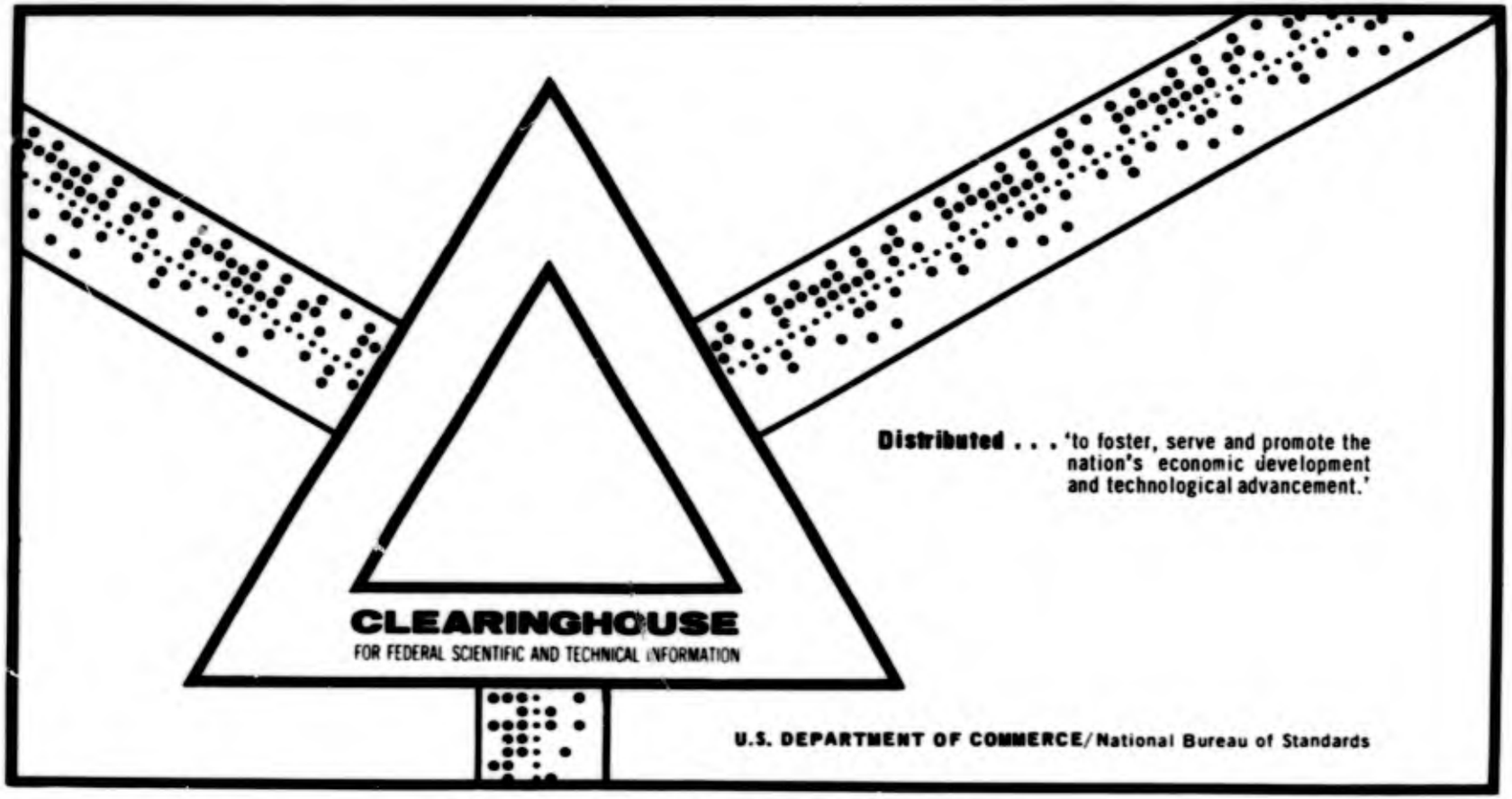
1969 RESEARCH CLERKSHIP REPORTS

John C. Cacioppo, et al

Naval Medical Research Unit Number 4
Great Lakes, Illinois

January 1970



NAVAL MEDICAL RESEARCH

UNIT No. 4

1969 RESEARCH CLERKSHIP REPORTS

MR 005.09-0009
MR 005.09-0096B
M4305.01-1012
MR 005.09.01-0087C

RU 70.4

JANUARY 1970

Distribution of this document is unlimited

BLANK PAGE

FOREWORD

It is the intent of this publication to familiarize those responsible for medical education with the results of the research clerks' 1915 program experienced at Naval Medical Research Unit No. 4 (NAMRU-4). The exposure of the clerks to research methods in arriving at the reports herein contained is believed to give them a greater appreciation of investigative work and its importance to the medical sciences. The environment in which the clerks work is best portrayed by a description of the research unit.

The mission of NAMRU-4 is to conduct research on the epidemiology, etiology, and methods of prevention and control of acute communicable diseases of the respiratory tract.

NAMRU-4 offers a unique opportunity to the research clerks in offering field trials, laboratory study, and clinical evaluation, singularly or in combination, through the fine cooperation of other adjacent Naval activities including the Recruit Training Command, Service School Command, and the Naval Hospital.

The permanent staff at the research unit consists of approximately 80 individuals who are about equally divided between civilian and military ranks. The scientists are widely recognized as experts in their fields.

The consultant staff and affiliations represent such organizations as the World Health Organization, National Institutes of Health, Universities of Chicago, Illinois, Michigan, Northwestern, Tennessee, South Dakota and Wisconsin, and many of the Commissions of the Armed Forces Epidemiological Board, including those on influenza, acute respiratory diseases, streptococcus and immunization.

The unit offers its resources to the clerks in the process of conducting their investigations. Their projects are frequently not a means to an end in themselves, but are pilot studies for much larger programs. The clerks not only have the satisfaction of completing their own projects, but also may have the opportunity of realizing that they played a small part in a much larger scheme while being introduced to the philosophy and principles of the research methods.

Robert O. Peckinpaugh
Captain, MC USN
Commanding Officer

PREFACE

This report includes papers presented by the research clerks assigned to the Naval Medical Research Unit No. 4 (NAMRU-4) in 1969, giving the details of the laboratory investigations which they undertook. These young officers in the Naval Reserve were in medical school when they reported for 60 days of active duty. Due to limitations of time, preliminary plans and scientific designs for the studies were made for the clerks by their preceptors. However, insofar as possible, the clerks selected the divisions in which they wished to work and they chose their specific projects from among numerous alternatives.

At NAMRU-4, the clerks experience the various important steps involved in research work, including: (1) review of the pertinent literature, (2) planning the work, (3) collection of specimens, (4) laboratory procedures, (5) collection of data, (6) analysis of data, and (7) preparation of the final report. Close supervision and direction were given the clerks during their work in order to insure their knowledge of the objectives and techniques needed to accomplish the study. Each paper was reviewed by the respective preceptors, but changes were kept to a minimum.

In order to broaden the clerks' experience, additional opportunities were offered. There were weekly seminars, a field trip, and a formal lecture series, given by members of the Scientific Department. The lectures included selected topics in biochemistry, biometrics, epidemiology, immunology, microbiology and virology which were intended to be of particular interest to the clerks.

It is hoped that the ideas and techniques learned were a useful supplement to the medical education of each officer, as well as a helpful introduction to service in the Naval Medical Corps.

David P. Johnson
Lieutenant Commander, MC USNR
Editor
1969 Research Clerkship Reports

TABLE OF CONTENTS

BLANK PAGE

THE EFFECT OF pH OF THE MEDIUM ON THE ISOLATION AND SUBCULTIVATION OF *MYCOPLASMA PNEUMONIAE**

Ensign John C. Cacioppo
Loyola University Stritch School of Medicine

Preceptor: William H. Kraybill
Mycoplasma Research Division

Mycoplasma pneumoniae is a capricious organism, notorious for its slow growth and difficulty of isolation on artificial media. Several factors can affect the isolation and propagation of this tiny organism from the human oropharynx. Among the essential factors are: type of media (agar or fluid); pH of the media; atmospheric environment (aerobic or anaerobic); and added serums or inhibitors. When a PPLO agar plate is inoculated with a throat culture, the resulting colonies may vary as to species of mycoplasma. Since at least 5 of these may be present, methylene blue chloride is added as an inhibitor to insure isolation and propagation of only the *M. pneumoniae* species (1).

The pH of the basal agar media as one of these essential determinants has been studied by Shepard and Lunceford (2). They found that human mycoplasma could be cultivated in agar or fluid media over a rather broad pH range, but the optimal level of pH for the medium appeared to be primarily species dependent. For example, *M. salivarium* grew best from pH 5.5 to 6.5. T-strain mycoplasmas were best recovered at pH 6.0 in agar. *M. pneumoniae* optimal isolation was dependent upon a pH of 8.0. Concerning pH in a more recent study, Kraybill and Crawford state: "The pH factor has been shown to be important for propagating and isolating mycoplasmas. We found that the optimum pH for isolation of *M. pneumoniae* was 7.0 to 8.0, while the optimum for oropharyngeal species other than *M. pneumoniae* was pH 6.0 to 7.0" (3).

During the past 20 years, *M. pneumoniae* has emerged as an important pathogen of the human respiratory tract. It was originally recovered from filtered throat washings of patients with cold-agglutinin-positive primary atypical pneumonia (PAP) (4). Pioneering in this field was W. D. Eaton, after whom this nonbacterial form of pneumonia was named (Eaton agent pneumonia), and the species involved, *M. pneumoniae*, was dubbed Eaton's agent.

- -

*From Research Project No. MR 005.09-0009, Bureau of Medicine and Surgery, Navy Department, Washington, D. C.

"In 1960, evidence of the disease obtained from Marine recruits by Chanock and associates indicated an etiological relationship of Eaton agent to cold-agglutinin-positive variety of nonbacterial pneumonia. Eaton's agent has since been identified as a pleuropneumonia-like organism (PPLO) by Chanock, Hayflick and Barile, which would place it in the genus Mycoplasma" (5).

Much of our understanding of M. pneumoniae and its association with PAP has come from studies of military recruits.

"The first large scale epidemiologic study of Eaton agent pneumonia was performed by Chanock et al in a military population at Parris Island, S. C., during a 1959 to 1960 epidemic of the illness. Sixty-eight percent of the PAP at that Marine camp was associated with Eaton agent infection as measured by fluorescent antibody test. Subsequent study of recruits from this population by Chanock, employing artificial media, revealed isolation of M. pneumoniae on agar plates in 12 of 13 patients with M. pneumoniae FA positive PAP" (6).

Recent studies on recruit population have established M. pneumoniae as the etiological agent in 10-20% of hospitalized PAP cases. However, this incidence of 10-20% is subject to seasonal variation (6).

The purpose of this investigation is to pinpoint the optimum pH for the agar media in the isolation and subcultivation of M. pneumoniae from recruits hospitalized with PAP.

MATERIALS AND METHODS

Medium for the isolation of M. pneumoniae was prepared according to the procedure outlined by Crawford (7). To make the plates selective for M. pneumoniae, 0.002% methylene blue chloride was added to the agar media to inhibit the growth of all other mycoplasma species of human origin (7). In order to avoid precipitation of the methylene blue chloride within the media, this dye was added after dissolving the basal PPLO agar in distilled water. The basal agar was adjusted with appropriate amounts of sterile NaOH (1N) and HCl (1N) to the following pH levels: 5.0, 6.0, 7.0, 8.0. This adjustment of pH was accomplished with a pH meter (Beckman) and a standard solution of pH 7.0 (Harleco). Horse serum containing penicillin (gamma globulin free), yeast extract, and fungizone were mixed together separately and adjusted with sterile base or acid to the aforementioned pH levels. Utilizing sterile lab techniques, this mixture was added to the basal agar media, and the plates were poured, labeled, and stored in a refrigerator at 36°F for 24 hours before use. To reduce the error of sampling, plates representing each of the 4 different pH levels were randomly selected and placed in groups of 4 before streaking. In the pneumonia wards of

a Great Lakes Naval Recruit dispensary, oropharyngeal cultures were obtained from PAP patients every 3 days. A total of 231 double throat swabs were collected from 90 hospitalized patients. Throat swab material was collected utilizing 2 throat swabs, simultaneously. Two plates were streaked directly with each swab, using a different aspect of the swab on each plate, according to the method of Crawford (7). The plates were sealed with paraffin, and aerobic incubation was maintained at 36°C.

A search for mycoplasma colonies was undertaken at 8, 16 and 24 days, using a standard dissecting microscope at a magnification of 40X. When necessary, confirmation of M. pneumoniae colony growth was accomplished under 100X magnification of a light microscope. Recognition of M. pneumoniae colonies was based on the characteristic granular, spherically shaped colony without the traditional fried egg appearance of other human mycoplasma species. The M. pneumoniae isolates were further identified by subcultivation using the paper disc growth-inhibition technique of Clyde (8).

