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Localization and Characterization of the Hippuricase Activity of Group B Streptococci

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Studies are presented on the isolation, localization, and characterization of hippuricase activity of group B streptococci. Washed, intact cells, live or heat killed at 56 C, exhibited hydrolysis of hippuric acid, but cell-free filtrates of the organism did not. Excellent hippuricase activity was recoverable from supernatant fluids of mechanically disrupted cells, and evidence suggests that it exists largely intracellularly. Characteristics of the hippuricase preparation are consistent with the view that the biologically active principle is an enzyme. A quantitative microtiter technique has been developed which is useful in titrating enzymatic activity and antibody neutralization. Sera from rabbits immunized with filtered preparations neutralized hippuricase activity.

It has been known for many years that group B streptococci are capable of hydrolysis of hippuric acid to benzoic acid and glycine (1). Before the development of serological grouping of streptococci, this ability to split sodium hippurate permitted investigators to differentiate *Streptococcus agalactiae*, a common cause of bovine mastitis, from other beta-hemolytic streptococci.

Little is known about the basis of this presumably enzymatic reaction which is characteristic of strains of group B streptococci of both human and animal origin. The active principle responsible for the splitting of hippuric acid is generally referred to as hippuricase, but it has not been isolated in pure form, and its biochemical characteristics are poorly defined. Whether it is released as an extracellular product or is associated with some cellular component of group B streptococci is uncertain. The antigenicity of the active principle has not been examined, nor is it known if infection with group B streptococci in man or animals results in development of neutralizing antibody to hippuricase.

Previous techniques for testing for hydrolysis of hippurate have employed whole cultures of streptococci grown for 24 h in a medium containing 1% sodium hippurate to which 7% ferric chloride was added. An insoluble, flocculent brown precipitate, ferric benzoate, is formed when one-fifth or more of the hippurate has been hydrolyzed (1). Another test employs the addition of 50% sulfuric acid to the whole-cell cultures with formation of a white precipitate if one-half or more of the hippurate has been split (1). These assays for detection of hydrolysis of hippurate are suitable for screening purposes, but do not lend themselves well to quantitation of enzymatic activity.

The present study has involved the isolation, characterization, and localization of this hippuricase activity of group B streptococci. A quantitative microtiter technique has been developed to detect liberated benzoate by ferric chloride precipitation. This test is useful for titrating both enzyme activity and antibody neutralization. Sera from rabbits immunized with soluble preparations of the enzyme have exhibited neutralization of hippuricase activity.

MATERIALS AND METHODS

Streptococcal strains. A group B beta-hemolytic streptococcus (GT-7909), type Ic, isolated from a urine specimen was used for these studies. This strain and other group B streptococcal strains, isolated from various body sites and fluids, as well as groups A, C, D, and G streptococci, were from our culture collection. In addition, group B streptococcal strains representing types Ia, Ib, Ic, II, and III were kindly provided by Rebecca Lancefield of the Rockefeller University.

Typing of group B streptococci. The capillary precipitin technique, using type-specific rabbit antisera prepared against types Ia, Ib, II, and III (antisera kindly provided by Harry Hill of our department), was performed on HCl extracts of all the group B strains (9). Extracts giving reactions with Ia and Ib antisera were designated Ic.

Media and reagents. Streptococci to be tested for

hydrolysis of sodium hippurate were grown in a peptone-pepsin medium containing 1% peptone (Difco), 0.5% pepsin (Nutritional Biochemicals Corp.), 0.003% calcium chloride (anhydrous), 1% sodium hippurate (Difco), and 1 drop of 1% ferric chloride per liter and was adjusted to pH 7.1 before sterilization. Todd-Hewitt broth (Difco) was used for growing larger volumes of organisms.

A 7% solution of ferric chloride was used to detect liberated benzoate. Buffer used was 0.01 M tris(hydroxymethyl)aminomethane (Tris) containing 0.003 M Mg So_4 (anhydrous) and 0.003 M $CaCl_2$ adjusted to pH 7.1.

