STUDIES ON MENINGOCOCCUS INFECTION

I. BIOLOGICAL PROPERTIES OF "FRESH" AND "STOCK" STRAINS OF THE MENINGOCOCCUS

BY GEOFFREY RAKE, M.B., B.S.

(From the Laboratories of The Rockefeller Institute for Medical Research)

PLATE 39

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A study has been made of stock and fresh strains of meningococcus obtained from various sources. The stock strains, isolated in the past from cases of meningococcal meningitis, have been grown on artificial media over a period of years, while the fresh strains have been recently isolated from the cerebrospinal fluid of patients or from the throats of carriers. This paper deals with certain differences in morphology and biological characteristics in these two groups. Differences in serological reactions, in antigenicity and in antigenic complexity will be discussed in subsequent papers.

Cultural Characteristics on Blood Agar Plates

Stock or Laboratory Strains.—Strains of meningococci, whatever their type, having been grown on artificial media over a period of years, show certain morphological characteristics by which they can be recognized. Stock strains, lightly sown on blood agar plates, present small grey or whitish colonies little over 1 mm.

1 For the stock strains, the author is indebted to the New York and the Massachusetts state boards of health, the National Institute of Health, Washington, and Dr. John Enders of Harvard Medical School. Certain of the fresh strains have been isolated from the throats of patients or normal individuals in The Rockefeller Institute. Others have been received through the kindness of the New York State Department of Health, the Departments of Medicine and Pediatrics of the Johns Hopkins and the Yale Medical Schools, the Willard Parker Hospital, New York, the National Institute of Health, Dr. O. D. Chapman of Syracuse, Mr. Eccles of the Harlem Hospital, New York, Professor R. McIntosh of the Babies Hospital, New York, Dr. G. M. Mackenzie of Cooperstown, Dr. John Norton of Detroit, and Dr. L. G. Zerfas of Indianapolis.
in diameter at the end of 24 hours' growth. These are somewhat drier in appearance than those of the freshly isolated spinal fluid strains and tend to remain discrete even when closely apposed. Under the binocular field microscope they appear domed with regular round margins and a smooth glistening surface (Fig. 1). Prolonged growth, up to 72 hours, gives rise to no secondary growth; the colonies become brownish in color and faintly granular, undergoing desiccation with the medium itself; subculture is now no longer possible.

**Fresh Strains from Cerebrospinal Fluid. Smooth Forms.**—The majority of strains freshly isolated from the cerebrospinal fluid form, after 24 hours' growth on blood agar plates, large flattened colonies which are pearly grey and lenticular with gradually shelving sides and a round and entire outline. The surface is smooth, or but slightly granular, and distinctly moist (Fig. 2). Single colonies may measure up to 4 mm. in diameter but there is a tendency for neighboring colonies to coalesce with the formation of a sheet-like growth. At the end of 36 hours some of these freshly isolated strains show colonies with a somewhat uneven surface due in large part, however, to the drying of the media and superimposed growth. At this time many of the strains have become transparent and glassy, sometimes with the formation of minute, glistening crystals on the colony surface such as have been observed by other authors (1, 2). At the same time, new and opaque, yellowish white growth appears at the margin or in the center of the colonies in the form of papillae or crescentic masses which grow over and obliterate the original growth. Transplantation from the original colonies is only rarely successful, but subculture can be made from the new or secondary growth during the first 48 hours after its appearance. Such subculture yields colonies somewhat more opaque and domed than those of the original culture but, while most are smaller and show less tendency to coalesce, there are some which, without any reference to distance from neighboring colonies, are larger and flattened, resembling those of the original culture and giving the plate a characteristic uneven growth. The uneven growth continues to appear for many subcultures, but the smaller colonies with their drier domed surfaces and their tendency to remain discrete become gradually more predominant, until finally the larger colonies disappear and all the colonies are of the small type, having become similar or closely similar to those of the stock strains. The secondary growth occurs in many strains for the first ten or even more subcultures, but then fails to appear any more. These small colonies, belonging to strains which may now be regarded as stock strains, remain viable for from 36 to 60 hours and are readily maintained on artificial media. While freshly isolated strains grow readily on primary culture and first subculture, this is no doubt due to a carrying over of some enriching substances present in the body fluids; and subsequent subcultures are often difficult or even impossible to make. Once, however, the strain has accustomed itself to artificial media, and especially when the secondary characteristics of morphology and behavior have developed, subculture onto solid media is simple, provided such contain some enriching substance and are sufficiently moist.
Rough Variants.—While the above description holds true for the majority of spinal fluid strains, notable exceptions have been encountered. Enders (3) has described a method for the development of rough variants from freshly isolated strains of meningococci. All his variants have been obtained from fresh Type II strains, no success being met with Type I or stock strains. The development of rough variants has been confirmed in this laboratory and extended to fresh Type I strains. Even before the appearance of Enders' paper, however, two stock strains were discovered, one of Type IV and the other a member of the group isolated by Branham and called by her Neisseria flavescens (4), which showed rough and smooth variants growing side by side in the same culture (5). More recently five spinal fluid strains have been encountered which agglutinated in all four monovalent sera; and all showed the presence of rough variants. Three of these strains proved on absorption to belong to Type II, one to Type I, while the fifth remained indeterminate. In four strains rough variants were obtained which, though less granular than those obtained in vitro, nevertheless differed in morphology and other respects from the smooth strains. In the Type I strain a typical rough variant was found. In some cases these variants formed the greater percentage of colonies on the plate; in others they were in the minority.

