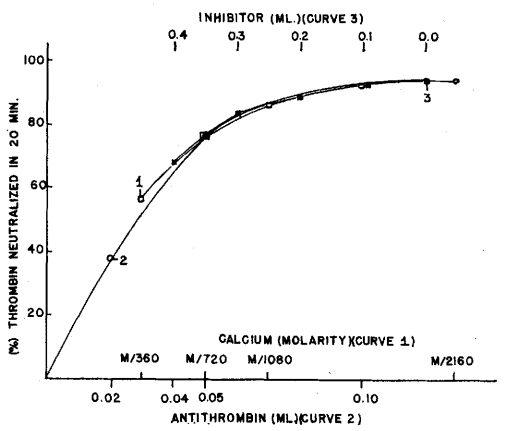

FIG. 3. Relationship of calcium concentration to antithrombin. Incubation mixtures consisted of: *Curve 1*: Plasma, calcium free .05 ml; $CaCl_2$ to give final concentration of 0-M/360 various ml; Thrombin, calcium free, .1 ml; Veronal buffer to .9 ml. *Curve 2*: Isolated antithrombin, 0-0.1 ml; Thrombin, calcium free, .1 ml; Veronal buffer, to .9 ml. *Curve 3*: Isolated antithrombin, 0.1 ml; Isolated inhibitor, 0-0.4 ml; Thrombin, calcium free, .1 ml $CaCl_2$ to give final concentration of M/360, .1 ml; Veronal buffer, to .9 ml. The reactions were started with addition of the thrombin.

cium substitutive effect. The chloride salts of the divalent cations Mg, Mn, Zn and Cu and of trivalent Fe were added to the calcium free plasma system in twice their physiologic concentrations. Only very slight inhibitor activity was detectable with each ion. The lack of effect of these various chloride salts suggests that the activation of the inhibitor is a specific function of calcium.

Discussion. These data indicate that while calcium does not influence the binding of thrombin by antithrombin, it is essential for the reaction between antithrombin and inhibitor. The schema proposed in the previous study(1) must now be modified to include calcium.

$$\text{Antithrombin} \leftrightharpoons \overset{\ulcorner \overset{+}{Ca} \urcorner}{\text{Inhibitor}} \rightarrow \text{Platelet factor}$$

In the resting state, antithrombin is bound by inhibitor in the presence of calcium, the divalent calcium perhaps acting as a bridge. When platelet disintegration furnishes platelet factor, it binds inhibitor releasing antithrombin. Whether calcium is required for the reaction between inhibitor and platelet factor is, for the moment, immaterial, since without calcium there is no binding of antithrombin by inhibitor, and consequently no platelet factor effect. Furthermore, it appears that calcium enters quantitatively into the reaction between inhibitor and antithrombin. The combined effect of calcium and platelets is exactly as anticipated from theory.

Summary and conclusions. 1. The reaction between antithrombin and inhibitor has a quantitative requirement for calcium. 2. Calcium is not the inhibitor of antithrombin. 3. Calcium is not required for neutralization of thrombin by antithrombin.

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Received January 24, 1966. P.S.E.B.M., 1966, v122.

Isolation and Characterization of a Bacterial Inhibitor from Human Throat Washings.* (31102)

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The antibacterial and virucidal activity of stimulated and unstimulated saliva in the human mouth has been confirmed by a number of investigators(1). The nature of the salival components that inhibit bacteria such as *Escherichia coli, Serratia marcescens,* and *Hae-*

* The opinions or assertions contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval service at large. From the Bureau of Medicine and Surgery, Navy Department Research Task, MR005.09-1200.06.

mophilus influenzae has not been clearly defined. They are extremely variable in activity in the same individual or among individuals, and in stability during common laboratory procedures involving steps such as centrifugation, filtration, and heating(1,2,3). In contrast the salival inhibitors of *Lactobacillus acidophilus, Corynebacterium diphtheriae,* and *Mycobacterium tuberculosis* are resistant to the above treatments(4,5). The recent studies on the antibody content of oral secretions(6) illustrate the complexity of population dynamics in the pharyngeal cavity.

The present investigation was prompted by repeated observations made in this and other laboratories during meningococcus surveys. Some inhibitor was apparently present at the site where swabs of the posterior pharynx were streaked onto agar surfaces. In many instances isolated colonies of *N. meningitidis* could only be obtained by further streaking of this planted material with a wire loop. Similar evidence of the presence of an antibacterial substance was obtained during unsuccessful attempts at isolation of bacteriophages for the meningococci from throat washings. The present report describes some of the chemical and biological properties of this meningococcal inhibitor in throat washings.

