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I. LOCALIZATION IN *PROTEUS VULGARIS*
AND
II. FAILURE TO DEMONSTRATE IN A GROUP A *STREPTOCOCCUS PYOGENES*

by

Donald Orval Miles

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TITLE

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PART I

INTRODUCTION

Evidence for localization of degradative enzymes of Gram-negative bacteria outside of the cytoplasmic membrane in what is called the periplasmic space has been indirect for the most part. A very strong case has been made for the localization of alkaline phosphatase of Escherichia coli in the periplasmic space (Malamy and Horecker, 1961, 1964; Heppel, 1967; and Brockman and Heppel, 1968). Several other enzymes including asparaginase, ribonuclease I, deoxyribonuclease, 5'-nucleotidase, acid phosphatase and cyclic phosphodiesterase have been reported to be localized in the periplasm of E. coli and certain other members of the Enterobacteriaceae (Neu and Chou, 1967; Neu, 1967, 1968; and Cedar and Schwartz, 1967). One criterion for suspecting localization of an enzyme outside of the membrane has been that 80% or more of total enzyme activity has been measured with intact cells even though the substrates were phosphate esters presumed not to penetrate the membrane barrier (Malamy and Horecker, 1961, 1964; Neu and Heppel, 1964b; and Heppel, 1967).

Release of quantitative amounts of an enzyme, when the cell wall was removed enzymatically by lysozyme in the presence of ethylenediaminetetraacetate

and sucrose to protect the osmotically sensitive spheroplasts, has also been used as supporting evidence for periplasmic localization (Malamy and Horecker, 1961, 1964; and Neu and Heppel, 1964).

Other evidence has included quantitative release of these enzymes by osmotic shock which leaves the cells intact and viable (Neu and Heppel, 1966; Heppel, 1967; and Neu and Chou, 1967). Somewhat more direct evidence for surface localization has been supplied by cytochemical studies with the electron microscope (Done et al, 1965; Kushnarev and Smirnova, 1966; and Wetzel et al, 1970). However, the methods employed have led to some controversy on interpretation (Wetzel et al, 1970; Cheng et al, 1970a; and MacAlister et al, 1972).

A new reagent developed by Pardee and Wantanabe (1968), diazonapthalene-disulfonic acid, which inactivates surface enzymes and does not penetrate the membrane, appears to be a very useful tool for enzyme localization studies.

No reports have been found for localization studies of cell-bound enzymes attacking substrates of larger molecular weights such as isomers of the chondroitin sulfates [molecular weights estimated to be 5 to 50 thousand (Mathews, 1967)]. An enzyme from Proteus vulgaris which degrades the chondroitin sulfates has been reported by Konetzka et al (1954) and Dodgson and Lloyd (1957). Extensive characterization of this enzyme has been done (Martinez et al, 1959a, 1959b;

Nakada and Wolfe, 1961; and Yamagata *et al*, 1968) with purified preparations. Solubilization of *P. vulgaris* chondroitinase has been by extraction of enzyme from acetone-dried cells (Dodgson and Lloyd, 1957), grinding lyophilized cells with glass beads (Martinez *et al*, 1959b), and by passage through a French pressure cell (Yamagata *et al*, 1968). The report of slow growth of *P. vulgaris* with only chondroitin sulfate as a carbon source by Martinez *et al*.(1959a) indicated that the substrate was accessible to the enzyme in actively growing cells. This also suggests that this enzyme is located outside of the permeability barrier or cytoplasmic membrane. The very slow growth reported by Martinez *et al* (1959a) could be due to the cell wall acting as a partial barrier to substrate penetration as suggested by Heppel (1967) for certain phosphatase substrates with *E. coli*.

The *P. vulgaris* enzyme which degrades the chondroitin sulfates is officially called chondroitin sulfate lyase (E.C. 4.2.99.6) by the International Union of Biochemistry (Herp *et al*, 1970). However, common literature useage has retained the name chondroitinase and this will be used in this thesis.

The following investigation describes studies which support the thesis that *P. vulgaris* chondroitinase is localized outside of the cell membrane and very likely in the periplasmic space.

