OBSERVATIONS ON THE GROWTH REQUIREMENTS OF NON-PATHOGENIC NEISSERIA IN SUPPLEMENTED CASEIN HYDROLYSATE AND CHEMICALLY DEFINED MEDIA

By

John L. Nemes

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CHAPTER I

HISTORICAL INTRODUCTION

The nutritional requirements of the genus Neisseria are poorly defined. The first member of this genus to be cultivated was N. gonorrhoeae, described by Neisser (1879) and grown by Leistikow and Loeffler (1882) on blood serum gelatin plates. Since this early performance and up through the definitive work of Wilson (1928), cultivation of the Neisseria has depended upon the addition of body fluids such as blood, serum, ascitic fluid or hydrocele fluid to a base of nutrient agar. Subsequently, many strains of Neisseria were cultivated successfully upon nutrient agar alone and this characteristic correlated with pigment production and biochemical differences has resolved the genus into the present classification as outlined in Bergey's Manual. Growth of organisms upon enriched medium has been considered a pre-requisite for pathogenicity of man and the organisms N. gonorrhoeae and N. meningiditis are, by this token, called pathogenic although the gonococcus will grow, often poorly, on nutrient agar. Contrariwise, the N. flavescens species of Branham (1930), although capable of good growth on nutrient agar and retaining the ability to give rise to meningitis under favorable conditions, was considered non-pathogenic under ordinary laboratory conditions.

Cultivation of Neisseria species in a natural medium with replacement of body fluids by simpler chemical compounds has been reported in the literature. The growth stimulation of N. gonorrhoeae by the addition of cystine has been reported by McLeod, Wheatley and Phelon (1927) and Boor (1942) in concentrations of 0.025-0.075 per cent with some stimulation at the 0.00125 per cent level and inhibition with concentrations above 0.10 per cent. Lankford (1944) found that the gonococci were stimulated by a five milligram per cent cystine addition to peptone-hemoglobin agar. Frantz (1942) reported good growth of the meningococci at a cystine level of 0.00012 per cent. Grossowicz (1945) reported inhibition of meningococci by cystine and the medium of Frantz' only suitable for growth when large inocula were used. Recently, Scherp and Fitting (1949) concluded that the medium of Frantz will support the growth of meningococci with small inocula, even in the absence of glucose. These authors conclude that the growth of meningococci in the medium of Frantz is not the "ready and profuse growth of all viable cells of the inoculum" but rather the "growth of at least one of the cells originally inoculated and the production of a trained culture.....of cells with the greatest synthetic powers" and the medium of Frantz, a deficient but adequate medium.

This conception of growth of variants in response to the medium composition may well be the explanation of the extreme variability of cultural characteristics of pigmented and biochemically active cultures of Neisseria, as reported by Gordon (1921) and Wilson (1928). Gordon had noted the reversion of type I N. catarrhalis- a rough, friable grey colony, to a type II- with similar physical characteristics but colored a pale yellow. Wilson had reported loss in yellow pigmentation and changes in biochemical fermentations upon five serial subcultures on ascitic agar. Lankford and Skaggs (1946) also have indicated the production of variants from cocarboxylase-deficient strains of N. gonorrhoeae, variants which grew well without a supplement of phosphothiamine. The variants were obtained by massive inocula or by cultivation in suboptimum amounts of cocarboxylase.

The influence of physical rather than chemical factors upon the growth of Neisseria strains in natural medium has been noted in the literature. Improvement of growth of Neisseria resulting from the presence of a ten per cent carbon-dioxide atmosphere over the natural medium has been reported by Wherry & Oliver (1916), Rockwell & McKhaven (1921) and others but has been denied by Cook & Stafford (1921) and Torrey & Buckell (1922). Scherp & Fitting (1949) reported the growth of three strains of meningococci in a synthetic medium containing glutamic acid and cystine as the only nitrogen and carbon sources in the presence of a three per cent carbon-dioxide atmosphere and the growth of one strain in the medium without supplemental carbon-dioxide. In the growth of a single organism without carbon-dioxide supplement, the possibility of carbon-dioxide accumulation from cellular metabolism of the inoculum was eliminated by the use of extremely minute inoculum. In these experiments, the role of carbon-dioxide was difficult to assess but possibly glutamic acid transamination to aspartic acid and the decarboxylation of aspartic acid to pyruvic acid would supply the carbon-dioxide required for growth of the one strain.