RESULTS

Total isolation of M. pneumoniae

Table 1 indicates the results of the isolation of M. pneumoniae from 90 hospitalized recruits who were diagnosed by their physicians as having PAP. Nine of the 231 culture trials on individual patients, or 4%, were positive for M. pneumoniae.

Analysis of pH influences

An analysis of the positive isolates with respect to the effect of pH of the media on the primary isolation of M. pneumoniae is also presented in Table 1. The greatest yield of M. pneumoniae cultures occurred in the media with a pH of 8.0. All 8 individuals, or 8.8%, who were shown to have M. pneumoniae in their oropharynx had growth at this pH level. M. pneumoniae failed to grow at pH 5.0 and 6.0, and only 2 isolates were discovered at pH 7.0. Cultures that were positive at pH 7.0 were also positive at pH 8.0.

Eighty-eight of the 90 patients were discharged from the hospital wards before the end of the study. M. pneumoniae was never recovered from the remaining 2 men. Additional admission cultural data were available from 5 of the 8 patients from whom M. pneumoniae was cultured. Three of these 5 were positive for M. pneumoniae on admission, however, 2 of the 3 were discharged with culturally negative throats. The other 2 of the 5 with admission data, and who were culturally negative on admission, acquired M. pneumoniae after 1 and 2 months on the pneumonia ward, being discharged with culturally positive throats. An additional 3 patients, for a total of 6, were discharged within a few days of their last positive subculture.

DISCUSSION

As a result of this study, several noteworthy facts emerged. In the range of pH tested, the optimal pH for successful M. pneumoniae isolation was shown to be 8.0. This was consistently noted in culturing various patients with the clinical diagnosis of PAP. All materials, not just the basal agar as in some earlier studies, underwent pH adjustments prior to mixing into the final media, thereby insuring the accuracy of the final pH of the media. Furthermore, the results of this study were consistent with data from the earlier studies of Kraybill and Crawford (9), Miller et al (5), Shepard and Lunceford (2), and Griffin and Crawford (6), in that the data revealed an 8.8% incidence rate associating M. pneumoniae and PAP. The most noteworthy point in this study is that it tested "wild strains" of M. pneumoniae, cultured under clinical field conditions where the pH factor may be critical. The pH data are consistent with previous observations reported by Shepard (2) who utilized laboratory-adapted strains of M. pneumoniae. Their highest recovery of M. pneumoniae was achieved at pH 8.0, as it was in the present investigation.

Previous studies by Kraybill and Crawford indicated that "wild strains" of M. pneumoniae grew equally well at pH 7.0 and 8.0. The inconsistency between their study and the present one might be due to either a strain variation of a slight variation in technique. The impact of this study on the previous one is to choose pH 8.0 over pH 7.0 for the isolation of M. pneumoniae from clinical throat swab material. Since 82% of the isolates obtained grew out at pH 8.0, the effect of higher pH levels of 8.5 and 9.0 should be explored.

By following the cultural status of a number of these patients, it was of interest to note that 2 patients acquired M. pneumoniae after extended hospitalization. The fact that 6 of the 8 patients, from whom M. pneumoniae was isolated, were discharged as carriers, leads one to speculate on their effect on non-recruit Naval personnel in future contacts.

SUMMARY

A study of the influence of pH on the ability to isolate M. pneumoniae on modified PPLO agar media was conducted using throat swab material obtained from PAP patients in pneumonia wards. M. pneumoniae was isolated from 8.8% of the 90 PAP patients. Of a total of 11 positive cultures obtained in the study, 9 (82%) were recovered at pH 8.0; 2 (18%) at pH 7.0; and none at pH 5.0 and 6.0. It was concluded that in the pH range studied, the optimal pH for the recovery for M. pneumoniae was 8.0.

REFERENCES

1. Kraybill, W. H. and Y. E. Crawford: Selective Medium and Color Test for Mycoplasma pneumoniae. Proc. Soc. Exp. Biol. & Med. 118: 965-970, 1965.

2. Shepard, M. C. and C. D. Lunceford: Effect of pH on Human Mycoplasma Strains. J. Bact. 89:265-270, Feb. 1965.

3. Kraybill, W. H. and Y. E. Crawford: Comparison of Two Agar Media for the Isolation of Mycoplasma from the Human Oropharynx. Ann. N. Y. Acad. Sci. 143(1):401-410, (July 28) 1967.

4. Chanock, R. M., H. H. Fox, W. D. James, R. J. White, and L. B. Senterfit: Epidemiology of M. pneumoniae Infection in Military Recruits. Ann. N. Y. Acad. Sci. 143:484-496, 1967.

5. Miller, L. F., M. Rytel, W. E. Pierce and M. J. Rosenbaum: Epidemiology of Nonbacterial Pneumonia Among Naval Recruits. JAMA. 185:92-99, (July 13) 1963.

6. Griffin, J. P. and Y. E. Crawford: Mycoplasma pneumoniae in Primary Atypical Pneumonia. JAMA. 193:1011-1016, 1965.

7. Crawford, Y. E.: A Laboratory Guide to Mycoplasma of Human Origin. BuMed Report No. MR 005.09-0024, RU 66.25, 1966, pp. 14.

8. Clyde, W. A., Jr.: Mycoplasma Species Identification Based Upon Growth Inhibition by Specific Antisera. J. Immunol. 92:958-965, 1964.

9. Kraybill, W. H. and Y. E. Crawford: Influence of pH on Recovery of Mycoplasma from Human Oropharynx. Bact. Proc. Rt. 1:138, 1966.

TABLE 1. Effect of pH of the medium on the isolation of *M. pneumoniae* from hospitalized PAP recruits Great Lakes, Illinois -- July-August 1969

pH of the PPLO Agar Medium	pH 5.0	pH 6.0	pH 7.0	pH 8.0
No. of Recruits with *M. pneumoniae* Isolates	0	0	1*	8*
No. of PAP Recruits Tested	90	90	90	90

*One recruit positive on two separate trials.

ALTERED CELL MEDIATED IMMUNOLOGIC REACTIONS DURING RECRUIT TRAINING INDUCED BY STRESS ASSOCIATED WITH RECRUIT TRAINING*

Lieutenant (junior grade) Thomas C. Cadier
Loyola University Stritch School of Medicine

Preceptor: Earl A. Edwards
Chief, Immunology Division

Respiratory disease presents a significant problem in Naval recruits (1-4). The incidence of infection has been noted to be particularly high during the first 2 weeks of training (1) and the role of "stress" during this interval, as an etiological host factor, has received much speculation (5-7). Stress is known to induce an increased production of adrenal steroids (5,8) which in turn are known to decrease the immune response (5,6,9) through a variety of mechanisms. In this study, some of those parameters which are altered by elevated plasma corticosteroids have been measured. Specifically, total and differential leukocyte counts and skin test reactions to antigen evoking delayed hypersensitivity were measured in recruits during their first 2 weeks of training. It was anticipated that this information might reveal a relative deficiency in the immune response among new recruits.

MATERIALS AND METHODS

As part of a larger study, 4 companies of Naval recruits were randomly selected, each in a different day of training, ranging from day 1, i.e., the 1st day on board, to day 15. This resulted in 70-120 volunteers for each day studied. Each recruit was then interviewed regarding present upper respiratory illness (URI)(cough, sore throat, nasal discharge or congestion), past history of frequent URI, present or past "athletes foot," history of mumps infection, present or past acne or boils, and allergy or sensitivity to eggs. Four antigen solutions were then administered intradermally, in 0.1 ml amounts with a 26 g needle, to each volunteer on the volar aspect of the left forearm in randomly varied positions.

(M) Mumps - skin test antigen, Eli Lilly and Co., diluted with saline to 10 complement-fixing units per cc.

---

*From Research Project No. MR 005.09-0096B, Bureau of Medicine and Surgery, Navy Department, Washington, D. C.

(K) Mumps placebo - egg albumin control with 1:10,000 thimerosal, Eli Lilly and Co.

(C) Candida - fungus extract prepared from Oidiomycin (Candida albicans) in 0.45% phenol preservation, Hollister-Stier Laboratories, diluted with saline to 1:100.

(T) Trichophyton - fungus extract prepared from Trichophyton cysseum, T. purpureum and T. interdigitale in 0.45% phenol, Hollister-Stier Laboratories, diluted with saline 1:50.

Five ml of blood was obtained from the antecubital vein of each individual with a B-D vacutainer containing 0.04 ml of 15% EDTA solution for determination of total leukocyte count and differential leukocyte count. Also, 10 persons were randomly selected from each company from which 25 additional ml of blood was taken for use in lymphocyte transformation studies (10). Oral temperatures were taken at this time.

At 24 and 48 hours after skin testing, each recruit was again questioned regarding the presence of URI symptomatology, any new boils or athletes foot. Oral temperatures were again taken. Skin test reactions were read as millimeters of induration and millimeters of erythema and appropriately recorded.

Since so many separate pieces of data were collected on each individual in the study, the following procedures were undertaken in order that comparisons could be made within the study group. Information on present respiratory illness was scored according to the empirical code shown in Table 1. Recruits with an illness score equal to or greater than 4 were designated as "sick" and those with a score of 3 or less were designated "well." For graphical comparisons, skin test measurements of erythema and induration were necessarily rounded off to the closest multiple of 5, plus or minus 2 mm, i.e., a reading of 0 through 2 mm equalled 0, 3 through 7 equalled 5 mm, etc. Further grouping was then undertaken for statistical comparison of reactions. Reactions of 0 through 7 mm of erythema or induration were termed negative, 8 through 17 mm were termed positive and greater than 17 were called strongly positive.

RESULTS

Only the results of the skin test findings were treated in this report. Readings of all 4 skin tests, control, candida, trichophyton and mumps were tabulated. Only the mumps skin test showed an appreciable number of positive reactions. Consequently, more thorough analysis of data on the trichophyton and candida skin tests was deferred and attention was centered around the mumps skin test results. If differences

in skin test responsiveness were to be found between the different companies of recruits in the study, it was felt that they would be most apparent when considering reaction to a test to which a large percentage of the volunteers reacted strongly. Companies were first compared without regard to past history of mumps or present respiratory illness score. It was found that, overall, men in companies later in training responded more strongly than the companies which had less time in training. The mumps history of the individuals in each of the study companies was then considered. There was no significant difference between the distribution of levels of induration and erythema when this factor was considered. That is, the skin test response of the group which denied mumps did not differ significantly from the group which gave a positive history. A comparison of these data is seen in Figure 1. This finding was borne out on the company level, except for day 15 company, which did have a higher percentage of positive responses among those who had a positive history for mumps.

The effects of present respiratory disease were next considered. The aggregate comparison of all "sick" recruits to all "well" recruits showed no significant differences in skin reactions between the 2 populations. This independence of present illness and skin reaction was also borne out within each company considered separately. That is, in a given company, the sick people responded to the same degree as the well ones. An intercompany comparison, however, revealed that a significant difference existed between the well recruits of one company and the well recruits of any other company tested, but there was no difference in the reactivity of the sick men. This comparison can be seen in Figure 2 where a sample comparison is graphed. The apparent difference between the sick recruits' response is not significant while the different response of the well groups is significant. It appears that illness interferes with the effect of time of training on delayed skin test responses.

DISCUSSION

The delayed-type hypersensitivity reaction is mediated primarily by local infiltration with lymphocytes in the areas of antigen injection (7). This reaction is inhibited by corticosteroids but not affected by antihistamines (11). The mechanism of action of increased levels of corticosteroids in reducing this antigenically stimulated inflammatory response probably has many facets. Twenty years ago, it was shown that elevated plasma adrenal steroids resulted in a lymphopenic response (12). It has more recently been shown that this lymphopenia is due to at least 3 factors: (1) karyorrhexis and lysis of lymphocytes, predominantly the small, more "mature" type; (2) inhibition of mitosis in lymphatic tissue; (3) inhibition of DNA synthesis in lymphatic tissue. These effects result in a decrease in circulating small lymphocytes and a relative increase in large lymphocytes (9). This effect is seen with blood levels of only 25 µg/100 ml in humans. This level is not sufficiently high to cause a decrease in plasma

antibody levels. The proportion of larger lymphocytes increases in a dose related fashion with the blood levels of corticosteroids and have been termed "stress lymphocytes" by some investigators (11). Finally, electron microscope studies have recently shown that the phagocytotic function of reticuloendothelial cells is inhibited by corticosteroids (13).

The results of this study coupled with the above-noted observations compel one to speculate that the stress of recruit training may be of sufficient magnitude to cause a relative decrease in the cell mediated reaction to antigens. This could conceivably contribute to a reduction in resistance to infections in a recruit who, under less stressful circumstances, would not become infected.