PART I

LITERATURE REVIEW

Chemistry of the Chondroitin Sulfates

Mucopolysaccharides are defined by Meyer (1966) as macromolecules isolated from proteolytic digests of connective tissues after extraction with alkali. Included in the mucopolysaccharides are the chondroitin sulfate isomers, keratosulfate, heparitin sulfate, heparin and hyaluronic acid. The mucopolysaccharides are all polyanionic in character and show considerable polymorphism and microheterogeneity in their occurrence in connective tissue (Meyer, 1966). Most of the mucopolysaccharides are heterologous polymers composed of alternate residues of a uronic acid and a hexosamine. The hexosamine residue is usually acetylated and there may be a sulfate ester present (Schiller, 1966). Extensive treatment of mucopolysaccharide structure, chemistry and metabolism can be found in Balazs (1970), Brimacombe and Webber (1964), Jeanloz (1970), Muir (1964) and Schiller (1966).

There are five isomers of the chondroitin sulfates which have been isolated and characterized. Chondroitin sulfates A, -C, -D and -E all contain a glucuronic acid residue and an N-acetylgalactosamine residue. They differ in placement and number of sulfate esters per repeating unit (Suzuki et al, 1968). Chondroitin sulfate A contains

one sulfate ester on the four carbon of the N-acetylgalactosamine while chondroitin sulfate C has one sulfate ester on the six carbon. Chondroitin sulfates D and E contain sulfate to hexosamine ratios of 1.45 and 1.55 respectively and they have some disaccharide repeating units with two sulfate ester groups. The D isomer is thought to have one sulfate ester on carbon six of the N-acetylgalactosamine as with the C isomer and some residues with a second sulfate ester on carbon two or three of the glucuronic acid residue. The E isomer is thought to have some residues with two sulfate esters on the four and six carbon atoms of the N-acetylgalactosamine residue or only one at either position. Chondroitin sulfate B, also called dermatan sulfate, has an L-iduronic acid residue instead of a D-glucuronic acid residue but otherwise is identical to the A isomer (Hoffman et al, 1960). However, Suzuki et al (1968) and Malmström and Fransson (1971) have shown evidence for additional sulfate in some preparations of chondroitin sulfate B.

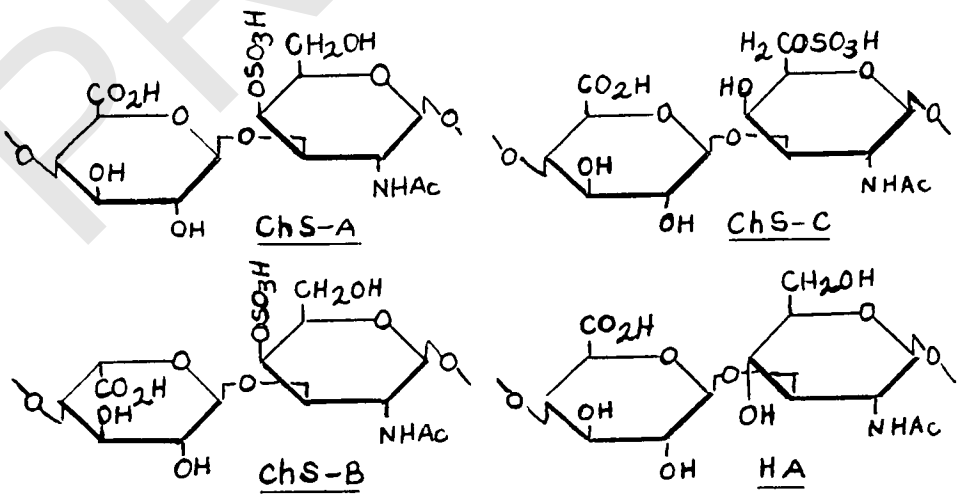

Figure 1. Structure of the chondroitin sulfates (ChS) and hyaluronic acid (HA).

There is also evidence for some D-glucuronic acid residues in addition to the L-iduronic acid residues in the B isomer (Fransson and Malmström, 1971).

Chondroitin sulfates A and C have the same glycosidic linkages as hyaluronic acid. The repeating unit is (1→3)-O-(2-acetamido-2-deoxy-β-D-galactopyranosyl-4 or 6-sulfate)-(1→4)-O-β-D-glucopyranuronosyl. Chondroitin sulfate B has the (1→4) bond alpha to the idopyranuronosyl moiety.

The chondroitin sulfates along with other mucopolysaccharides are components of the extracellular, amorphous ground substance which surrounds the collagen and elastin fibers and the cells of connective tissue of many animals. Usually more than one type is found in a specific tissue. The physiological roles played by these compounds are not completely known but may include induction of calcification (Balazs and Rogers, 1965), control of metabolites, ions and water and healing of wounds (Jeanloz, 1970). These compounds are most easily isolated and separated by their differential solubility of complexes formed with quaternary ammonium salts, such as cetylpyridinium chloride. The resulting preparations show polydispersity of molecular weights as do other polysaccharides.