These rough strains derived from various sources, while not identical in morphology, show nevertheless many essential resemblances. The colonies present a surface which is, in varying degree, roughened, and a margin which is irregular in outline; they are drier than the smooth variants. The rough variants discovered in the stock strains are the most granular of all variants yet studied. They have a surface often so wrinkled as to resemble that of a typical colony of Neisseria sicca (Figs. 3 and 4) and in keeping with this they emulsify with difficulty in saline and exhibit spontaneous agglutination even when the greatest care is taken in adjusting the pH. The rough colonies developed in vitro from Type II strains appear on first dissociation at the end of 24 hours' growth as very minute, colorless, discoid colonies, less than 1 mm. in diameter, with a flat, granular surface and an irregular margin (Fig. 5). At the end of 48 hours they are larger, up to 2 or 3 mm., with a raised, even more roughened surface and great irregularity of outline. Repeated subculture from these variants gives colonies which are larger, 2 or 3 mm. in diameter, at the end of 24 hours, but otherwise present all the appearances of the primary rough variant (Fig. 6). The variants obtained from Type I differ somewhat from those of the Type II strains. The minute discoid form has not been seen. The colonies in one instance show smooth and rounded margins but a plateau shape, with a finely granular surface and numerous deep pits. In other instances they resemble the larger colonies of the Type II R variant. The rough variants discovered thus far in spinal fluid strains have all been of the general type described for the later subcultures of Type II in vitro variants. It is to be noted that three out of five of these spontaneously occurring variants have been in Type II strains.

Among the features which rough variants have in common is their relative
instability when subcultured on the usual artificial media (blood agar plates, serum dextrose slants and semisolid ascitic fluid agar stabs). Just as the freshly isolated smooth variants tend to pass over gradually into what may be termed the stock variant, so also the rough variants, in spite of careful picking and transfer of single colonies, show an ever present tendency to pass over into the stock variant. Certain media, as 5 per cent rabbit serum peptone water or 5 per cent glycerin egg slants (6), maintain these rough variants for a while, but it is wise to make careful selection every few weeks of rough colonies plated out on 10 per cent chocolate rabbit's blood hormone agar in order to keep pure and characteristic rough types.

Other features common to the rough variants are some degree of instability and difficulty of even emulsion in normal saline; broad agglutination in phosphate buffer solutions at varying pH; and widely unspecific agglutination in serum, each monovalent type serum showing an equal or nearly equal agglutinative titre.

**Mucoid Forms.**—A few spinal fluid strains have been encountered which, while presenting colonies on primary isolation of normal or slightly moister appearance, develop on subculture mucoid colonies adhering firmly to the surface of solid media and tending to form glutinous strings when suspended in saline, in place of the usual homogeneous suspension. All such strains isolated to the present time, save one, have been of Type II. The factors underlying this mucoid character are as yet undetermined and apparently have little to do with the salt content of the media used, but such strains appear to be less satisfactory antigens for the production of monovalent serum than normal ones.