Preparation of group B streptococcal cell walls and cell membranes. Generally, 500 ml of Todd-Hewitt broth was inoculated and grown at 37 C for 18 h, and the cells were harvested by centrifugation at 10,400 $\times$ g for 30 min in a Sorvall refrigerated centrifuge. The cells were washed three times in Tris buffer (pH 7.1) and resuspended in 60 ml of the same. The cells were disrupted for 50 min on a mechanical shaker (H. Mickle, Gomshall, Surrey, England). Complete disruption of cells was assessed by Gram staining of the preparation. After centrifugation at 3,500 $\times$ g at 4 C for 60 min, the cell walls were washed three times and then were resuspended in 60 ml of Tris buffer. The cell wall supernatant fluid was centrifuged again at 3,500 $\times$ g for 2 h to remove cell wall particles. The resulting supernatant fluid was centrifuged for 2 h at 27,000 $\times$ g at 4 C and saved for further testing, and the pellet containing the membrane fraction was recovered. The membrane fraction was washed three times in Tris buffer and resuspended in a small volume of the same buffer.

Analytical methods. Trypsinized, group B streptococcal cell walls, also treated with ribonuclease (RNase) and deoxyribonuclease (DNase) (7), were assayed for rhamnose (4), as were untreated lyophilized membranes obtained by the above method.

The cell wall supernatant fluid recovered was analyzed for protein content with the Folin phenol reagent by the method of Lowry et al. (11), for deoxyribonucleic acid by the diphenylamine reaction (2), and for ribonucleic acid by the orcinol reaction (12).

Testing for hydrolysis of sodium hippurate. The organisms to be tested were inoculated into 4 ml of peptone-pepsin medium containing 1% sodium hippurate and grown at 37 C for 48 h, at the end of which time 1 ml of 7% ferric chloride was added. In addition, cultures grown in Todd-Hewitt broth, both nonheat killed and heat killed for 60 min at 56 C, were centrifuged, and the cells were harvested. Washed cells were resuspended in Tris buffer, and 1 ml was combined with an equal volume of sodium hippurate (final concentration 1%) and incubated for 24 h, and 0.5 ml of 7% ferric chloride was added. Supernatant fluids of whole-cell cultures and cell-free supernatant fluids obtained by filtration with a membrane filter with an average pore diameter of 0.45 μm (Millipore Corp., Bedford, Mass.) were tested similarly at a pH of 6.8 and also adjusted to pH 7.1.

Microtiter technique for assaying hippuricase activity. The microtiter equipment (Cooke Engineering Co., Alexandria, Va.) employed was the same as that described by Edwards for the determination of microantistreptolysin O titers (5). For assaying of enzyme activity in various cell fractions, 0.025 ml of Tris buffer was added to each well of a vertical row in a microtiter plate and then 0.025 ml of the fraction being tested was added to the first (top) well, and serial twofold dilutions were made descending vertically and were followed by addition of 0.025 ml of Tris buffer. Sodium hippurate, 0.05 ml, was added to each well in a final concentration of 1%, and the plate was incubated for 24 h at 37 C. Then 0.025 ml of 7% ferric chloride was added, and the resulting brown precipitate was read immediately and graded on a 0 to 4+ scale. For antibody detection, 0.025 ml of Tris buffer was added to each well, followed by 0.025 ml of undiluted serum to the first well, and serial twofold dilutions were made and 0.025 ml of a standard enzyme dilution was added. The plate was covered and incubated for 1 h at 37 C, the substrate (sodium hippurate) was added to give a final concentration of 1%, and the mixture was incubated for 24 h at 37 C. Immediately before a reading, 0.025 ml of ferric chloride was added, and the resulting precipitate was graded on a 0 to 4+ scale. The end point, indicating neutralization of hippuricase activity, was read as the last well giving a 0 to 1+ reading followed by a 3 to 4+ precipitate in subsequent wells. A substrate buffer control without serum or enzyme and an enzyme control without serum were always included.

Preparation of enzyme for antibody tests. The intracellular content (supernatant fluid from cell wall-membrane preparation) was adjusted to pH 7.1 and filtered through a membrane (average pore diameter of 0.45 μm; Millipore Corp.). Bovine serum albumin (0.1%) was added to enhance stability before filtration. The working enzyme dilution was determined by titrating the activity, by determining the end point dilution, and by using a twofold higher concentration in the test for antibody neutralization.