SUMMARY

Mumps skin test response was studied in recruits in an attempt to correlate relative immunosuppression in the 1st two weeks of training. The longer a company has been aboard, the more strongly it responded, as a population, to the mumps skin test. There was no significant difference on the aggregate level or company level between the reactions of recruits who denied mumps and those who claimed to have been infected, except in the company which had been on board for 15 days. This more seasoned company had a greater response in that segment which gave a positive history for mumps. When present illness was considered, the response of the aggregate of all well people did not differ from the response of the "sick" aggregate. This was also borne out at the company level. However, when comparing companies, well recruits responded more strongly than their well counterparts in companies selected earlier in training. All sick recruits in the study responded essentially the same.

Suppression of the cell-mediated reaction among new Naval recruits, manifested only in well individuals, has been demonstrated. There may be some relationship between this suppression and a generalized immunosuppression, possibly mediated by elevated plasma corticosteroid levels, in Naval recruits. In this context, stress may validly be implicated as an etiological factor in recruit respiratory disease.

TABLE 1. Respiratory illness scoring code

FEBRILE RESPONSE	SYMPTOMS
<99.5 = 0	
99.6 - 100.6 = 2	NASAL COMPLAINT ONLY = 1
100.8 - 101.6 = 4	THROAT COMPLAINT ONLY = 1
100.8 - 102.6 = 6	CHEST COMPLAINT ONLY = 1
102 8 - 103.6 = 8	
>103.8 = 10	

≥4 → "SICK"	ANY 2 OF ABOVE = 4
≤3 → "WELL"	ANY 3 OF ABOVE = 6

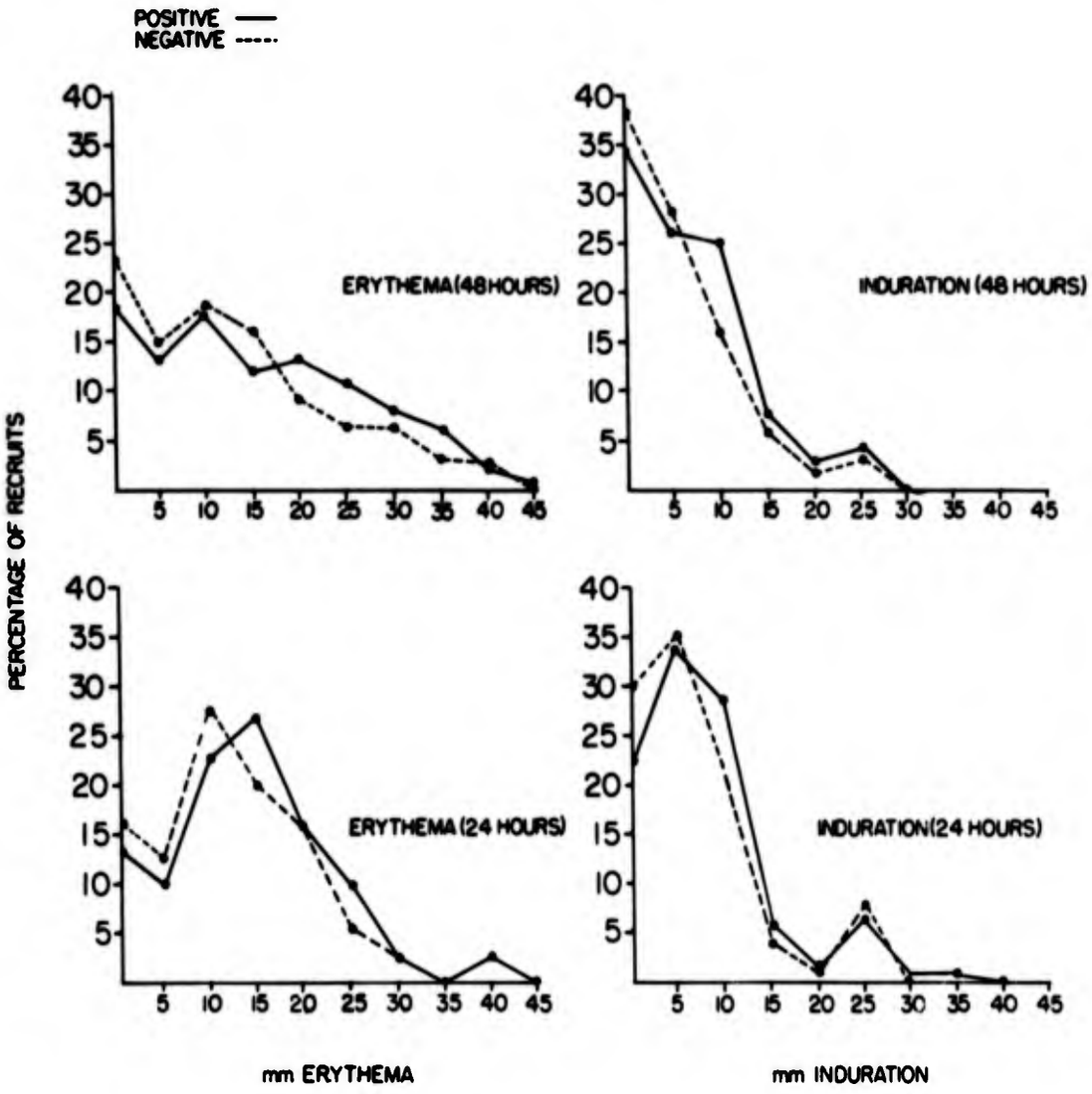

POSITIVE
NEGATIVE
PERCENTAGE OF RECRUITS
ERYTHEMA(48HOURS)
INDURATION (48 HOURS)
ERYTHEMA (24 HOURS)
INDURATION(24 HOURS)
mm ERYTHEMA
mm INDURATION

Fig. 1

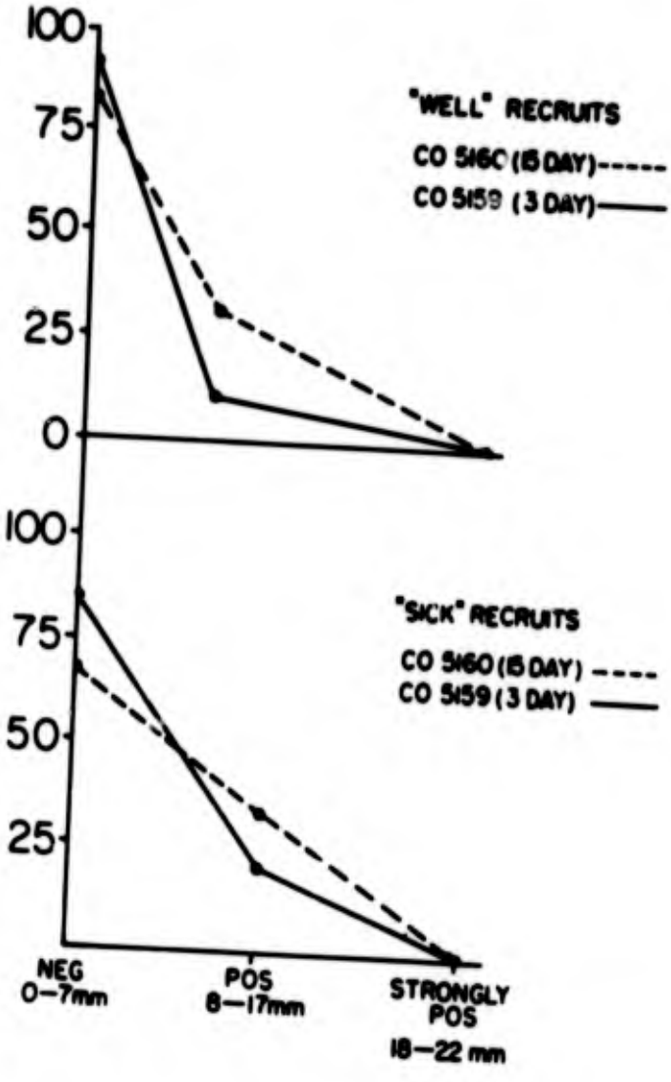

100
75
50
25
0
"WELL" RECRUITS
CO 5160 (15 DAY)-----
CO 5159 (3 DAY)——
100
75
50
25
"SICK" RECRUITS
CO 5160 (15 DAY) ----
CO 5159 (3 DAY) ——
NEG
0–7mm
POS
8–17mm
STRONGLY
POS
18–22 mm

Fig. 2

REFERENCES

1. Miller, L.F., Rytel, M.W., Pierce, W. E. and Rosenbaum, M.J.: Epidemiology of Non-bacterial Pneumonia among Naval Recruits. JAMA. 185:92-99, 1963.

2. Andrewes, C. H.: The Complex Epidemiology of Respiratory Virus Infections. Science. 146:1274-77, 1964.

3. Lytle, R. I., Rytel, M. W. and Edwards, E. A.: Correlation of Immunological and Biological Factors in the Host with Susceptibility to Respiratory Infections. J. Inf. Dis. 116: 67-74, 1966.

4. Miller, L. F.: Acute Respiratory Disease in Naval Personnel. Milit. Med. 129:526-32, 1964.

5. Wintrobe, M. M.: Clinical Hematology, Ed. 6, Lea and Febiger, Philadelphia, 1967.

6. Beisel, W. R. and Rapoport, M. I.: Inter-Relations Between Adeno-cortical Functions and Infectious Illness. New. Eng. J. Med. 280:541-46, 1969.

7. Pierce, W. E. and Miller, L. F.: The Effect of Delayed Immunizaticns on Acute Respiratory Disease in Navy Recruits. Amer. J. Public Health. 55:60-67, 1965.

8. Cooper, C. E. and Nelson, D. H.: Adrenocortical Tropic Hormone Levels in Plasma of Pre-Operative and Surgically Stressed Patients. J. Clin. Invest. 41:1599-1605, 1962.

9. Dougherty, T. F., Berliner, M. L., Schneebeli, G. L. and Berliner, D. L.: Hormonal Control of Lymphatic Structure and Function Ann. N. Y. Acad. Sci. 113, Art. 2:825-43, 1964.

10. Wirschhorn, K.: Immune Response and Mitosis of Human Peripheral Blood Lymphocytes In Vitro. Science. 142:1185-86, 1963.

11. Miller, S. E. ed.: Clinical Pathology, Ed 7, Williams and Wilkins, Baltimore, 1966.

12. Dougherty, T. F. and Frank J. A.: The Quantitative and Qualitative Responses of Blood Lymphocytes to Stress Stimuli. J. Lab. Clin. Med. 42:530-37, 1953.

13. Wiener, J., Cottrell, T. S., Margareteu, W. and Spiro, D.: An Electron Microscopic Study of Steroid Induced Reticuloendothelial Blockade. Amer. J. Path. 50:187-94, 1967.

THE DETERMINATION OF BACTERICIDAL ACTIVITY IN NAVY RECRUITS WITH NEISSERIA MENINGITIDIS INFECTIONS*

Ensign Michael P. Gerberi
Loyola University Stritch School of Medicine

Preceptor: Earl A. Edwards
Chief, Immunology Division

The epidemiological findings of several investigators report that resistance to Neisseria meningitidis infection is directly related to the bactericidal antibody level. "Sera from individuals susceptible to N. meningitidis do not appear to lack factors other than antibody which are necessary for bactericidal activity, however, such individuals are capable of responding immunogically during the course of infection" (1). A complement-fixation (CF) antigen and a hemagglutination (HA) antigen have been shown to possess the sensitivity and the specificity required of serologic tests in determining seroresponse to N. meningitidis infections (2). However, the sequential relationship between acquisition of N. meningitidis and the level of bactericidal or HA and CF antibody has not been determined.

The objective of this research was to test the serum collected from recruits for bactericidal antibody. The occurrence of bactericidal antibody was compared to the acquisition of the organism and the appearance of both HA and CF antibody. This same comparison was made on serum collected from recruits who have had meningococcal disease.

MATERIALS AND METHODS

The bactericidal test procedures employed were basically those described by Roberts (3) with certain modifications. The media used as a solvent for the tests consisted of 99 parts Hanks salt solution (4) and 1 part of a gelatin solution prepared from 0.1% gelatin (Difco Laboratories, Detroit, Michigan). The strain of bacteria was a group C (PTS5) obtained from Dr. N. A. Vedros, NBL, Oakland, California.

