin is in all respects identical with ChS-B of pig or bull skin[1]). Extracts prepared from the adapted bacteria with ChS-B of pigskin as substrate cause an increase of reducing value, a rise in the carbazole value in the first 4 h of incubation and a parallel increase in the absorption at 230 mμ. After 4 h, carbazole color and U.V. absorption decrease parallel to each other and disappear practically on exhaustive digestion. The primary reaction consists of a cleavage of the hexosaminidic bonds with the production of Δ 4–5 unsaturated uronides. A second adaptive enzyme causes the cleavage of the unsaturated uronides in an as yet undefined manner leading to free N-acetyl-galactosamine. The enzyme responsible for the secondary reaction is inactivated by warming the extracts to 56° for 5 min. Such heated extracts yield a mixture of unsaturated (sulfated and desulfated) di- and oligosaccharides identical with the pattern produced from ChS-A and C with the unadapted enzyme. The heat-labile enzyme, which is present in the adapted and absent in the unadapted bacteria, also cleaves the Δ 4–5 unsaturated disaccharides prepared from hyaluronate or chondroitin[3] causing the disappearance of carbazole color and yielding free N-acetyl-hexosamines. It does not cleave the normal saturated disaccharides. These experiments suggest that ChS-B contains the same β-4 hexosaminidic and β-3 uronidic linkages that are found in chondroitin sulfate A and C and that the different uronic acids known to be present, L-iduronic in the former and D-glucuronic in the latter, yield identical Δ 4–5 unsaturated uronides. The ChS-B adapted enzyme has only a negligible action on chemically desulfated ChS-B consistent with the findings on the heparin adapted enzyme. Like the extracts of the latter, the β-adapted extracts hydrolyse the hexosaminidic bonds of the ChS-A and C and also hydrolyse the ester sulfate groups of their oligosaccharides.

Adaption to ChS-B thus leads to the elaboration of a number of consecutive steps, first to the cleavage of the galactosaminidic bonds of the sulfated polymer by an elimination reaction, and second to the cleavage of the unsaturated uronides leading to free N-acetyl-galactosamine. Attempts to separate the various enzymic steps are in progress.

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The possible role of the ribonucleic acid (RNA) of the pH 5 enzyme in amino acid activation

Recently HOAGLAND[1], and HOAGLAND, KELLER AND ZAMECNIK[2] discovered an enzyme (the pH 5 enzyme) in the soluble fraction of rat liver, which catalysed the activation of several amino acids. They[3] further succeeded in preparing a cell-free system from rat liver, which contained microsomes and the pH 5 enzyme and gave active incorporation of ^{14}C-amino acids into the microsome protein. In these reports[3,4], they emphasized the role of the microsome RNA in the protein synthesis by this enzyme system, but they made no reference to that of the RNA in the soluble fraction. The present communication deals with the possible role of the RNA of the pH 5 enzyme in amino acid activation, and the possibility of complex formation between this RNA and amino acid is also discussed.

The pH 5 enzyme, prepared from young rabbit liver according to the method of HOAGLAND *et al.*[2], was incubated with 100 μg crystalline RNase (Worthington Biochem. Corp.) per ml at 37° for 30 min (pH — 7.8). Its pH was then adjusted to 5.1 with dilute acetic acid, the precipitate was washed with acetate buffer and then dissolved in 0.1 *M* tris-buffer, pH 7.8. As a control, the same amount of the pH 5 enzyme was used, which was treated in the similar manner but without RNase. The amino-hydroxamate formation was measured by the procedure of HOAGLAND *et al.*[2], using acetohydroxamate as a standard.

As shown in Table I, when the pH 5 enzyme pretreated with RNase was incubated with ATP and the amino acid mixture, the amount of the hydroxamate formed by the amino acids was decreased, as compared with that in the control experiment. The results suggested that the RNA of the pH 5 enzyme might participate in the mechanism of amino acid activation. Recently HOLLEY[6] reported that the pH 5 enzyme brought about the conversion of radioactive AMP into ATP, which occurred only in the presence of L-alanine and further that this conversion was inhibited by RNase.

TABLE I

THE EFFECT OF RNASE ON AMINO HYDROXAMATE FORMATION BY THE pH 5 ENZYME

Expt. No.	*Enzyme pre-treated without RNase* — *Hydroxamate formed (μmoles per flask)*			*Enzyme pre-treated with RNase* — *Hydroxamate formed (μmoles per flask)*			*Relative RNA content of the enzyme (% of control value)*
	With amino acid mixture	*Without amino acid mixture*	*Difference*	*With amino acid mixture*	*Without amino acid mixture*	*Difference*	
31	0.16	0.05	0.11	0.06	0.01	0.05	50
32	0.21	0.07	0.14	0.07	0.04	0.03	52
33	0.15	0.01	0.14	0.07	0.03	0.04	53
Mean value	0.17	0.04	0.13	0.07	0.03	0.04	52

Incubation at 37° for 1 h. The same reaction mixture was used as that of HOAGLAND *et al.*[2].