Concentration of enzyme preparation. A soluble preparation (supernatant fluid after cell disruption) exhibiting hippuricase activity was concentrated approximately sixfold in a collodion membrane (S & S Collodion Bag Filter, A. H. Thomas Co.) under vacuum suction and dialyzed against Tris buffer, pH 7.1, at 4 C for 18 to 24 h.

Trypsin effect on hippuricase activity. The soluble enzyme preparation was adjusted to pH 8.5, mixed with three-times-crystallized trypsin (Worthington Biochemical Corp.) at a concentration of 0.1 mg/ml, and incubated for 30 min at 37 C.

Rabbit immunization. Adult New Zealand rabbits (approximately 2.5 kg) were immunized subcutaneously with a soluble, filtered preparation of enzyme plus incomplete Freund adjuvant on a two-times-weekly schedule for 4 weeks, followed by a rest period of 2 weeks, and then were given a second series of immunization. Each injection contained an emulsion of 0.5 ml of the solubilized, filtered enzyme preparation plus 0.5 ml of mineral oil and 0.25 ml of Aquaphor. The animals were bled prior to immunization and 1 week after each series of injections.

RESULTS

Testing of various streptococcal groups for hydrolysis of sodium hippurate. Figure 1

shows broth cultures of representative strains of groups B, A, C, D, and G streptococci. The first vial illustrates the typical reaction of a group B culture. An insoluble, not readily sedimented, brown precipitate, ferric benzoate, appeared immediately after addition of ferric chloride to the whole culture, indicating that the hippurate had been split. The other vials, containing streptococci of other common groups and the blank or medium control, gave a negative reaction represented by a sedimented precipitate and clear amber supernatant fluid. This nonspecific precipitate, which tends to be an orange color, settled out rapidly, but was readily resuspended. The reactions shown in the vials were quite stable over a period of many weeks at 4 C (Fig. 1).

A total of 64 group B strains and 37 strains of other groups were examined (Table 1). All group B strains, of both human and bovine sources, tested to date give a similar reaction readily distinguished from that of other groups.

Localization of hippuricase activity of group B streptococci. Whole-cell cultures of group B streptococci exhibited ability to split sodium hippurate, but it was not clear whether this was due to an extracellular product released into the medium or to enzymatic activity of cell-bound or intracellular origin. Unfiltered supernatant fluids of group B broth cultures as well as cell-free filtrates failed to give a positive reaction when incubated with hippurate substrate. In contrast, washed whole cells, both live or heat killed, exhibited hydrolysis of hippurate.

Mechanically disrupted group B streptococcal cells yielded a supernatant fluid with excellent hippuricase activity. Biochemical characterization of the final supernatant fluid (from a 27,000 $\times$ g spin) having enzymatic activity revealed 820 μg of protein per ml, 135 μg of deoxyribonucleic acid and 730 μg of ribonucleic acid per ml.

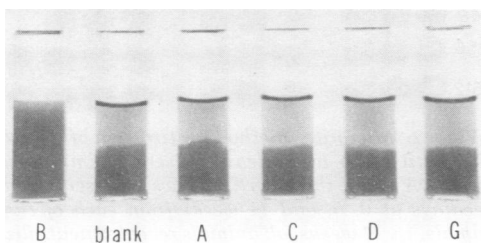

FIG. 1. *Test for hippuricase activity of various streptococcal groups by using addition of ferric chloride to broth cultures of organisms. First vial shows an insoluble brown precipitate, a typical reaction of a group B culture. The blank and other streptococcal groups give negative reactions.*

TABLE 1. *Capacity of various streptococcal groups to hydrolyze sodium hippurate*

Serological group	No. strains tested	No. of strains showing hydrolysis
A	3	0
A variant	1	0
B	64[a]	64
C	14	0
D	12	0
F	1	0
G	3	0
H	1	0
L	1	0
O	1	0

[a] The distribution of types of group B streptococci was as follows: Ia, 9 strains; Ib, 1 strain; Ic, 22 strains; II, 7 strains; III, 12 strains; and 13 untypable strains.