Preparation of bacterial suspensions

Meningococci were maintained by subculture on Mueller-Hinton agar (Difco). Culture plates were incubated on 37°C in the presence of

---

*From Research Project No. M4305.01-1012 , Bureau of Medicine and Surgery, Navy Department, Washington, D. C.

moisture and CO_2 (candle jar). In preparation for each test, an 18 hour agar culture was transferred to 125 ml Erlenmeyer flasks containing 25 ml of Eugonbroth (Baltimore Biological Laboratory, Inc., Baltimore, Maryland). Broth cultures were placed in a Psychotherm Incubator Shaker (170-200 rotations/min) and incubated aerobically at 37°C for 4 hours to obtain the bacteria in log phase growth (3).

Serum

Normal serum was collected from a surveillance company of Navy recruits at Great Lakes Naval Training Center at 0, 5 and 9 week intervals. Serum collected from recruits with clinical meningitis was taken at about weekly intervals for 8 weeks. The serum was separated from clotted blood and stored at -20°C until tested.

Bactericidal test

Test tubes size 10 x 75 mm were used. To each tube was added 0.7 ml gel-Hanks medium, 0.1 ml bacterial suspension diluted to a suitable number of organisms by the Nephelometric Method (5), 0.1 ml serum (human inactivated), and 0.1 ml of fresh complement in the form of rabbit serum. In addition to the test samples, 2 controls were run to determine any degree of toxicity to the bacteria by the inactivated human serum or by the rabbit serum. To the serum control test tube was added 0.8 ml gel-Hanks medium, 0.1 ml bacterial suspension, and 0.1 ml serum. A similar mixture was used in the complement control, however, 0.1 ml rabbit serum was exchanged with the human serum.

At intervals of 0 and 120 minutes, 0.2 ml samples were removed from the test suspensions and colony plated. After 18 hours incubation at 37°C in the presence of CO_2 and moisture, the bacterial colonies were counted.

The bactericidal activity of the rabbit serum used as the source of complement was determined to prevent it from coloring the final results of the bactericidal tests to be performed. Serial dilutions of the rabbit serum were made and 0.1 ml was added to tubes containing 0.8 ml of gel-Hanks medium and 0.1 ml bacterial suspension.

RESULTS

It was determined, as shown in Table 1, that an undiluted sample of normal rabbit serum showed little, if any, bactericidal activity, whereas a 1:5 dilution showed no activity. Due to the uncertainty of the activity of the undiluted serum, the 1:5 dilution was used in the bactericidal tests.

The results of bactericidal activity in sera of 5 recruits are shown in Table 2. Two men, 00905 and 00916, showed definite bactericidal activity while the remaining 3 indicated no bactericidal activity. Recruit 00905 demonstrated bactericidal activity without the

presence of complement and without having either a positive CF or HA test, nor did he become a carrier. Recruit 00916 showed bactericidal activity only in the presence of complement and had positive CF and HA tests.

Table 3 contains the results of the bactericidal activity of an individual against a heterologous C strain of meningococcus over a period of 10 weeks. Prior to the 5th week when he acquired a C strain as a carrier, no bactericidal activity and negative CF and HA tests were demonstrated. In a matter of days after acquisition of the meningococcus in a carrier state, CF and HA tests were positive and the bactericidal test demonstrated a phenomenon which was difficult to interpret. Neither growth nor killing of the organism occurred, rather a bacteriostatic effect was observed. It was thought that perhaps if the bactericidal activity was not maximal, a small percentage of the organisms could survive and increase in number after 2 hours of incubation to approximately the original colony count. In order to determine this, a test on the effect of time on bactericidal activity was performed using a serum which showed definite bactericidal activity. As can be seen in Table 4, the bactericidal activity was maximal for all practical purposes after a period of 60 minutes and maintained a high degree of killing for a minimum of 4 hours.

Table 5 describes bactericidal tests performed with sera from meningococcal patients. In the first case, the acute stage serum and the 1st week serum demonstrated strong bactericidal action. In the 2nd case, only the acute serum showed any bactericidal action. Both men's sera became bacteriostatic within the week following the bactericidal activity. As in the Weekly Acquisition of Meningococcus (WAM) study test, positive CF and HA tests appeared at the same time as the bacteriostatic effect.

A final test was performed comparing the bactericidal activity of serum from a recruit with infectious meningitidis against a heterologous C strain (Table 5, case 1) and the homologous C strain of meningitidis which he himself acquired. It can be seen in Table 6 that the bactericidal activity was identical for all practical purposes against both strains in every sequential sera tested.

DISCUSSION

The relationship between bactericidal antibodies and the observed bacteriostatic phenomenon, which occurred consistently in our tests, is undetermined. Since the CF and HA tests appear concurrent with the bacteriostatic effect, it is probable that the latter represents antibody activity. However, clear cut evidence of bactericidal activity has been demonstrated by sera 00905 (Table 2) with no positive CF or HA indicated. This would tend to suggest that the bactericidal activity is independent of either CF or HA antibodies. These observations, however, do not shed light on the question of whether or not the bacteristatic and bactericidal activity are manifestations of the same antibody system.

Several factors may have had a definite effect on the outcome of the tests performed. One factor, the significance of which has not been clearly ascertained, is the use of heterologous strains of meningococcus. In the test which we performed comparing bactericidal activity against homologous and heterologous C strains of meningitidis, the data indicated no specificity for either strain by the bactericidal antibody. However, in similar tests performed by Muehl on several samples of sera of meningococcal patients, the results were varied and inconsistent (6). Some sera demonstrated bactericidal action against a heterologous strain, but not against a homologous strain. Other sera showed no bactericidal action against either strain or showed activity against the homologous but not the heterologous strain.

It was demonstrated by Goldschneider et al (1) that there was a 15% decrease in bactericidal activity against a heterologous C strain, as compared with bactericidal activity against homologous strains. Furthermore, they were able to demonstrate bactericidal activity after more than 2 weeks subsequent to acquisition of the meningococcus only with the homologous strain.

Another important factor to be considered is the source of complement. Although it is demonstrated that some sera possess powerful bactericidal activity in the absence of complement (Table 2 - 00905 and Table 5 - Acute), the bactericidal action of normal serum appears to be dependent upon the presence of complement. Fresh rabbit serum has approximately the same degree of bactericidal action against meningococcus as adult human serum (7). But for practical purposed, the bactericidal test employing human defibrinated blood as complement with a resistant strain was found to be the best method for estimating bactericidal antibody (8).

Thomas and Dingle (7) found that if a generally susceptible strain of meningococcus is employed, bactericidal antibody can be demonstrated in the presence of a non-bactericidal complementing serum. However, if a resistant strain is used, a difference between the effectiveness of complement from different species becomes apparent such that rabbit complement is effective while human complement is not (7). No source of complement seems to be satisfactory for all the conditions which may exist in vitro.

SUMMARY

It is difficult to demonstrate consistent bactericidal activity in the carrier state or in a sequential study of the carrier and disease states employing a heterologous strain of bacteria. This indicates the need for further study contrasting the bactericidal action against homologous vs. heterologous strains in a sequential situation. This investigation also shows the need for the further determination of an adequate source of complement to test human bactericidal activity. Concerning the association of specific agglutinins and CF antibodies with the presence of bactericidal antibody, no definite quantitative relation

can be determined from the results. Generally, these antibodies coincide, but it is clearly demonstrated that strong bactericidal activity can and does occur in the absence of positive CF and HA determinations.

TABLE 1. Control test on bactericidal activity of rabbit serum used as the source of complement

Dilution	0 Hour		2 Hour	
	Plate A	Plate B	Plate A	Plate B
Whole serum	4	2	1	1
1:5	61	58	49	57
1:10	71	65	63	60
1:20	96	83	87	94
1:40	33	40	43	47
1:80	30	30	24	33

TABLE 2. Bactericidal activity in recruit sera of a surveillance company

Recruit Number		0 Hour	2 Hour	CF*	HA**	BT***
	Complement Control	96	209			
		56	101			
00891	Serum Control (1st wk)	97	105	-	-	-
	Test "	85	113			
	Serum Control (9th wk)	84	76	+	+	+
	Test "	79	78			
00905	Serum Control (1st wk)	76	2	-	-	-
	Test "	81	4			
	Serum Control (9th wk)	86	0	-	-	-
	Test "	87	0			
00916	Serum Control (1st wk)	50	56	-	-	-
	Test "	56	90			
	Serum Control (9th wk)	62	0	+	+	+
	Test	46	3			
00921	Serum Control (1st wk)	90	46	-	-	-
	Test "	58	66	+	+	+
	Serum Control (9th wk)	36	44			
	Test "	70	60			
00930	Serum Control (1st wk)	86	108	-	-	-
	Test "	80	100			
	Serum Control (9th wk)	88	72	+	+	+
	Test "	62	38			

* CF - Complement Fixation Test (9)
** HA - Hemagglutination Test (10)
*** BT - Bacteriology Throat Culture

TABLE 3. Bactericidal activity of serum from a recruit(28) in a WAM* study

Week No.		0 Hour	2 Hour	CF	HA	BT
	Complement Control	119	256			
1	Serum Control	70	236	-	-	-
	Test	75	297			
4	Serum Control	85	307	-	-	-
	Test	70	328			
5	Serum Control	113	255	-	-	+
	Test	83	313			
6	Serum Control	70	84	+	+	+
	Test	82	88			
7	Serum Control	82	83	+	+	+
	Test	75	92			
8	Serum Control	70	88	+	+	+
	Test	77	76			
9	Serum Control	87	120	+	+	+
	Test	73	133			
10	Serum Control	50	75	+	+	+
	Test	37	72			

* WAM - Study of Weekly Acquisition Rate of Meningococcus in Navy Recruits

TABLE 4. The effect of time on bactericidal activity of serum

	TIME (MINUTES)								
	0	10	20	30	45	60	90	120	240
Complement Control	87	78	89	98	78	84	105	102	250
Serum Control	31	22	22	12	5	3	0	1	0
Test	35	24	23	14	6	1	0	0	0

TABLE 5. Bactericidal activity in the diseased state

Stage of Disease		0 Hour	2 Hour	CF	HA
	Complement Control	51	121		
CASE 1					
Acute	Serum Control	0	0	-	-
	Test	1	0		
1st Week	Serum Control	41	35	-	+
	Test	52	0		
4th Week	Serum Control	45	46	+	+
	Test	34	30		
5th Week	Serum Control	31	28	+	+
	Test	33	34		
6th Week	Serum Control	33	29	-	+
	Test	31	28		
10th Week	Serum Control	34	23	-	+
	Test	47	30		
	Complement Control	67	269		
CASE 2					
Acute	Serum Control	92	4	-	-
	Test	90	6		
1st Week	Serum Control	95	107	+	+
	Test	124	119		
3rd Week	Serum Control	89	104	+	+
	Test	91	84		
4th Week	Serum Control	87	97	+	+
	Test	98	107		
6th Week	Serum Control	87	134	+	+
	Test	117	133		

TABLE 6. A comparison of bactericidal activity in the diseased state against homologous and heterologous C strains of *Neisseria meningitidis*

Stage of Disease		Heterologous Strain		Homologous Strain	
		0 Hour	2 Hour	0 Hour	1 Hour*
Acute	Serum Control	0	0	33	6
	Test	1	0	42	12
1st Week	Serum Control	41	35	238	234
	Test	52	0	190	81
4th Week	Serum Control	45	46	212	186
	Test	34	30	263	273
5th Week	Serum Control	31	28	177	223
	Test	33	34	208	168
6th Week	Serum Control	33	29	216	239
	Test	31	28	177	190
10th Week	Serum Control	34	23	191	192
	Test	47	30	193	201

* This test was performed after the time study shown in Table 5 so that 1 hour was considered enough time to demonstrate bactericidal activity.

ACKNOWLEDGEMENTS

I would like to express my appreciation to Mr. Edwards for his guidance and his invaluable instruction on the basic immunological principles involved. I would also like to thank the members of the Immunology Division, especially Pat Muehl, for their constant cooperation and assistance.

REFERENCES

1. Goldschneider, Irving, Gotschlich, Emil C., and Malcom S. Artenstein: Human Immunity to the Meningococcus. J. Exp. Med. 129:1307, 1969.

2. Edwards, E. A., Driscoll, W. S., Muehl, P. M., Lytle, R. I. and L. F. Devine: Hemagglutination and Complement Fixation Antigens from Neisseria meningitidis. I. Some Laboratory Characteristics. Meningococcal Progress Report. Naval Medical Research Unit No. 4, 1968.

3. Roberts, Richard B.: The Interaction in vitro Between Group B Meningococci and Rabbit Polymorphonuclear Leukocytes. J. Exp. Med. 126(5):795-818, November 1967.

4. Paul, John: Cell and Tissue Culture. Williams and Wilkins Co., Balt., (Table I, p. 86), 1961.

5. Davidsohn, Israel and Benjamin Wells: Clinical Diagnosis by Laboratory Methods. W. B. Saunders Co., Phil., 1966, p. 807.