Material and methods. Throat washings. The majority of throat washings were obtained from military recruits gargling before breakfast with 10 ml sterile Brain Heart Infusion (BHI) (Difco) containing 5% Bovine Serum Albumin. The material was immediately frozen at −20°C and stored at this temperature until used. Other throat washings were obtained from cadre and laboratory personnel with the above broth or with phosphate buffered saline (pH 7.2) and stored in the same manner. All specimens were routinely filtered through 0.22 μ Millipore filters immediately after thawing to remove bacteria. Occasionally this was accomplished by centrifuging the specimens at 36,000 $\times$ *g* for 10 minutes at 4°C immediately after thawing. The inhibitory activity of the supernatant was similar to that encountered in filtered specimens.

Testing of throat washings. Strains of *N. meningitidis* were isolated from patients with meningococcal infection. Other non-meningitidis *Neisseriae, H. influenzae* (*str* 186), *Aerobacter aerogenes, E. coli, Staphylococcus aureus,* and *Streptococcus pyogenes,* were obtained from departmental stock cultures. Bacterial lawns of the test microorganisms were prepared on the appropriate agar medium by streaking the surface of the plate with cotton swabs previously immersed in young broth cultures. The plates were incubated 1-2 hours at 37°C until the surface was dry. The throat washings were then applied by dipping sterile sensitivity discs into the test washing and aseptically laying them onto the bacterial lawn. Spotting with capillary pipette or using penicillin assay wells gave similar results. The degree of inhibition was determined after 18 hours at 37°C by measuring the zone of inhibition (in mm) from the edge of the disc.

Physical-chemical procedures. Pancreatin, lipase, and penicillin were obtained from Nutritional Biochemicals Co., 2$\times$ crystallized trypsin from Armour, and pronase from Kakken Chemical Corp. Fractionation was carried out using Sephadex G-25 (coarse), which had been washed 3 times with deionized water, to eliminate the finely suspended particles. A column, 35 cm in length by 2 cm in width, was packed by gravity flow and equilibrated with a sodium chloride (1.0 M) Tris (hydroxymethyl amino methane) (0.1 M pH 8.0) buffer. Elutions were made with a Tris HCl buffer. The void volume of the column as determined with dextran blue was 110 ml. The eluates were collected in a refrigerated fraction collector (Chromatography Corp.) and stored at 4°C until tested.

Results. Throat washings collected over a 4-month period were tested, and the number of specimens that inhibited growth of *N. meningitidis* is shown in Table I. The highest

TABLE I. Inhibitory Activity of Throat Washings Against *Neisseria meningitidis,* Group B.

Date	No. of specimens	Inhibitory activity % Pos.	Inhibitory activity Range of zones of inhib. (mm)
Dec. 1964	46	93	20–30
Jan. 1965	76	33	4– 8
Feb. 1965	21	20	1– 3
Mar. 1965	35	15	1– 2

TABLE II. Isolation of Inhibitory Substance in Throat Washings of Carriers and Non-Carriers of Meningococci.

	No. of specimens	No. specimens pos. inhib.*
Carriers†	40	11
Non-carriers	40	17

* Inhibitory for Group B *N. meningitidis.*
† All cultures identified as Group B *N. meningitidis.*

percentage of specimens with the greatest degree of inhibitory activity was obtained in December 1964. The percentage of positive specimens and degree of inhibitory activity then decreased over the next 3 months. The results in Table I represent inhibition of a Group B meningococcus, resistant to 10 mg% sulfadiazine, isolated from a patient with this disease but similar activity was observed against meningococci of Groups A and C.

Zones of inhibition roughly corresponded to inhibitory activities determined by dilution. For example, washings producing zones of inhibition >20 mm inhibited meningococci in dilutions of 1:1000. Those with zones of inhibition of 1-2 mm inhibited meningococci in dilutions of 1:10 but not 1:20. It was noticed in 2 out of several hundred tests that the colonies of meningococci were greater in number at the edge of the inhibition zone than in the remainder of the plate. This phenomenon is similar to one described with several antibiotics.

The inhibitor was bactericidal rather than bacteriostatic. We were unable to obtain viable meningococci by transfer to broth of portions of the agar within the inhibition zone.