**Fresh Carrier Strains from the Nasopharynx of Healthy Persons.**—While the foregoing description includes all the more usual forms observed in freshly isolated spinal fluid strains, it is not applicable to the majority of strains recovered from the nasopharynx of normal individuals at least. Thus, of fourteen strains obtained from the throats of normal healthy individuals with no history of meningococcal infection, only two, both typical Type II strains, have resembled the smooth spinal fluid strains on primary isolation. The others have presented colonies which resemble rather the stock strains. Thus, they are often somewhat more opaque and white; small and of uniform size; slightly domed and rather dry; and tend to remain discrete. Some authors have refused to admit these atypical nasopharyngeal strains to the group of true meningococci (7, 8), while others insist that such strains must be regarded as true meningococci in spite of atypical agglutination reactions (9, 10). The whole question is discussed elsewhere (11). Here it need only be pointed out that the atypical strains have as much, if not more claim to be regarded as true meningococci as have atypical strains of pneumococci to belong to that group.

**Staining Reactions**

All the strains of meningococci examined have been characteristically Gram-negative. Freshly isolated strains when emulsified in saline
and smeared on a slide appear as small groups, diploid forms or single organisms. Using a slight modification of the method of capsule demonstration described by Baker (12), it has been possible to show a clear zone or "capsule" around organisms of the freshly isolated strains; in stock strains this "capsule" is narrower and rough variants show no such structure. It has not been possible, however, with any of the methods used to demonstrate the presence of a true capsule comparable to that of the pneumococcus. While with the Gram stain the organisms of the stock strains are of the same size or but slightly smaller than those of the fresh strains, the rough variants are considerably larger and show a tendency to be in groups of four (tetrads) or more organisms. These facts concerning the rough variants have been noted by Enders (3). It would seem probable that the larger forms (not the giant forms) sometimes seen in the smears of original spinal fluid cultures represent rough variants. Such, in colony form, are apt to be overlooked on the plate and are probably soon lost as a result of their instability on ordinary culture media.

**Growth in Broth**

Another feature serving to differentiate fresh and stock strains is their behavior in broth. It is generally believed that it is difficult to obtain plentiful growth of meningococci in any fluid medium. While this is in large part true of the stock strains, fresh strains will grow readily in Huntoon's hormone broth even without gelatin or dextrose. Growth is in the form of a heavy pellicle on the surface of the broth which breaks up when disturbed, sinking to the bottom of the flask to be replaced by a new pellicle on the surface. This form of growth occurs not only in small amounts of broth in a tube, but also in large amounts; i.e., 900 cc. in a flask. Stock strains, however, show as a

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2 To this extent the claim to have demonstrated a capsule, made in a previous paper (5), must be modified. The "capsule" or clear zone which can be demonstrated readily by Baker's India ink method would appear to represent some difference between fresh, stock and rough organisms in or around the bacterial cell. However, it has not been possible to stain any structure outside the bacterial cell, using the usual methods of capsule staining, and it seems certain that the organism does not possess a capsule of the same kind as that found on the pneumococcus or H. influenzae.
rule scanty, diffuse and granular growth when planted in small amounts of broth and in larger amounts grow poorly if at all, since the organisms are apparently readily "drowned." Rough variants grown in 5 per cent rabbit's serum peptone water have a diffuse granular growth.

**Acid Agglutination**

It has not proved possible to obtain the same clear-cut results with the acid agglutination range of meningococci as with that of many other organisms (13, 14). Nevertheless, it has been possible to show differences between fresh, stock and rough strains.

While, as pointed out above, the rough variants form unstable suspensions in normal saline, *i.e.* are "salt-sensitive," freshly isolated spinal fluid strains containing no rough variants emulsify readily and form smooth suspensions in normal saline at the usual pH of 5.5 to 6.0. Nasopharyngeal strains and stock strains usually behave in the same manner as the spinal fluid strains, but on occasion form unstable suspensions. These peculiarities are closely bound up with the acid agglutination ranges of the strains in question.