6. Muehl, P. M.: Personal Communication, August 25, 1969.

7. Thomas, Lewis and John H. Dingle: Investigations of Meningococcal Infection. III. The Bactericidal Action of Normal and Immune Sera for the Meningococcus. J. Clin. Invest. Vol. 22, pp. 375-385, 1943.

8. Thomas, Lewis, Smith, Webster and John H. Dingle: Investigations of Meningococcal Infection. II. Immunological Aspects. J. Clin. Invest. Vol. 22, pp. 361-373, 1943.

9. Edwards, E. A. and L. F. Devine: A Genus-Specific Complement-Fixation Antigen for Neisseria meningitidis. Proc. Soc. Exp. Biol. and Med. 126:1168-1173, 1968.

10. Edwards, E. A. and W. S. Driscoll: Group-Specific Hemagglutination Test for Neisseria meningitidis Antibodies (32593). Proc. Soc. Exp. Biol. and Med. 126:876-879, 1967.

GEL DIFFUSION STUDIES OF NONGROUPABLE MENINGOCOCCI*

Lieutenant (junior grade) Philip M. W. Pailey
Marquette University School of Medicine

Preceptor: Commander Leonard F. Devine, MSC USN
Chief, Bacteriology Division

In 1956, Jyssum employing double-diffusion in gels, found 5 of 13 strains of meningococci isolated from spinal fluid which failed to agglutinate with antisera prepared to groups A, B, C and D (1). He called these strains type N. Branham, however, found these strains would react with her group B antisera.

In 1961 and 1963, Slaterus reported 3 new groups which he labeled X, Y and Z (2,3). These 3 groups and a 4th strain (Z') were not agglutinated by antisera to meningococci of serological groups A, B, C and D. Antisera to the Z' strain agglutinated group Z cells, but Z' cells were not agglutinated by Z antisera. Evans, Artenstein and Hunter, in 1968, reported 3 new groups, Bo, 29E and 135 (4). The Bo strain was described as being identical with the Y strains of Slaterus.

A number of meningococcal strains isolated from recruit carriers at Great Lakes Naval Training Center could not be typed by the conventional slide-agglutination technique. A study of the precipitation lines formed in gels using the double-diffusion method was undertaken to determine if this method could elucidate the relationships of these strains to the standard known strains of meningococci.

MATERIALS AND METHODS

Serogroups and source of strains

A	MK01 isolated from spinal fluid and received from W. Sanborn, NAMRU-3, Cairo, United Arab Republic.
B	Will-B isolated from spinal fluid at Great Lakes.
B	Grubbs-B isolated from nasopharynx of carrier at Great Lakes.
C	PTS5 received from N. Vedros, NAMRU-1, Berkeley, California
Bo	Bo(WR) received from M. Artenstein, Walter Reed Army Institute of Research (WRAIR), Washington, D. C.
Bo	Bo-6 received from N. Vedros, NAMRU-1, Berkeley, California.

*From Research Project No. M4305.01-1012, Bureau of Medicine and Surgery, Navy Department, Washington, D. C.

Serogroups and source of strains (Cont'd)

135	135(WR) received from M. Artenstein, WRAIR.
X	X(WR) Slaterus strain "type" X received from M. Artenstein, WRAIR.
Z	Z(WR) Slaterus strain "type" Z received from M. Artenstein, WRAIR.
Z	17 strain isolated from nasopharynx of carrier at Great Lakes.
29E	29E(WR) received from M. Artenstein, WRAIR.
29E	112 strain isolated from nasopharynx of carrier at Great Lakes.
Lact. 25	Isolated from nasopharynx of carrier at Great Lakes.
Lact. 27	Isolated from nasopharynx of carrier at Great Lakes
Flor. 350	Isolated.
Flor. 351	
RAS-7	Isolated from nasopharynx of carrier at Great Lakes.
RAS-10	Isolated from nasopharynx of carrier at Great Lakes.
RAS-10	331 strain isolated from nasopharynx of carrier at Great Lakes.
RAS-10	351 strain isolated from nasopharynx of carrier at Great Lakes.
RAS'	11 strain isolated from nasopharynx of carrier at Great Lakes.
RAS'	469 strain isolated from nasopharynx of carrier at Great Lakes.
Nongroupable (NR)	1 strain isolated from nasopharynx of carrier at Great Lakes.
Nongroupable	4 strain isolated from nasopharynx of carrier at Great Lakes.
Nongroupable	5 strain isolated from nasopharynx of carrier at Great Lakes.
Nongroupable	6 strain isolated from nasopharynx of carrier at Great Lakes.
Nongroupable	8 strain isolated from nasopharynx of carrier at Great Lakes.
NR	12 strain isolated from nasopharynx of carrier at Great Lakes.
NR	132:48-Col. 3 strain isolated from nasopharynx of carrier at Great Lakes.
NR	266 strain isolated from nasopharynx of carrier at Great Lakes.
NR	408 strain isolated from nasopharynx of carrier at Great Lakes.
NR	737 strain isolated from nasopharynx of carrier at Great Lakes.
Salmonella B	#8759 received from American Type Culture Collection, Rockville, Maryland.
Salmonella D	#12840 received from American Type Culture Collection, Rockville, Maryland.

Preparation of antisera

All locally obtained antisera were prepared according to the method of Edwards and Devine (5).

The antisera were tested against their homologous antigens to determine the efficacy of each individual lot. The antisera used in the determination of the unknown strains were chosen from these tests on the basis of high titer and minimal nongroup-specific precipitation lines.

Description of antisera

Serogroup and source of strains used from immunization:

A	Lot #4 received from the Neisseria Repository, NAMRU-1, Berkeley, California.
C	Prepared locally to strain PTS5 in rabbit #156.
Bo	Prepared locally in rabbit #232 and absorbed with RAS-10 and RAS' cells.
29E	Prepared locally in rabbit #79.
W135	Prepared locally in rabbit #344.
X	Prepared locally in rabbit #106.
Z	Prepared locally in rabbit #239.
Z	Prepared locally in strain 17 in rabbit #194.
NR 408	Prepared locally in rabbit #230.
NR 6	Prepared locally in rabbit #41.
NR 12	Prepared locally in rabbit #620.
NR 266	Prepared locally in rabbit #244.
NR 737	Prepared locally in rabbit #276.
Flor. 351	Prepared locally in rabbit #303.
RAS-10	Prepared locally in rabbit #300.
RAS-10	Prepared locally in rabbit #362 and absorbed with RAS' cells.
RAS'	Prepared locally to strain 11 in rabbit #359 and absorbed with RAS-10 cells.

Absorption of antisera

Antisera were absorbed according to the method of Devine and Hagerman (6).

Antisera absorption were done *in situ* in some experiments by a modification of the technique of Björklund (7). The routine test was preceded by adding meningococcal cells to the central well and replacing them with more cells after 8 hours. These were removed by suction 16 hours later. Antiserum was added in the central well, and bacterial cells in the peripheral wells.

Gel diffusion tests

"Ionagar" no. 2 was obtained from Oxoid Division of Oxo, Ltd., London, England. A 1% agar gel with a final concentration of 0.01%

merthiolate and 0.014% sodium borate as preservative was used for double-diffusion tests. A standard 1 X 3 inch microscope slide was overlayed with a thin layer of agar and dried and then overlayed with 2.5 ml of agar. Upon drying, an immunodiffusion set, 6800A-8, using die 6866A circular pattern of 1.5 mm outside diameter well cutters 6818A was used to make all the wells (L.K.B. Instruments, Inc., Washington, D. C.). The usual procedure consisted of placing the desired antisera in the center well 1 hour prior to the addition of the whole cell antigens. The antisera were replenished once immediately before the thick suspensions of meningococcal cells in 0.15 M NaCl containing 0.01% merthiolate were placed in the peripheral wells. Precipitation lines were observed at 24 and 48 hours.

RESULTS

Meningococcal group-specific antigen-antibody precipitation lines were found in gel-diffusion tests for serogroups A, C, 29E, Bo, 135, X and Z. Nongroup-specific precipitation lines were found in most of the tests, but did not interfere with the detection of the group-specific lines, since they could be removed by absorption of the antisera with various meningococcal whole cell types (6). Representative results are shown in Figure 1.

The addition of antisera and antigens to the gels produced well-defined antibody-antigen precipitation lines for the known serological groups A, C, 29E, Bo, 135, X and Z when the reactants were added within an hour of each other. However, characteristic lines of precipitation were not consistently observed following addition of reactants at these time intervals when studying strains of the RAS category. A study to determine the optimum time for the addition of sera and cells for getting the best lines of precipitation was performed using high titered, undiluted RAS-10 (#721) antisera. The most characteristic precipitation lines for the RAS-10 strain were observed after 2 or more additions of antisera 24 hours after the whole cell antigens were introduced.

Studies of antigen-antibody precipitation lines using antisera of generally recognized serological groups against meningococcal cells showed that some of the nongroupable strains could be identified on this basis. However, antisera to the unknown strains would not form precipitation lines in gels with the whole cell antigens of the standard known groups in a reciprocal manner, even though the nongroupable antisera were shown to be of sufficiently high titer against its own antigen. Two strains, NR 6 and Flor. 351, were tentatively identified as group C, Fig. 2 and Fig. 3, respectively. Strain 737 was tentatively identified as group A, Fig. 4. The NR 266 strain formed very faint precipitation lines with the Z antisera and no lines of identity with any of the other known serogroups. This suggested that NR 266 may be a Z subgroup, however, more investigation of this strain is needed. The NR 408 strain was tested against all known type antisera and failed to produce any group-specific precipitation lines.

One strain, NR 22, formed group-specific precipitation lines with standard Bo antisera. The reciprocal NR 22 antisera formed similar lines with Bo whole cell antigen thereby confirming the identification, Fig. 5.

A positive test for the identification of a nongroupable strain was considered to be perfect continuity between the precipitation lines of the unknown strain and the precipitation lines of the standard known group in adjacent wells, Fig. 3.

One strain, 132:48-Col. 3, was agglutinated by antisera to B, Bo, 135, L form of B, Bc (absorbed with RAS-10 and RAS' antigens), RAS-10 (absorbed with RAS' antigen), and RAS' (absorbed with RAS-10 antigen) in the slide agglutination test. This strain was also shown to form precipitation lines with all the above antisera using the gel diffusion method. Antisera to strain 132:48-Col. 3 were not prepared and therefore was not tested against the known group whole cell antigens.

Gel diffusion analyses of certain meningococcal strains that could not be typed using routine slide-agglutination tests indicate that the gel-diffusion method can be of considerable value in the serological identification of unknown strains.

None of the precipitation lines demonstrated for the 132:48-Col. 3 whole cell antigen with the various known standard antisera have been shown to be group-specific. More study of this strain is necessary, therefore, to establish the relationship of this strain to the groupable strains.

Results of this study indicate that the gel diffusion method was normally quite sensitive in detecting the standard known serotypes. This method is not completely satisfactory for the positive identification of specific antibody-antigen precipitation lines with the strains in the RAS category. Our study showed that the RAS-10 whole cell antigen had to be introduced to the gel medium at least 24 hours prior to the addition of the antisera. In the past, RA specific precipitation lines have been noted only immediately surrounding the antigen wells and occasionally have been thought to be present with the wells only.

These findings indicate that the RAS-10 specific antigen has a very small diffusion coefficient with respect to the specific antigen of the standard known serotypes. The smaller diffusion coefficient might suggest that the RAS specific antigen is more highly branched and may be a protein polysaccharide complex of greater molecular weight with antigenically active polysaccharide sites.

It became apparent that many of the nongroupable strains were not completely homologous to any of the standard serotypes and were in fact subtypes whose antigenicity had been slightly altered. The gel diffusion technique, however, is a good qualitative method for detection of homologous or closely related antigens.

SUMMARY

Double-diffusion in gels was employed for the detailed study of 5 nongroupable meningococcal strains previously studied by the routine slide-agglutination method. These strains were isolated from recruit carriers at Great Lakes Naval Training Center. Two of the strains were tentatively identified as group C; 1 strain was tentatively identified as group A; and 1 strain was tentatively identified as group Z. The remaining strain was positively shown to be a Bo. The unusual precipitation patterns of the RAS-10 strain were studied and found to vary with respect to the time of addition of the whole cell antigen. The gel-diffusion technique was of considerable value in the elucidation of the relationships between the nongroupable strains and the known standards.