Group B cell walls and membranes were obtained by differential centrifugation. Chemical determinations on these preparations revealed 23% rhamnose content for the walls and 10% for the membranes. The rhamnose value for the walls corresponds with other published information (3). The exaggerated value for the membranes (expected value $<$ 1%) indicated contamination of this preparation with cell wall material. It may reflect the limitations of differential centrifugation for separation of walls and membranes. Prolonged mechanical disruption of the cells (50 min) may break the cell walls into pieces of various size, some of which are sedimented at 27,000 $\times$ g. In addition, since the quantity of membranes obtained was very small, they were not treated with RNase and DNase prior to lyophilization and rhamnose determinations.

Samples of the final supernatant fluid from the Mickle preparation and samples of washed and unwashed cell walls and membranes obtained by high-speed differential centrifugation were tested simultaneously using the microtiter technique to assay and quantitate enzymatic activity. In Table 2 results of serial twofold dilutions of each cell fraction are presented. Activity of the supernatant fluid of Mickle disintegration is present to dilutions of 1:32. Unwashed cell walls exhibit minimal activity, most likely representing carry-over of supernatant fluid, which is not detectable after washing three times. In contrast, unwashed cell membranes, wash fluid, and washed membranes have titratable activity to a 1:2 dilution, suggesting that the active principle may be bound to some extent to cell membranes. These results indicate that hippuricase activity is intracellular and largely free of the sedimentable cell wall and cell membrane fractions since it is recov-

TABLE 2. *Titration of hippuricase activity of various cell fractions*

Cell fraction	Titration						
	u[a]	1:2	1:4	1:8	1:16	1:32	1:64
Mickle supernatant fluid (from 27,000 × *g* spin)	+	+	+	+	+	+	−
Unwashed cell walls	+	−	−	−	−	−	−
Washed cell walls	−	−	−	−	−	−	−
Unwashed cell membranes	+	+	−	−	−	−	−
Wash fluid	+	+	−	−	−	−	−
Washed cell membranes	+	+	−	−	−	−	−

[a] u, Undiluted.

ered in highest titer from the supernatant fluid of mickled cells.

Characteristics of hippuricase. Using the described microtiter technique for enzyme titration, it was possible to study some physical and chemical characteristics of our preparation. The supernatant fluid obtained from mechanical disintegration of cells shows no precipitation, and filtration yields a sterile preparation with little or no appreciable loss of titratable enzymatic activity.

The activity of the preparation is not decreased if incubated at 37 C prior to testing, but incubation at 56 C or 65 C for 30 min abolishes activity. Thus, in contrast to the heat stability of the whole cell preparations, the activity of the soluble preparation is heat labile. Over a period of several weeks of storage at 4 C or −20 C, activity diminishes slowly.

The activity of the preparation was examined over a pH range of 5.0 to 10.0. Optimum enzymatic activity is apparent at a pH range of 7.1 to 9. Adjustment of the enzyme preparation to pH 5 yields no titratable activity, and at pH 10.0 there is a significant reduction. The effect of the proteolytic enzyme trypsin on the hippuricase preparation was complete abolishment of activity. Dialysis of the hippuricase solution for 18 h at 4 C revealed it to be nondialyzable. Concentration of the soluble preparation under vacuum suction in a collodion membrane designated to retain proteins of approximately 70,000 to 100,000 molecular weight resulted in a sixfold increase in titratable enzyme activity.

Antigenicity of hippuricase. Evidence was obtained that hippuricase preparations were antigenic. Figure 2 illustrates the development of neutralizing activity in serial bleedings of three rabbits immunized with the sterile soluble enzyme preparation plus incomplete Freund adjuvant. For the first 2 animals one can see that there is neutralization of enzyme activity up to a serum dilution of 1:32 at 11 weeks from the onset of immunization. In the third animal by 11 weeks there is neutralization to a serum dilution of 1:64. Further immunization trials performed in these animals and others in an attempt to produce hyperimmune sera occasionally resulted in neutralizing activity of higher magnitude (up to 1:128), but the levels decline somewhat between booster injections.

Utilizing the microtiter method to titrate enzyme activity, we find the filtered soluble preparation is not inhibited by normal human serum, normal rabbit serum, or group B antiserum. Furthermore, enhanced enzymatic activity was not observed when a fixed concentration of immune rabbit serum (dilutions of 1:2, 1:32, and 1:128 tested separately) is added to serial twofold dilutions of the enzyme.