Another physical factor resulting in the stimulation of growth of the gonococcus and meningococcus was the addition of various forms of particulate carbon as reported by Glass and Kennett (1939). The stimulatory effect was not produced by all carbon particles and was not due to soluble organic matter contained in the carbon powders. The development of the starch agar medium of Mueller and Hinton (1941) seemed to indicate that commercial agar contained an inhibitory compound which reduced the growth of the gonococcus in the absence of starch. Ley and Mueller (1946) suggested the function of starch as an adsorption agent– the inhibitory fraction of agar identifiable as a fatty acid. These authors reported that oleic, stearic and butter fatty acids in 0.5 microgram per ml concentrations were sufficient to inhibit gonococcal growth. Gordon (1921), however, used an oleate-hemoglobin agar for the cultivation of the gram negative cocci and reported satisfactory growth of all strains. Also, oleic acid was used as a biotin substitute for Clostridium sporogenes by Shull, Thoma and Peterson (1948), the lactic acid bacteria by Williams, Broquist and Snell (1947) and Lactobacillus arabinosus by Lardy, Potter and Elvehjem (1947) and concentrations of 0.5 to 5.0 micrograms per ml were reported as optimal for these fastidious organisms.

The growth of Neisseria species in partially-defined media* has been studied in recent years with particular emphasis upon the nitrogenous requirements of these organisms. The addition of nitrogenous supplements to a natural medium in order to promote growth of Neisseria was reported by Lankford (1944) using either cystine, cysteine or glutathione supplements to peptone hemoglobin agar to stimulate the growth of the gonococcus. Boor (1942) noted the growth of the gonococcus in a tryptic digest of casein when cystine was added. The addition of nitrogenous supplements to promote growth in a semi-synthetic medium with vitamin-free casein hydrolysate as the nitrogen source was noted by Lankford, Scott, Cox and Cooke (1943). All strains of gonococci grew in the partially-defined medium when a thermolabile substance was added, in small quantities, in liver extract. Later, Lankford and Snell (1943) reported this thermolabile substance to be glutamine, not replaceable by either glutamic acid or

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* Media containing chemically defined inorganic salts and vitamins but casein hydrolysate as nitrogen source.

glutathione. Gould, Kane and Mueller (1944) reported an unknown factor present in meat infusion which, upon addition to their partially-defined medium, stimulated a two-fold increase in colony size of N. gonorrhoeae. Gould (1944) noted the gradual dependence of stock strains of gonococci upon glutathione, when grown in a partially-defined medium. Freshly isolated strains did not require glutathione but all gonococcus strains, on continued subculture, changed to glutathione dependent variants. The glutathione was not replaceable by either cystine or cysteine. Landy and Gerstung (1945) supplemented a partially-defined medium with tryptone, cystine and glutamine and successfully cultured all N. gonorrhoeae strains tested.

The growth of Neisseria in a synthetic medium has been reported in recent years. In contrast to the complex protein mixtures employed in the past, the reported nitrogenous requirements of the Neisseria were few and simple. Frantz (1942) cultivated meningococci in a medium containing d-glutamic acid and cystine as nitrogen sources. Grossowicz (1945) reported the growth of meningococci in this medium only under strictly controlled conditions and with relatively large inocula. Grossowicz, in turn, proposed a medium containing sodium glutamate as the sole nitrogen source and was able to maintain undiminished growth throughout ten serial subcultures. Gould, Kane and Mueller (1944) proposed a medium for the cultivation of N. gonorrhoeae containing glutathione, glutamic acid and histidine as nitrogenous sources but indicated the medium was far from optimal for the growth of the gonococcus. Welton, Stokinger and Carpenter (1944) prepared a medium for the cultivation of N. gonorrhoeae using, as nitrogen sources, glycine, cystine, indole-3-acetic acid, glutamic acid, leucine, arginine, histidine, methionine and proline. Upon occasion, this medium was supplemented with glutamine and choline and all strains of N. gonorrhoeae, stock and recent isolations, grew well.