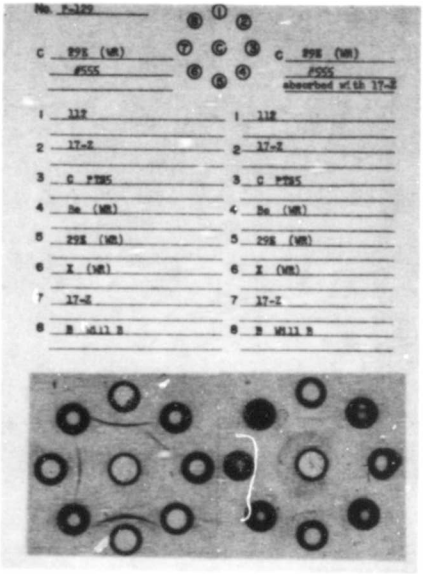


Fig. 1. a. Group-specific and nongroup-specific precipitation lines between 29E antisera and selected whole cell antigens in gels.

b. Group-specific precipitation lines between 29E (absorbed) antisera and selected whole cell antigens in gels.

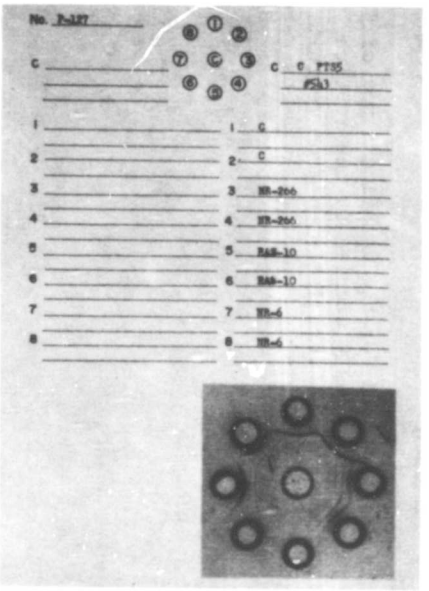


Fig. 2. Precipitation lines of identity between C antisera and C - NR 6 whole cell antigens in gels.

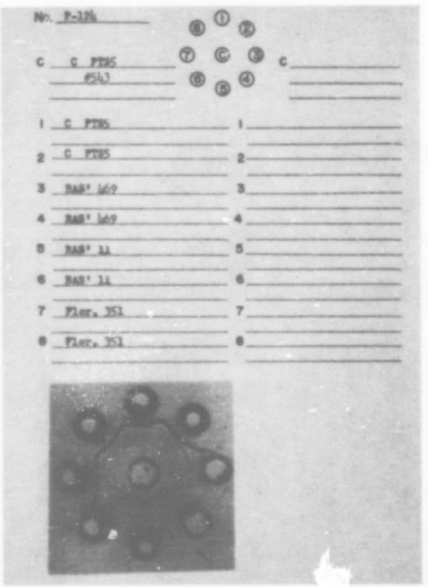


Fig. 3. Precipitation lines of identity between C antisera and C - Flor. 351 whole cell antigens in gels.

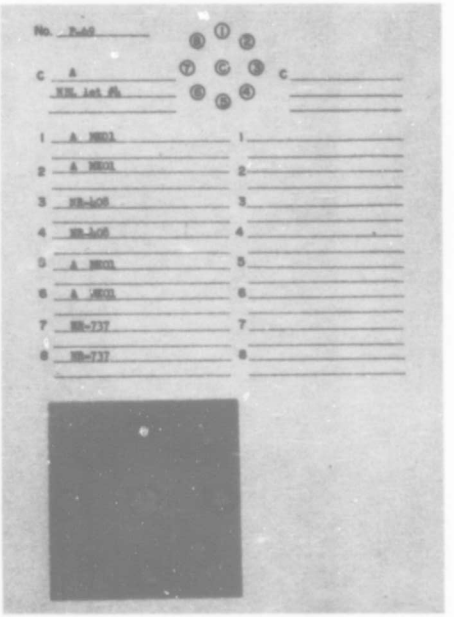


Fig. 4. Precipitation lines of identity between A antisera and A - NR 737 whole cell antigens in gels.

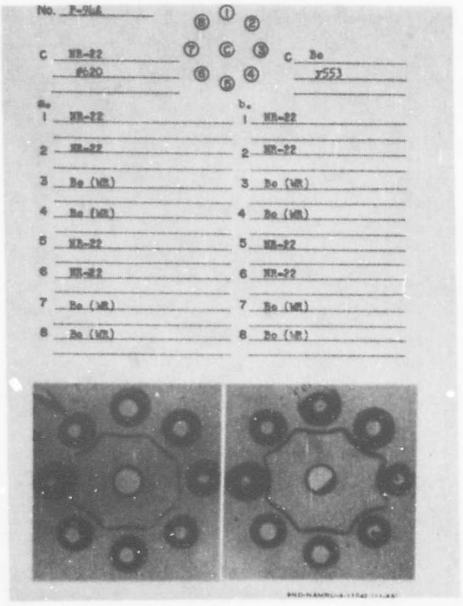


Fig. 5. a. Precipitation lines of identity between NR 22 antisera and NR 22 - Bo whole cell antigens in gels.

b. Precipitation lines of identity between Bo antisera and Bo - NR 22 whole cell antigens in gels.

REFERENCES

1. Jyssum, K.: Serologic Relationship Among Meningococcal Strains in Norway. J. Immunol. 76:433-440, 1956.

2. Slaterus, K. W.: Serological Typing of Meningococci by Means of Micro-precipitation. Antonie van Leeuwenhoek J. Microbiol. Serol. 27:304-315, 1961.

3. Slaterus, K. W.: Types of Meningococci Isolated from Carriers and Patients in a Non-epidemic Period in the Netherlands. Antonie van Leeuwenhoek J. Microbiol. Serol. 29:265-271, 1963.

4. Evans, J. R., M. S. Artenstein, and D. H. Hunter: Prevalence of Meningococcal Serogroups and Description of Three New Serogroups. Am. J. Epidemiol. 87:643-646, 1968.

5. Edwards, E. A. and L. F. Devine: A Genus Specific Complement Fixation Antigen from Neisseria meningitidis. Proc. Soc. Exp. Biol. and Med. 128:1168-1173, 1968.

6. Devine, L. F. and C. R. Hagerman. NAMRU-4, Great Lakes, Illinois, to be submitted for publication.

7. Björklund, B.: Specific Inhibition of Precipitation as an Aid in Antigen Analysis with Gel Diffusion Method. Proc. Soc. Exp. Biol. and Med. 79:319-324, 1952.

COMPLEXITY OF BIOLOGICAL FLUIDS AS RELATED TO DISEASE STATES*

Ensign Richard C. Smith
Creighton University School of Medicine

Preceptor: Ralph I. Lytle
Chief, Biochemistry Division

In recent years there has been a great deal of interest in the immunologic properties of extravascular fluids, particularly colostrum, perspiration and saliva. The hypothetical effectiveness of these fluids in providing "local" immunity and enhanced protection from foreign antigens at the epithelial-endothelial barriers has been the primary motivation for identification of immunoglobulins and studies of their secretion.

Considerable progress has been made during the last 20 years in fractionating large molecular materials of extravascular secretions by physicochemical methods. These include various forms of electrophoresis on different media, column chromatography on such substances as DEAE- and CM- Sephadex columns, partition paper chromatography, immunodiffusion, and ultracentrifugation. Of these methods, electrophoresis has been most successful in achieving a reasonable degree of resolution. The success of electrophoresis in the study of secretions has been achieved principally through the use of appropriate media, and correlation of these results obtained with the information yielded by other methods.

Parotid saliva was studied by Patton and Pigman (1,2) and Zipkin et al (3) using free-boundary electrophoresis. Seven peaks were observed, 5 of which were seen distinctly in every case. Two major peaks accounted for 75% of the total components. Patton and Pigman (1) reported 10 to 12 electrophoretic components, of which some were minor and inconstant, whereas, ultracentrifugal patterns showed only 3 or 4 components. The components appeared to be different from those in serum. Several materials moved to the cathode at pH 8.5, 1 of which may have corresponded to lysozyme. Several fast moving anodic components of low concentration, having a mobility higher than that of serum albumin, were also observed. They probably corresponded to some acidic mucopolysaccharides. The parotid and submaxillary secretion showed some similarities but both appeared to be different from the sublingual saliva.

Ferguson et al (4) found several protein and carbohydrate staining components in the human parotid saliva, some of which had mobilities of serum proteins. Especially distinct protein bands had the mobility

- -

*From Research Project No. MR 005.09.01-0087B, Bureau of Medicine and Surgery, Navy Department, Washington, D. C.

of gamma-globulin. No correlation of gamma-globulin concentration with the salivary flow rate could be shown.

Extensive work on fractionation of human parotid saliva by paper electrophoresis was performed by Mandel and Ellison (5). Six to 8 fractions were usually detected in parotid saliva. The most rapidly migrating anodic protein component spread over a wide area and was of relatively low concentration. Bands 3, 4 and 6 represented major fractions (Fig. 1). Band 1 contained little carbohydrate, was frequently poorly resolved, and represented serum albumin, as shown by immunological tests. Band 2 probably was an alpha or a beta globulin and also contained little carbohydrate. Bands 3 and 4 contained gamma globulin, as shown by immunochemical criteria. Amylase, a small amount of fucose, and a large amount of hexosamines were present as well. Band 7 migrated most rapidly to the cathode and contained very little carbohydrate. Mandel and Ellison concluded that, although parotid glands are considered to be serous in nature, they regularly secrete mucoid material containing glycoproteins of cathodic mobility, which include hexoses, hexosamines, and fucose.

It was not possible to detect, by paper electrophoresis, anodic components of a higher mobility than serum albumin, which Patton and Pigman found by free-boundary electrophoresis. Tamarin (6) showed that parotid saliva, when concentrated by prevaporation and submitted to paper electrophoresis, separated into 10 components.

Hoerman and Berzinskajs (7) collected parotid saliva by the Curby modification of the Lashley parotid cups. Nine to 12 distinct protein zones were readily detected in 10 samples of parotid saliva after staining with amido black. Meyer and Lamberts (8) have more recently utilized acrylamide gel and Wool Fast Blue-BL for the electrophoresis of parotid saliva. They have observed up to 40 components in patterns from individual parotid saliva specimens (Fig. 2).

Ellison et al (9) and Mandel and Ellison (10) have used immunoelectrophoresis for identification of the protein components of human parotid saliva. They utilized immune sera directed against saliva and individual serum proteins. Seven proteins, immunochemically similar to plasma protein, were detected. The differences in salivary protein concentration were rather small in various individuals. Fibrinogen could not be detected, but albumin, orosomucoid, ceruloplasmin, beta-lipoprctein, transferrin and beta 2-macroglobulin were found. The highest concentration of protein was that of gamma-globulin.

The present study attempts to further define the composition of parotid saliva relating various immunoglobulins detected by immunologic methods to the patterns obtained during the separation of proteins by acrylamide gel electrophoresis, and to localize immunoglobulins to a specific portion of the electrophoretic pattern.

MATERIALS AND METHODS

Collection and processing of specimen

Acute and convalescent specimens of parotid saliva, serum, and nasal washings were collected from 50 Navy recruits.

Parotid saliva was collected in modified Lashley cups and cooled during collection to about 4°C. Salivary flow was increased by the use of lemon-flavored troches. Each specimen was measured, then dialyzed 2-4 hours in distilled water to remove salts. 0.5 ml of each sample was removed for total protein studies.

The organic components in parotid saliva are so diluted that separation is difficult to visualize after staining. Concentration was accomplished by placing each sample (9-12 ml.) in 30 ml. vaccine bottles, and lyophilizing for 12 hours.

Electrophoretic separation

Gel-strip preparation was adapted from the methods of Meyers and Lamberts (8). One hundred gm of acrylamide and 2.668 gm of methylene bisacrylamide were dissolved in tris-EDTA-boric acid buffer containing 1.2 ml of N,N,N,N,-tetramethylethyldiamine (TEMED), and the volume adjusted to 2 liters. Two hundred mg of ammonium persulfate was added to 200 ml of acrylamide solution to initiate polymerization, and the resulting mixture was poured into a "Plexiglas" mold, designed to produce a 265 x 50 x 3 mm gel strip. Polymerization was accomplished after about 30 minutes at room temperature.

For electrophoresis, 6 mg of each lyophilized sample was dissolved in 0.1 ml tris-EDTA-boric acid buffer. Seven to 8 spaced incisions were made across the midpoint of a gel-strip, and wicks measuring 3 x 14 mm (Schleicher and Schuell No. 470) were soaked in the samples for 5 minutes. The gel-strip, sandwiched between "Parafilm" sheets, was then placed in a horizontal water-cooled electrophoresis unit. Electrical contact between the gel-strip and the bridge buffer was maintained by paired paper wicks (Schleicher and Schuell No. 470). Electrophoresis was carried out for 5.0 hours at a field strength of 550 volts and a current of 5.0 ma.