Chondroitin sulfate A has been isolated and identified in cartilage, bone (Balazs and Rogers, 1965), cornea, notochord of lamprey and sturgeon and skin (Mathews, 1967). Chondroitin sulfate B has been found in skin, tendon and aorta (Mathews, 1967). Chondroitin sulfate C has been

demonstrated in cartilage, umbilical cord and skin (Mathews, 1967). The determined molecular weight for purified chondroitin sulfates A and C has been reported to be 5 to 50 thousand and for chondroitin sulfate B to be 15 to 40 thousand (Mathews, 1967).

An enzyme which catalyzes the depolymerization of the chondroitin sulfates is referred to as a chondroitinase while one that catalyzes the release of the sulfate ester is called a chondrosulfatase. Early studies on degradation of the chondroitin sulfates utilized increase in reducing end groups as a measure of depolymerization (Miyazaki,1934; Reggianini, 1950; Konetzka et al, 1954). Another method employed the polyanionic nature of undegraded chondroitin sulfate which would coprecipitate with acidified human plasma to produce turbidity (Schultz-Haudt and Scherp,1956). Reduction in turbidity production indicated breakdown of the polymer. Later, Dodgson and Lloyd (1958), Martinez et al (1959a) and Yamagata et al (1968) used the Morgan and Elson (1934) chemical reaction, as modified by Reissig et al (1955), for free N-acetylhexosamine to follow degradation of chondroitin sulfate polymers. This reaction has been shown by Jeanloz and Tremege (1956) and Suzuki and Strominger (1960) to be negative for breakdown products containing sulfate esters on the four carbon of the N-acetylgalactosamine residue. The substitution at the four carbon apparently stabilizes the molecule to alkali hydrolysis and the subsequent color development with

Ehrlich's reagent cannot occur (Aminoff et al, 1970). Recently a colorimetric procedure has been developed for assaying chondroitinase activities on chondroitin-4-sulfate substrates by Hascall et al (1972). With the determination that bacterial enzymes degrading hyaluronic acid (Linker et al, 1956 and Ludowieg et al, 1961) and the chondroitin sulfates (Linker et al,1960; Nakada et al, 1960; and Nakada and Wolfe, 1961) act as lyases and produce unsaturated end products, a new method of enzyme assay became available. The unsaturated end products absorb strongly in the ultraviolet region of the spectrum enabling direct spectrophotometric assay in the 230-250 nm range (Nakada et al, 1960). Chondrosulfatase activity can be measured by following release of inorganic sulfate as done by Dodgson et al (1957) or by use of [^{35}S] radioactively labeled substrate as done by Lloyd et al (1967) and Yamagata et al (1968). Additional discussion of certain aspects of chondroitin sulfate chemistry will be found in the next section (p.9).

The chondroitin sulfates are hydrolyzed by mammalian testicular hyaluronidase but not by bacterial hyaluronidases as produced by Streptococcus pyogenes or Staphylococcus aureus. The non-sulfated compound, chondroitin, formed by mild acid hydrolysis of the chondroitin sulfates, is attacked by the bacterial hyaluronidases. The bacterial chondroitinases also will depolymerize hyaluronic acid polymers.

Condroitin Sulfate Degradation by Bacterial Enzymes

Evidence for bacterial degradation of chondroitin sulfate as demonstrated by the formation of inorganic sulfate by putrefactive bacteria was presented originally by Neuberg and Rubin (1914). Later an enzyme concentrate called chondrosulfatase was obtained from an organism resembling Pseudomonas fluorescens (Neuberg and Hoffmann, 1931a). Neuberg and Hoffmann (1931b) found that Pseudomonas aeruginosa and Proteus vulgaris could also degrade chondroitin sulfate.

Miyazaki (1934) found that Bacillus pyocyaneus (Pseudomonas aeruginosa) as well as Streptococcus hemolyticus and Staphylococcus flavus could hydrolyze chondroitin sulfates as judged by the change in reducing power. He did not find any chondrosulfatase activity as indicated by a lack of formation of inorganic sulfate.

Reggianini (1950) also reported hydrolysis of chondroitin sulfate by three strains of Proteus vulgaris by increase in reducing substance. One strain produced appreciable quantities of N-acetylgalactosamine by the sixth day. Romanelli (1951) demonstrated enzymes capable of hydrolyzing hyaluronic acid and chondroitinsulfuric acid in two of five strains of Erysipelothrix rhusiopathiae. One of these which was especially active was percutaneously infectious for swine.