Circumstances prevented determination of possible correlation between the presence of meningococci in the throat of the individuals in Table I and the presence of inhibitory material. Such a study was, however, carried out on a group of 80 men in April 1965. The results are shown in Table II. Of the 40 recruits that were carrying Group B *N. meningitidis,* 11 (27.5%) had some inhibitory material in their throat washings, while of 40 non-meningitidis carriers (based on one culture of the pharynx) 17 (42.5%) had inhibitory material. This difference is not considered to be significant. Furthermore, the degree of inhibition of meningococci by throat washings of the 2 groups was similar (1-3 mm). *N. perflava* was isolated from all 40 recruits in which *N. meningitidis* was absent.

Action of inhibitor against various bacteria. Selected throat washings that inhibited growth of the meningococci and others that did not, were tested for activity against various other bacteria. Results are shown in Table III. As can be noted, specimens active against *N. meningitidis* were just as active against other *Neisseriae, A. aerogenes,* and slightly less active against *S. aureus* and *H. influenzae.* If the throat washings showed no inhibition of growth of the meningococci, they also were inactive against the above bacteria. None of the throat washings tested had any effect on the growth of *E. coli.*

Effect of heat and enzymatic treatment of throat washings. Selected throat washings were pooled and aliquots treated by heat and various enzymes. Results are shown in Table IV. Heating the specimen at 100°C for 1 hour completely destroyed the inhibitory activity against the meningococci. None of the enzymatic treatments listed had any effect. Most of the recruits had not previously received large doses of sulfonamides for chemoprophylaxis or other antibacterial drugs, thus residual drug could not be held responsible for

TABLE III. Inhibitory Activity of Throat Washings Against Various Bacteria.

Test organism	Inhib. activity* (mm)	Test organism	Inhib. activity (mm)
Neisseria meningitidis	20	*Hemophilus influenzae*	10
" *flavescens*	23	*Aerobacter aerogenes*	22
" *perflava*	21	*Staphylococcus aureus*	5
" *sicca*	25	*Streptococcus pyogenes*	20
" *catarrhalis*	22	*Escherichia coli*	0

* Average of 3 tests of throat washings active against *N. meningitidis.* Throat washings inactive against *N. meningitidis* were inactive against the other test bacteria.

TABLE IV. Effect of Heat and Enzymatic Treatment on Inhibitory Activity of Throat Washings* Against *N. meningitidis.*

Agent	Concentration (per ml)	Temp (°C)	Time (hr)	Inhibitory activity† (mm)
None				20
Heat		56	1	20
"		75	.1	18
"		100	1	0
Trypsin	5 mg	37	1	17
Pronase	.35 "	56	17	18
Pancreatin	1 "	37	1	19
Lipase	1 "	37	.5	20
Penicillinase	40,000 units	40	1	16

* Final dilution 1:2. † Average of 2 tests.

the inhibitory activity of the throat washings. Furthermore, one of the test meningococci (Group B) was resistant to >10 mg% sulfadiazine. The possibility that lysozyme was responsible for the inhibitory activity was ruled out by the early observation that the test strains of meningococci used in this study were resistant to >100 μg/ml lysozyme.

Fractionation of throat washings by gel filtration. Two highly inhibitory throat washings obtained by gargling with BHI broth were pooled and fractionated on Sephadex G-25 (coarse). A representative separation of one of 3 pools is shown in Fig. 1. The reactive fractions were located in tubes 34 through 44 (total eluate volume of 238 to 308 ml). Activity in these fractions indicated that the inhibitory material had a molecular weight of 5000 or less. Fractions 35-40 were pooled and dialyzed against normal saline at 4°C. Approximately 75% of the inhibitory activity was recovered in the saline solution outside the dialysis bag. The dialyzable material was concentrated 4-fold by lyophilization with no loss in activity. The reconstituted material was found to be Biuret positive and Molisch negative.

Pooled non-inhibitory washings were also separated on Sephadex G-25 (coarse), and all fractions were found to be inactive against the meningococci.

Discussion. The origin and nature of most bacterial inhibitors present in the oral cavity are unknown. It is generally believed that the non-filterable, heat labile antibacterial substances present in unstimulated saliva originate from the intrinsic microflora. On the other hand, the filterable, heat stable bacterial inhibitors found in stimulated saliva are considered to originate from salivary secretions(1). The throat washings in the present study might be considered as unstimulated saliva. Data presented here indicate that the physico-chemical properties of the meningococcal inhibitor are different from previously reported antibacterial substances in unstimulated saliva. Antibacterial activity was not affected by filtration, centrifugation, or treatment with proteolytic or lipolytic enzymes. However, activity could be destroyed by heating at 100°C. Preliminary studies on crude fractions obtained by gel filtration indicate that the inhibitor is of low molecular weight (5000 or less), dialyzable, negative for carbohydrates, and contains peptide bonds. The material is probably a peptide but its detailed chemical nature and relationship to the many naturally occurring peptides with antibacterial activity(7) require further investigation.