Staining

Initially, the strip was stained for 1 hour with a 1% Wool Fast Blue - BL solution. Excess stain was removed by allowing the gel to stand in a 7% glacial acetic acid overnight. Subsequently, it was found that better resolution resulted when the mixture of Meyer and Lamberts (8), containing 0.5% (w/v) Wool Fast Blue in methanol-water-glacial acetic acid (5:5:1), was used. It was therefore indicated that an optimum time-concentration relationship exists for the staining and destaining methods in acrylamide electrophoresis.

Isolation of parotid IGA (membrane ultrafiltration)

Parotid saliva, 200 ml, was collected from the same individual and processed as previously mentioned. Ultrafiltration was attempted by Diaflo membranes (Amico Corporation). Centriflo membrane filter cones were employed with the Diaflo membrane ultrafiltration technique for concentration and purification of small samples of biological fluids. XM-100, XM-50, and PM-30 membranes were used, (Fig. 3), which separated molecules of 100,000, 50,000 and 30,000 M.W., respectively.

Column chromatography

Columns containing Sephadex G-100 and G-200 were also used for separation, from which nine fractions were obtained. These were evaluated for the presence of IgG, IgA, and IgD by immunodiffusion. Subsequently, all fractions were studied in parallel by acrylamide gel electrophoresis.

RESULTS

The total protein level found in parotid saliva had a range of 2.62 mg/ml with a high of 3.355 mg/ml, a low of 0.735 mg/ml, a mean of 1.786 mg/ml and a standard deviation of 5.60 mg/ml. The method of Lowry was used and results were in agreement with those of Wolf and Taylor (11). Slab electrophoresis provided an ideal method for the resolution of parotid fluids. The number of identifiable components ranged from 7 to more than 20 (Fig. 4). This technique did not yield satisfactory resolution of serum and nasal washings (Fig. 5).

Attempts to isolate the IgA by filtering cones resulted in both the filtrate and non-filtrate being positive for IgA (Fig. 6 and 7). This method was therefore abandoned in favor of column chromatographic means of separation. Again, filtrates and non-filtrates were collected and the IgA molecule appeared in filtrates supposedly consisting of molecules having a molecular weight less than 100,000 (Fig. 6 and 7).

Separation by Sephadex G-100 resulted in partial separation of parotid fluids. Good electrophoretic resolution and separation were obtained (Fig. 8). Fractions II and III contained both IgA and IgG as determined by gel-diffusion techniques. The various fractions were placed in the peripheral wells while the IgA and IgG were placed in the center wells (Fig. 9). IgD was not identified as a component of any chromatographic fraction. Fractions II and III were run against antiserum to IgA, IgG and IgD (Fig. 10). Fractions II and III were placed in the center wells while the antisera were placed in the peripheral wells. Fraction II was positive for IgA and IgG but was negative for IgD. Fraction III was strongly positive for IgA with trace amounts of IgG, and negative for IgD. Fractions II and III were diluted out and titered. A 10.0 mg sample was diluted initially with 0.1 ml of tris-EDTA-boric acid buffer and subsequent doubling dilutions with saline from 1:2 to 1:256 were done (Fig. 11). Fraction II was positive for IgA in a 1:2 dilution, whereas fraction III was faintly positive for IgA in a 1:16 dilution. Therefore, fraction III is positive for IgA at a concentration of at least 75 µg/ml.

DISCUSSION

The best resolution was obtained by the use of Meyer and Lamberts' solution of Wool Fast Blue - BL. The best destaining technique was that of the 1% glacial acetic acid solution.

The inconsistent separations observed when using the ultramembranes were probably related to the fact that the molecular weight specified by the membrane assumes a spherical molecule. Since the IgA molecule is probably not spherical, it passed easily through the membranes which were designed to screen it out. It should be noted that much difficulty was experienced in using the XM-50 membrane, as compared to the PM-30 or XM-100.

Good resolution was accomplished in determining the protein distribution in saliva from acute and convalescent Naval recruits. IgA was isolated in 2 of 9 fractions from Sephadex-G 100 Chromatography. The electrophoretic pattern indicated that the IgA component has a slow rate, and therefore remained close to the origin. This seems logical, in that IgA has a high molecular weight and would be expected to migrate very little. It was also found that IgA could be identified, both qualitatively and quantitatively, by immunodiffusion on Ouchterlony plates.

Under the conditions of this study, IgA is best isolated by column chromatography. Sephadex G-100 is most useful in the primary separation of salivary components whereas, through the use of Sephadex G-200, it is possible to separate IgA from IgG.

SUMMARY

Various protein components of parotid saliva were separated by acrylamide-gel electrophoresis. The immunoglobulin IgA was isolated and identified, both qualitatively and quantitatively, by immunodiffusion. Sephadex G-100 and G-200 provided excellent vehicles for the fractionation of the parotid proteins and, when combined with gel-diffusion techniques, provided an excellent means for separation and identification of IgA.

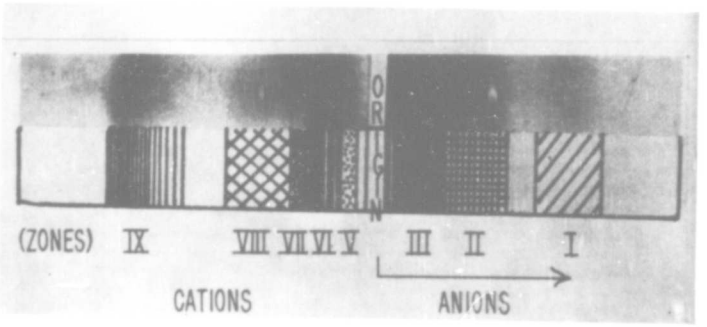


Fig. 1

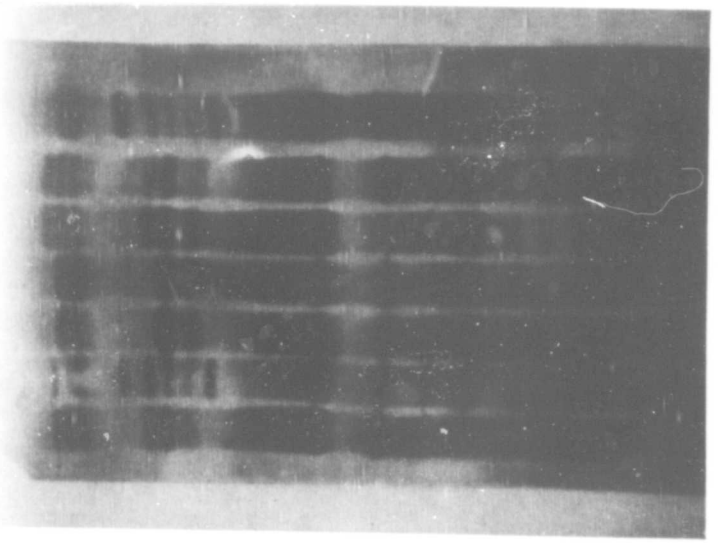


Fig. 2

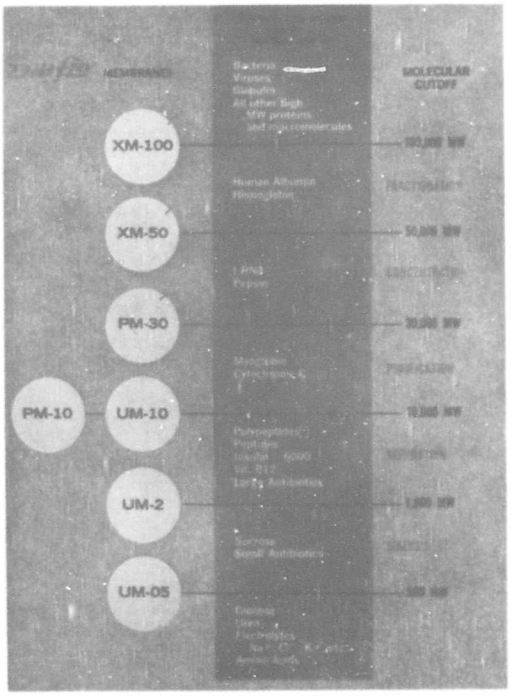

MEMBRANE
MOLECULAR CUTOFF
XM-100
XM-50
PM-30
PM-10
UM-10
UM-2
UM-05

Fig. 3

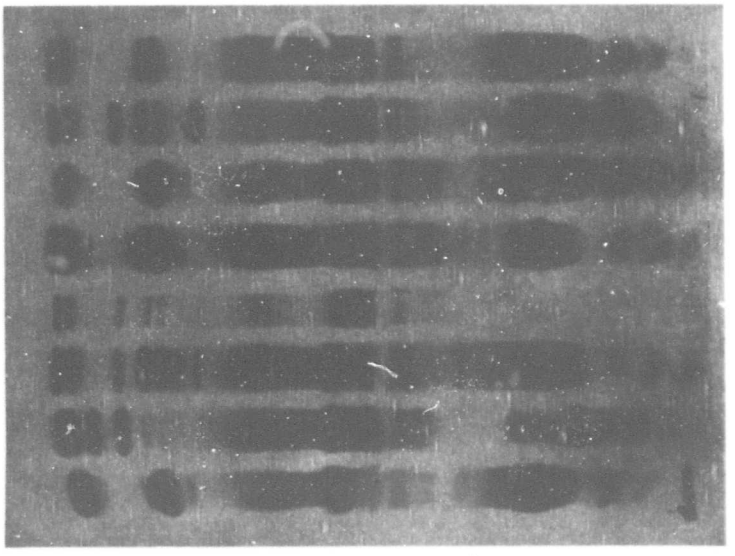


Fig. 4

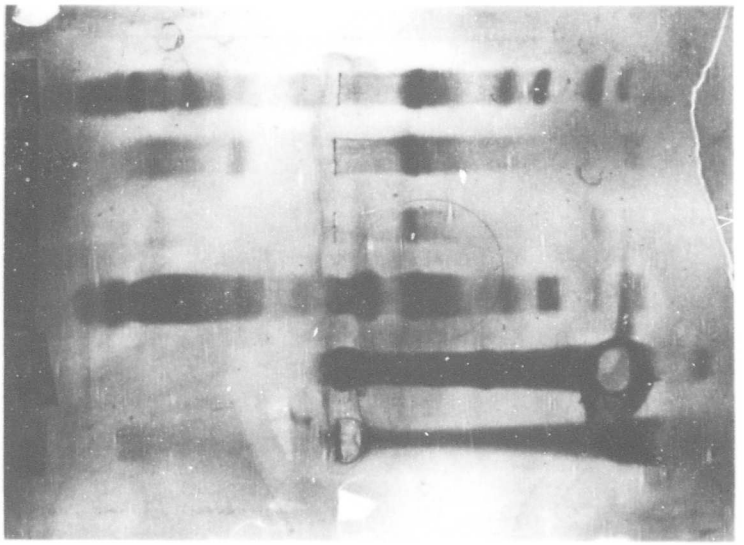


Fig. 5

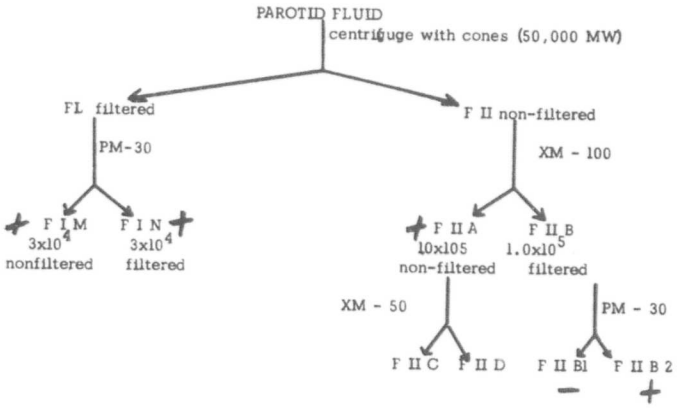

PAROTID FLUID
centrifuge with cones (50,000 MW)
FL filtered
F II non-filtered
PM-30
XM - 100
F I M
3x10^4
nonfiltered
F I N
3x10^4
filtered
F II A
10x105
non-filtered
F II B
1.0x10^5
filtered
XM - 50
PM - 30
F II C
F II D
F II B1
F II B2
−
+