Hippuricase preparations from group B types Ia, Ib, Ic, II, and III were neutralized equally well by antiserum (neutralizing titer of 1:64) from an animal immunized with an enzyme preparation from strain GT-7909, type Ic.

DISCUSSION

Before the introduction of serological methods to identify streptococci of various types, Ayers and Rupp (1) showed that differentiation of beta-hemolytic streptococci of bovine origin from those of human origin could be made by their ability to hydrolyze hippuric acid. This

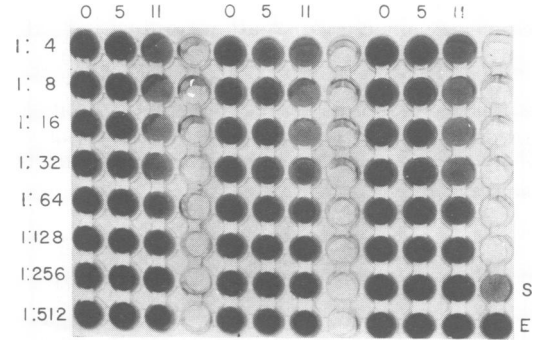

FIG. 2. *Microtiter method for titration of neutralizing activity to hippuricase in sera of immunized rabbits. A set of three vertical rows represents serial bleedings at 0, 5, and 11 weeks from each of three animals. By 11 weeks, all animals revealed neutralization of enzymatic activity (demonstrated by wells with no precipitate). Serum dilutions are designated on left (1:4–1:512) and substrate (S) and enzyme (E) controls are in lower right-hand corner.*

ability to hydrolyze hippuric acid is not confined to group B streptococci, for such activity apparently may occur in some other bacteria, in fungi, and in such organs as kidney and liver (10). There are suggestions from the work of Gilbert and Frobisher (8) that a few gram-negative organisms as well as some strains of staphylococci may possess the ability of hydrolyzing sodium hippurate. Thus a wide distribution of hippuric-acid splitting enzymes appears to exist in nature, but the biological significance of hippuricase is unclear. Moreover, the range of specificity of these enzymes in acting on substrates other than hippuric acid may differ from species to species.

In succeeding years little attention has been given to understanding the basis of this biological property of group B streptococci or where this enzymatic activity is localized in the organism.

The present study confirms the association of this activity with group B streptococci. It rules out release of hippuricase extracellularly into the medium since supernatant fluids of broth cultures of the organisms as well as cell-free filtrates are not capable of hydrolysis of sodium hippurate. Washed cells, live or heat-killed, on the other hand reacted with substrate in a manner similar to whole-cell broth cultures. Mechanical disruption of streptococcal cells revealed that excellent hippuricase activity was recoverable from the supernatant fluids of disrupted cells. Assays of cell fractions indicate that the enzymatic activity is not cell wall associated, but suggest that there may be a slight tendency for membrane association of the hippuricase. Recovery of a high-titer, solubilized preparation having hippuricase activity is feasible without use of extraction procedures to dissociate it from cell membranes or cell walls, suggesting that it exists largely in a free state within the streptococcal cell.

Development of a microtiter technique to detect enzymatically liberated benzoate from hippurate, using ferric chloride precipitation, offered a considerable advantage over the traditional testing in tubes. It also facilitated quantitative examination of the various fractions obtained from mechanical disruption of cells. Adaptation of this technique as an antibody assay permitted detection and titration by neutralization of this activity using sera from immunized animals. Other tests to detect liberated benzoate such as formol titration were not used because of their cumbersome nature (6).

Our bacteriologically sterile preparation having hippuricase activity displays trypsin sensitivity, heat lability, and requires a pH range of 7.1 to 9 for optimum activity. These properties are consistent with the possibility that the biologically active substance recovered is an enzyme.

The enzyme preparation appears to be antigenic in rabbits as evidenced by the capacity of sera from immunized animals to neutralize hippuricase activity. Hippuricase preparations from the various group B types were similarly neutralized by an antiserum obtained by immunization with one of these preparations, suggesting that they share an immunological identity. There may be antigenic differences among hippuricases obtained from group B strains isolated from animal versus human sources, but these have not yet been compared. This evidence of antigenicity and the development of a quantitative technique for measuring antibody provide further stimulation for examining animal and human sera after natural infections due to group B streptococci.

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