It has frequently been observed in this and other laboratories that isolation of the meningococci from the nasopharynx of healthy carriers is extremely variable. There are intermittent periods during a survey when isolations of the microorganisms from the nasopharynx of a known carrier are negative. This has usually been attributed to the technique of sampling but the phenomenon occurs so frequently that there may be other contribut-

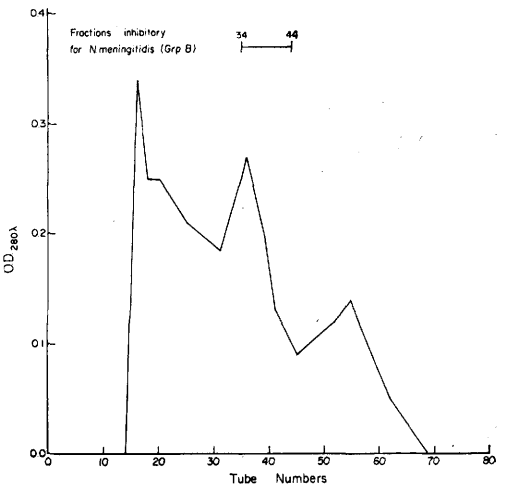

FIG. 1. Gel filtration of throat washing on Sephadex G-25 (coarse). Volume of each fraction equals 7 ml.

ing factors. The data here presented failed to establish a relationship between the presence of meningococci in the throat and the inhibitory activity of the throat washings. This might be explained by recent evidence (8) that numerous meningococci in the human pharynx are intracellular. However, the anti-meningococcal activity of some of the throat washings tested was of such a high degree that the role of this substance in the initial colonization and subsequent elimination of the meningococcus from the pharyngeal cavity should be considered.

It was interesting to note that of the 4 groups of men examined which are shown in Table I, the recruits in December 1964 were experiencing 4-5 times as much upper respiratory viral infections as the other groups. The anti-meningococcal activity of the throat washings of the recruits in December was the highest of any group tested. The possible relationship of this observation with the increasing evidence (R. O. Peckinpaugh, *personal communication*) that meningococcus carrier rates in military camps with a high incidence of upper respiratory infection are much lower than camps with a low incidence, requires further study.

Summary. A substance has been detected in the throat washings of military recruits that is bactericidal for Groups A, B, and C. *N. meningitidis*, related *Neisseriae*, and other Gram-negative and Gram-positive bacteria. The inhibitor appears to be different in its chemical properties from previously reported bacterial inhibitors present in unstimulated saliva. The inhibitor has been characterized as a low molecular weight (5000 or less) substance which is dialyzable, resistant to proteolytic and lipolytic enzymes, Biuret-positive, and Molish-negative. Activity of the inhibitor against the meningococci was destroyed by heating at 100°C for one hour.

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Received January 24, 1966. P.S.E.B.M., 1966, v122.

Quantitative Studies on γ_2 Anti-Dinitrophenyl Antibodies.* (31103)

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It was shown that guinea pig, slow moving, 7S γ_2 antibody can both sensitize heterologous skin for PCA and can fix complement but cannot sensitize homologous skin for PCA(1, 2,3). These experiments were designed to quantitate the complement fixing biologic activity of γ_2 anti-dinitrophenyl (aDNP) antibodies by determining the minimum amount of γ_2 antibody needed to lyse sensitized tanned sheep erythrocytes.

Materials and methods. Passive cutaneous anaphylaxis (PCA). PCA was carried out as previously described(2,4).

Passive lysis. Boyden's technique(5) was used to sensitize sheep erythrocytes. Stavitsky's method(6) applied to dinitrophenylated antigens as previously described(3) was followed. In brief, sheep erythrocytes were tanned and coated with DNP-bovine serum albumin, 37 groups/molecule (G/M) bovine

* Supported by Nat. Inst. Health, Grant AI-03075-06 and Health Research Council, City of New York, Contract I-140.

† Health Research Council Career Scientist.