Few reports have been published about the vitamin requirements of the genus Neisseria. Lankford, Scott, Cox and Cooke (1943) reporting on a thermolabile substance stimulatory for the growth of the gonococcus, attempted replacement of the substance with nine vitamins and eleven other nutriles but were unable to effect enhancement of growth in their partially-defined medium. The thermolabile substance was reported as glutamine in a later publication (Lankford and Snell, 1943). Gould, Kane and Mueller (1943) noted "a factor (or group of factors) present in meat infusion, not essential for growth but which cause a marked stimulation of growth" and later, "no one of a large number of vitamins, bacterial growth factors or other substances tested were able to replace the meat infusion factor, either singly or in various combinations." Landy and Gerstung (1945), in a study of N. gonorrhoeae, used a vitamin mixture including thiamin, riboflavin, pyridoxine, calcium d-pantothenate, nicotinamide, biotin and folic acid in a partially-defined medium but attempted no determination of minimum nutrile requirements of these organisms. Grossowicz (1945) used thiamin in a synthetic medium for the growth of N. meningitidis but noted that metabolism, not total growth, was influenced by the thiamin in the medium. Ordal and Busch (1946) reported a biotin requirement of four strains of N. sicca and stimulation of ten additional strains by biotin with maximum growth obtained at concentrations of 0.05-0.1 microgram of biotin per liter of medium.

Thus, considerable information is available on the growth requirements of the pathogenic Neisseria - N. gonorrhoeae and N. meningitidis, and growth of selected strains of these species in synthetic media have been noted above. Limited information, however, is available on the growth requirements of the non-pathogenic Neisseria species - species other than N. gonorrhoeae and N. meningitidis, although the reports in literature implied a more complex growth requirement of these species in addition to a lack of interest in these non-pathogenic Neisseria. In view of the limited information available on the growth requirements of the genus Neisseria, other than N. gonorrhoeae and N. meningitidis, the present study was undertaken with these objectives: (1) to define the minimal vitamin requirements of the non-pathogenic Neisseria and (2) to attempt to cultivate these Neisseria species in a completely synthetic medium.

CHAPTER II

CULTURAL AND BIOCHEMICAL CHARACTERISTICS OF THE GENUS NEISSERIA

A. Morphological Characteristics

The classification of the gram negative cocci of the normal nasopharynx has received considerable attention in the past and two divergent views have attained prominence. The first view is embodied in the current Bergey's Manual of Determinative Bacteriology and represents an attempt to classify the gram negative cocci, the Neisseria, as separate entities with species differentiation among the existing and reported cultures. The second view was suggested by Wilson (1928) as follows:

> ".... there is, at present, little justification for the recognition of separate species among the gram negative cocci...with the possible exception of N. catarrhalis. We should, therefore, combine the remaining types into a single species, with some appropriate name such as N. pharyngis,....."

This view had considered the variations in colonial morphology and pigmentation reported by Elser and Huntoon (1909), Gordon (1925) and Wilson (1928) and had adopted the conservative attitude in taxonomy.

The organisms studied in this investigation were of the genus Neisseria, chromogenic and non-chromogenic, aerobic, non-pathogenic and characterized as "growing well on ordinary culture media." Although three of the strains (N. flavescens 9746, 155, 157) were isolated from definite cases of meningitis, the entire group of organisms studied were considered non-pathogenic and no strains of either N. gonorrhoeae or N. meningitidis were included. The culture designations and the sources of the cultures are listed in Table 1.

Table 1

Source and Designation of Cultures

Culture Designation	Code Numbers	Source of Culture
N. catarrhalis N. sicca N. perflava N. flava	100 22, 1485 876, 1927 55	Stock culture University of Maryland
N. catarrhalis N. sicca N. perflava N. flavescens	7900, 8176, 8193 7902, 9913 7925 9746	American Type Culture Collection
N. flavescens	155, 157	Dr. S. E. Branham National Institute of Health
N. catarrhalis N. perflava	S1, S2, S7, S8, S9 S6 S3, S4	Dr. Spaulding Temple University
N. perflava	2AMS, 3AMS	Army Medical School
N. sicca N. perflava N. flava	X, 14, 19, 24, 32, 35, 36 12 4	Human throat isolations University of Maryland
Unclassified	B, C, E, F, J, K, L, N, O, P, R, S, T, U, V, W GP3, GP4, GP6, GP8, GP11, GP13, GP14, GP16	Guinea pig nasopharynx isolations University of Maryland
	Calf Sheep	Throat of Gurnsey calf Throat of sheep

The morphological and biochemical tests used in this study were restricted to tests necessary for identification of the organisms under study.