Fig. 6

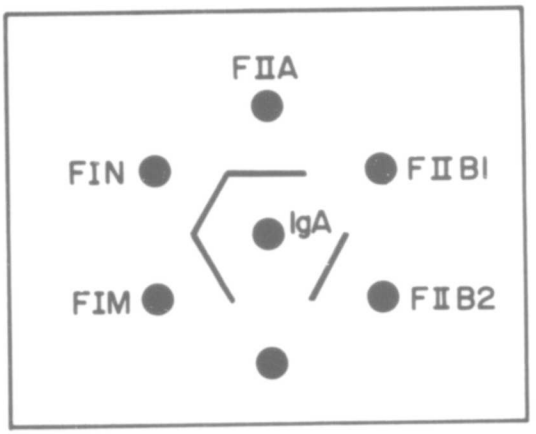


Fig. 7

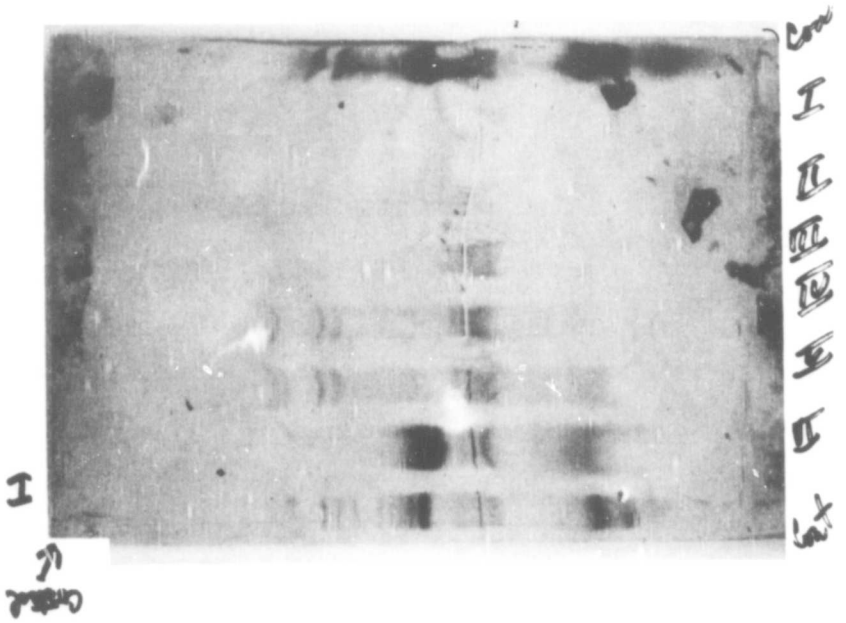


Fig. 8

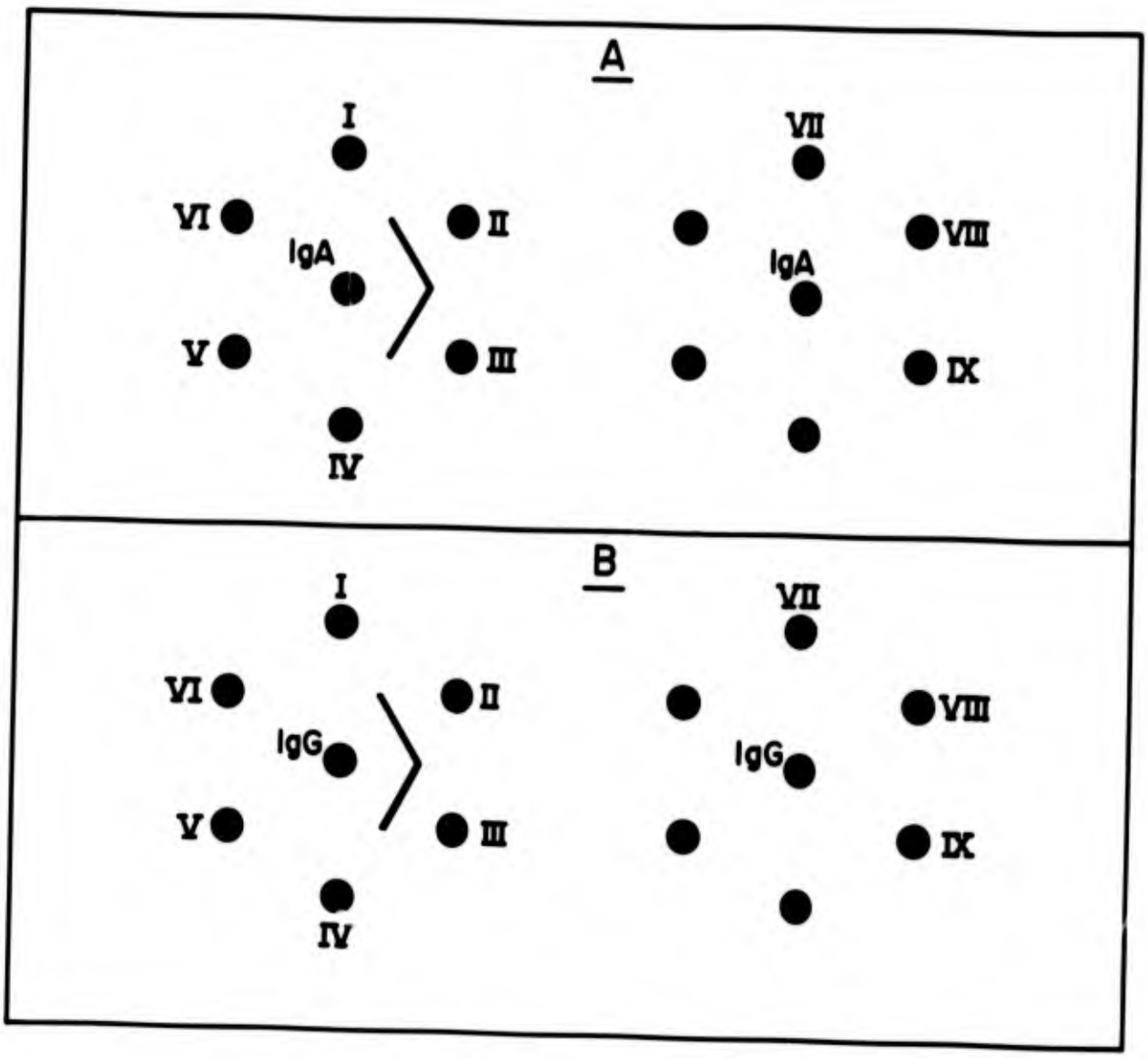

A
I
VII
VI
II
VIII
IgA
IgA
V
III
IX
IV
B
I
VII
VI
II
VIII
IgG
IgG
V
III
IX
IV

Fig. 9

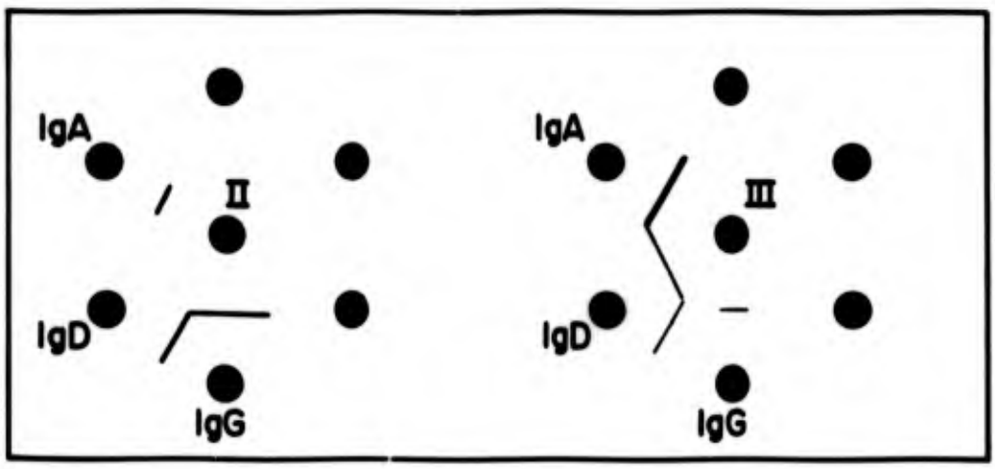


Fig. 10

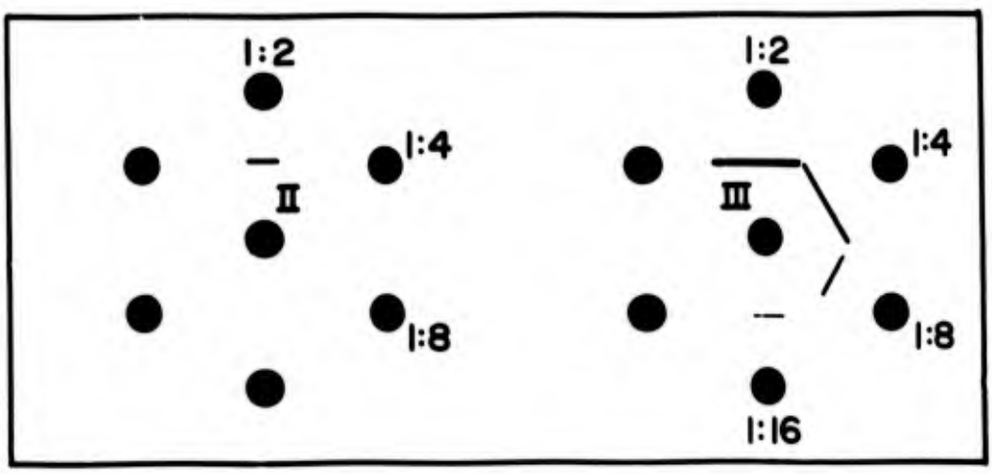


Fig. 11

REFERENCES

1. Patton, J.R. and W. Pigman: Electrophoretic and ultracentrifugal components of human salivary secretions. J. Am. Chem. Soc. 81:3035, 1959.

2. Patton, J.R. and W. Pigman: Amylase in electrophoretic and ultracentrifugal patterns of human parotid saliva. Science. 125: 1292-93, 1957.

3. Zipkin, I., E. R. Adamick and H. A. Saroff: Boundary electrophoresis of human parotid saliva. Proc. Soc. Exp. Biol. & Med. 95:69-71, 1957.

4. Ferguson, M. H., H. P. Krahm and J. A. Hildes: Parotid secretion of protein in man. Can. J. Biochem. Physiol. 36:1001, 1958.

5. Mandel, I. D. and S. A. Ellison: Characterization of salivary components separated by paper electrophoresis. Arch. Oral. Biol. 3: 77, 1961.

6. Tamarin, A.: Contempary salivary gland research. J. Am. Den. Res. 68:833-37, 1966.

7. Hoerman, K. C. and V. J. Berzinskas: On the zone electrophoresis of human parotid saliva in starch gels. J. Lab. Clin. Med. 53: 64-68, 1959.

8. Meyer, T. S. and B. L. Lamberts: The use of Wool Fast Blue - BL for the electrophoresis of human parotid saliva proteins in acrylamide gel. Arch. Oral. Biol. 13:839-40, 1968.

9. Ellison, S. A., P. A. Mashimo and I. D. Mandel: Immunochemical studies of human saliva. I. The demonstration of serum proteins in whole and parotid saliva. J. Am. Den. Res. 39:892-98, 1960.

10. Mandel, I. D. and S. A. Ellison: The proteins of human parotid and submaxillary saliva. Ann. N. Y. Acad. Sci. 106:271-77, 1963.

11. Wolf, R. O. and L. L. Taylor: A comparative study of saliva protein analysis. Arch. Oral. Biol. 9:135-140, 1964.

UNCLASSIFIED

Security Classification

DOCUMENT CONTROL DATA - R&D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author) Naval Medical Research Unit No. 4 Great Lakes, Illinois 60088	2a. REPORT SECURITY CLASSIFICATION Unclassified 2b. GROUP

3. REPORT TITLE

1969 Research Clerkship Reports

4. DESCRIPTIVE NOTES (Type of report and inclusive dates)

Final

5. AUTHOR(S) (Last name, first name, initial)

Cacioppo, John C.; Cadier, T. C.; Gerberi, Michael P.; Pailey, Philip P.W.; Smith, Richard C.

6. REPORT DATE	7a. TOTAL NO. OF PAGES	7b. NO. OF REFS
January 1970	49	50

8a. CONTRACT OR GRANT NO.	9a. ORIGINATOR'S REPORT NUMBER(S)
b. PROJECT NO. MR 005.09-0009; MR 005.09-0096B; M4305.01-1012; MR 005.09.01-0087B	RU 70.4
c.	9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.	

10. AVAILABILITY/LIMITATION NOTICES

Distribution of this document in unlimited.

11. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY
	Bureau of Medicine and Surgery Navy Department Washington, D. C.

13. ABSTRACT

This report contains the final reports of the research projects done by the research clerks assigned to the Naval Medical Research Unit No. 4 during the summer of 1968. The titles of the projects are as follows:

1. The Effect of pH of the Medium on the Isolation and Subcultivation of Mycoplasma pneumoniae.
2. Altered Cell Mediated Immunologic Reactions During Recruit Training Induced by Stress Associated with Recruit Training.
3. The Determination of Bactericidal Activity in Navy Recruits with Neisseria meningitidis Infections.
4. Gel Diffusion Studies of Nongroupable Meningococci.
5. Complexity of Biological Fluids as Related to Disease States.

DD FORM 1 JAN 64 1473

UNCLASSIFIED

Security Classification