A suspension of approximately 2,000,000,000 organisms per cc. is prepared. The organisms are emulsified directly from a blood agar plate at the end of 18 hours' growth. They are not washed since the tendency to spontaneous agglutination is found to be markedly increased by washing. 0.5 cc. is mixed with an equal amount of each of ten samples of sodium phosphate buffer solution ranging in pH from 2.15 to 6.3. A control tube of suspension and normal saline is added. Certain precautions must be adopted with the saline used for the suspension and the control. Owing to the absorption of CO₂ from the atmosphere, salt solution prepared from ordinary distilled water may have a pH of 6.0 or even 5.5. Such a pH is within the acid agglutination range of many strains and will cause agglutination in the control tube. In most instances the report of salt sensitiveness in a strain is due to this factor. It may be overcome if distilled water which has been triply distilled from glass is used, and the saline is boiled shortly beforehand to expel the CO₂. Many rough strains, however, have an acid agglutination range extending beyond the pH of any unbuffered saline and such are of necessity salt-sensitive. Buffer solution and suspension of organisms are thoroughly mixed together by agitation and the tubes placed in the water bath at 56°C. for 3 hours. They are then removed and those tubes in which agglutination has occurred are noted.

By this method it has been determined that freshly isolated strains show a narrow agglutinative zone usually between pH 3.0 and pH 5.0.
Repeated subculture broadens this zone, at first largely on the acid side, and the stock cultures show a considerably broader zone which, while quite irregular, tends to lie between pH 2.1 and pH 5.6. Rough strains usually show some agglutination over the whole range (pH 2.1 to pH 6.3) and agglutination is complete in most of the tubes.

These results are of particular interest in view of those reported by Gibbard (15) in connection with electrophoresis of meningococci. He was able to show a difference in potential difference or charge carried on the bacterial cell between fresh and stock strains. The P.D. was higher in the fresh strains, being from 36.0 to 42.0; it fell off rapidly with subculture; and stock strains showed a P.D. between 25.6 and 30.5. The range of variability was greater in stock than in fresh strains, and the P.D. tended to be greater in Types I and III than in II and IV.

**Behavior in Defibrinated Blood**

It has been noted above that attempts to obtain rough variants from stock strains and from most Type I strains have been unsuccessful. Rough variants are obtained after the method of Enders (3), which consists, in brief, of repeated subculture of freshly isolated strains of the organisms in defibrinated rabbit's blood, together with daily plating and selection. The strain is planted in about 0.5 cc. of defibrinated blood and slowly revolved at 37°C. for 15 hours. At the end of this time the tube is removed, a loopful plated on chocolate hormone agar and 0.1 cc. transplanted to a fresh tube of defibrinated blood with which the process is repeated. The plates are examined at the end of 24 hours' growth, preferably under a slightly increased CO₂ tension (about 10 per cent), for the presence of rough variants. In some strains these appear immediately, in others not until 20 or 25 successive subcultures have been made. Even if rough variants do not appear, the strain loses the characteristics of a fresh strain and takes on those of the stock type. Now the difficulty in obtaining rough strains from stock strains lies in the fact that the latter die out after the first two or three subcultures; *i.e.*, before the rough variants develop. This observation led to the idea that growth in defibrinated rabbit's blood might offer an additional method of differentiating stock and fresh strains. During the investigation of this point, a paper appeared by Wright and Ward (16), in which such a method was adopted with strains of H.
influenzae, and a subsequent review of the literature has revealed papers by Heist and the Solis-Cohens (17) and by Matsunami (18), dealing with the action of specimens of human or rabbit blood, or of different parts of one blood on a single strain or on different strains of meningococci cultured therewith. These authors suggest (16, 17) that the ability of a strain to grow out in defibrinated blood is associated with its virulence. A virulent strain, being better able to withstand the normal defense mechanism of the body, can presumably resist and overcome the phagocytes still present and active in freshly drawn defibrinated blood, and hence will survive and grow in the blood better and in higher dilutions than an avirulent strain. The greatest dilution at which subculture can be obtained would thus be taken to yield a relative indication of the virulence of the strain.