Method of isolation. Human throat and animal throat isolations were performed either by cough inoculation of trypticase soy agar plates or streak inoculation of the same medium with swabbings from the nasopharyngeal region of the subjects. Subsequent technique involved morphological examination of suspected Neisseria colonies by gram stain and purification of colonies which exhibited characteristic morphology. Cultures which exhibited facultative or microaerophilic growth in cystine trypticase agar (CTA) deeps were discarded. All incubations were at 33-35 C for 24-48 hours.

A description of the methods used in the isolation of Neisseria strains from the nasopharyngeal region of guinea pigs was given by Pelczar, Hajek and Faber (1949).

Stock cultures. Stock cultures of the Neisseria strains were maintained by stab inoculation into cystine trypticase agar (CTA) deeps and incubation at 35 C for 24 hours. Cultures were stored at 30 C in cotton-stoppered tubes and transfers made at three week intervals.

Storage of cultures at room temperature materially shortened the life span of the cultures during the winter months whereas higher storage temperatures resulted in more rapid desiccation of the medium. Thus, 30 C approximated the ideal storage temperature and, at this temperature, recourse to rubber-stoppered tubes or starch medium was found to be unnecessary for the maintenance of the stock cultures during this investigation.

Preparation of inocula. Inocula for morphological studies were prepared by cultivation of the organisms through two serial transfers on trypticase soy slants and inoculation of the test media by needle.

Colony morphology and staining reactions. Colony morphology and staining reactions were determined by streak plate inoculation upon trypticase soy agar with macroscopic observation of colony morphology after a 24 hour incubation period. The staining reactions were determined by gram's method (Hucker modification) on the above colonies with staining times of one minute each for the primary stain and mordant, one-half minute for the ethanol decolorizer and one-half minute for the safranin counterstain.

Pigmentation. Pigmentation was determined by two methods. As recommended in Bergey's Manual (1948) inoculations were made upon Loeffler's blood serum slants and macroscopic inspection of chromogenesis was effected by removal of colonial growth and smearing upon a sheet of white paper. The second method of observation was the suspension of slant growth from trypticase soy agar in five ml portions of physiological saline, centrifugation, resuspension in a second aliquot of saline and recentrifugation to obtain packed cells. The color of the packed bacterial cells was noted as evidence of chromogenesis. For the identification of the organisms under study, chromogenesis as determined on Loeffler's blood serum slants was used as the standard method.

The morphological reactions of the cultures of Neisseria studied in this investigation are summarized in table 2.

Table 2

Morphological and Colonial Characteristics of Sixty Strains of Non-pathogenic *Neisseria*

Culture	Gram Stain	Chromogenesis: Loeffler's slant	Chromogenesis: Packed cells	Colony Morphology
100	-	light grey	light grey	smooth, moist, entire
7900	-	light grey	light grey	smooth, moist, entire
8176	-	grey	grey-white	smooth, moist, entire
8193	-	grey	pink	smooth, moist, entire
81	-	grey	light grey	smooth, moist, entire
82	-	grey	light grey	smooth, moist, entire
86	-	grey	white-cream	smooth, moist, entire
87	-	grey	light grey	smooth, moist, entire
88	-	grey	white-grey	smooth, moist, entire
89	-	grey	light grey	smooth, moist, entire
14	-	light grey	white-grey	veil-like, spreading
19	-	light grey	white-grey	veil-like, spreading
22	-	grey	grey	smooth, glistening, entire
24	-	grey	grey	smooth, spreading, flaky
32	-	grey	white-grey	irregular, undulate
35	-	light grey	white-grey	small, beady, glistening
36	-	grey	white-grey	smooth, moist, entire
1485	-	light grey	light pink	smooth, convex, entire
7902	-	light grey	white-grey	irregular, slightly rough
9913	-	light grey	white-grey	small, convex, glistening
X	-	light grey	light pink	irregular, undulate
12	-	caramel	yellow	smooth, glistening, entire
876	-	dark caramel	yellow	smooth, glistening, entire
1927	-	light caramel	cream	slightly irregular, undulate
7925	-	light caramel	light yellow	irregular, undulate
83	-	light caramel	light yellow	smooth, glistening, entire
84	-	light caramel	light yellow	smooth, glistening, entire
2AMS	-	light caramel	light yellow	smooth, glistening, entire
3AMS	-	light caramel	light yellow	smooth, glistening, entire
4	-	yellow	light yellow	smooth, glistening, entire
55	-	yellow	yellow	smooth, moist, entire

Table 2 (continued)