The following method has been adopted in this laboratory to investigate fresh and stock strains. A suspension of meningococci is prepared in normal saline containing 2,000,000,000 organisms per cc. The strains are investigated as soon after isolation as possible and cultures on blood agar plates not older than 18 hours are used in preparing the suspension. Neglect of these factors will result in the use of strains and cultures which have lost some of their vitality. 1 cc. of the standard suspension is added to 9 cc. of normal saline, giving a suspension of 200,000,000 organisms per cc., and the dilution process is repeated to give regular dilutions down to 200 organisms per cc. Eight small sterile tubes are prepared, each containing 0.9 cc. of defibrinated rabbit's blood; and 0.1 cc. of the eight suspensions of meningococci is added to tubes numbered from 1 to 8. A control tube of defibrinated blood alone, No. 9, is included. Thus No. 1 contains 200,000,000 organisms and Tube 8, 20 organisms each in 1 cc. of diluted defibrinated blood. All manipulations are carried out as rapidly as possible and with precautions for sterility. The tubes are now corked and sealed with wax, and placed on a slow rotator for 15 hours at 37°C. At the end of incubation, the tubes are examined and a loopful of each is planted on a blood agar plate. Growth on the plate is recorded at the end of 24 hours, under an increased CO₂ tension of about 10 per cent. 2,000,000,000 organisms per cc. is taken as standard; therefore, Tube 1 is 10⁻¹ and Tube 8 is 10⁻⁸. If only the first tube shows growth, the strain is designated as 10⁻¹; if the first three show growth, as 10⁻³, and so on. As a rule, designation of viability of a strain can be made as soon as the tubes are removed from the rotator, for it has been noted that when growth has occurred the defibrinated blood is dark brown in color, owing apparently to methemoglobin formation. The designation obtained by this means is usually but not invariably the same as that obtained by growth on the blood agar plate.
Using this method, fresh spinal fluid and nasopharyngeal strains, stock strains and rough variants have been examined. The results are tabulated in Table I. From this it can be seen that quite distinct differences are apparent in the several groups. Spinal strains show the highest viability. They may be divided into three groups: (1) those with high viability; (2) those with medium viability; and (3) those with low viability. When this is done, it is immediately apparent that all strains in Group 1 are of Type II; Group 2 contains the Type

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* Atypical.

I-III strains; while the strains of low viability either agglutinate broadly in all sera or contain rough variants and are indeed not typical fresh spinal strains. Of the carrier strains, only one showed a high viability and this, Strain 15, as has been pointed out above, was morphologically and culturally a typical spinal strain of Type II, which renders its high viability not surprising. The remainder of the carrier strains are of low viability. The stock strains, though giving characteristic agglutinations of Types II or I-III, have uniformly low viability, as have the rough variants.
SUMMARY

Freshly isolated strains of meningococci present a number of characteristics which can be shown to differ not inconsiderably from those of stock strains long maintained on artificial media. Rough variants of the different types can be demonstrated, either arising spontaneously in vivo or in vitro, or evoked in the laboratory by the method described by Enders. Neither the freshly isolated strains—which are smooth—nor, in most cases, the rough variants of them are stable, both showing a tendency to pass over into the stock form or variant. The stock strains in the course of transformation from the freshly isolated strains show changes in morphology and cultural characteristics, and in viability in defibrinated blood.

BIBLIOGRAPHY

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EXPLANATION OF PLATE 39

Fig. 1. Stock strain of N. intracellularis, 18 hour growth on blood agar plate. × 4.
FIG. 2. Fresh strain of *N. intracellularis*, 18 hour growth on blood agar plate. × 4.

FIG. 3. Rough colony of *N. flavescens* (Branham) obtained by punctate inoculation of a blood agar plate. × 9½.

FIG. 4. Rough colonies of Type IV *N. intracellularis* evenly sown on blood agar plate. × 11.

FIG. 5. Minute discoid colonies of rough variant developed *in vitro* from a fresh Type II *N. intracellularis* strain. × 12.

FIG. 6. Rough and smooth colonies of a fresh Type II *N. intracellularis* strain undergoing dissociation *in vitro*. × 12.
Photographed by Louis Schmidt

(Rate: Meningococcus infection, 1)