Morphological and Colonial Characteristics of Sixty Strains of Non-pathogenic <u>Neisseria</u>

Culture	Gram Stain	Chromogenesis: Loeffler's slant	Chromogenesis: Packed cells	Colony Morphology
155	-	yellow	light yellow	small, smooth, circular
157	-	yellow	light yellow	small, smooth, circular
9746	-	yellow	light yellow	small, smooth, circular
B	-	light caramel	white-pink	smooth, moist, entire
C	-	light caramel	light pink	smooth, moist, entire
D	-	light caramel	light grey	smooth, moist, entire
F	-	light yellow	light cream	smooth, glistening, entire
J	-	light caramel	light grey	smooth, moist, entire
K	-	light caramel	white-grey-	smooth, moist, entire
L	-	light caramel	light pink	smooth, moist, entire
N	-	light yellow	yellow	smooth, glistening, entire
O	-	light yellow	yellow	smooth, glistening, entire
P	-	light yellow	yellow	smooth, glistening, entire
R	-	light caramel	white-grey	smooth, glistening, entire
S	-	light caramel	white-grey	smooth, glistening, entire
T	-	light caramel	white-grey	irregular, undulate
U	-	light caramel	light grey	smooth, moist, entire
V	-	dark caramel	yellow	smooth, glistening, entire
W	-	dark caramel	yellow	smooth, glistening, entire
OP3	-	dark brown	yellow	smooth, glistening, entire
OP4	-	yellow	light yellow	smooth, glistening, entire
OP6	-	light yellow	yellow	smooth, glistening, entire
OP8	-	dark brown	yellow	smooth, glistening, entire
OP11	-	yellow	yellow	smooth, glistening, entire
OP13	-	yellow	light yellow	smooth, glistening, entire
OP14	-	yellow	yellow	smooth, glistening, entire
OP16	-	brown	yellow	smooth, glistening, entire
Calf	-	light yellow	light grey	smooth, moist, entire
Sheep	-	light yellow	light yellow	smooth, moist, entire

__Results.__ Organisms grown on Loeffler's blood serum slants produced small colonies after a 48 hour period and the pigments of the colonies almost uniformly presented a light yellow-brown appearance. This yellow-brown color was called caramel in this study. When smeared upon paper, however, the caramel color of the colonies often appeared as light grey and it was concluded that the yellowish cast of the medium was responsible for the caramel color. Washed, packed cells of the same organism, however, often presented a variety of hues and in the non-chromogenic __N. catarrhalis__ species, grey, cream and pink colors were observed. No culture showed a complete absence of color although many strains were light grey in color when examined as packed cells.

The presence of greenish-yellow colonies were noted among the chromogenic strains when cultivated upon trypticase soy slants but the greenish cast never appeared upon the petri plate colonies. It is assumed that the thin growth of the chromogenic organisms, and non-chromogenic strains as well, upon the slant refracted the incident light and the refracted light gave an iridescent appearance to the colonies. The greenish, iridescent hues were noted when members of the __N. catarrhalis__ group were grown upon freshly-prepared agar slants and the tubes rotated in the sunlight. The green tint never appeared either upon the opaque Loeffler serum slant or in the packed cellular mass.

__Pigment isolation.__ Confirmatory evidence of the absence of green pigment in __Neisseria__ species was obtained by isolation of pigments from the bacterial cells. The packed cellular mass from the pigment determination was washed thoroughly with ten ml of petroleum ether. To the ether suspension, an equal volume of ethanol was added and the mixture allowed to remain undisturbed. After an hour, the ethanol layer was removed from the test tube and passed through a chromatographic absorption column charged with equal weights of filter-cel and magnesium oxide. By this technique, _N. perflava_ (2AMS) yielded two pigments: a light pink pigment near the top of the absorption column and a light yellow pigment midway between the extremities of the column. No evidence of a green tint was evident upon inspection of the absorption column either under sunlight or ultra-violet light.

Although all strains of _Neisseria_ grew well in the stock culture medium (CTA), pigmentation was not easily observable. Non-chromogenic _Neisseria_ grew as a grey-white mass either at the point of inoculation or spread over the entire surface of the deep culture tube. Chromogenic strains grew with equal facility although the intensity of pigmentation varied with the separate strains. Several strains produced pigment during the first 48 hours of growth and through subsequent storage at 30 C; the intensity and shade of pigment increased to a deep brown color at about a 14 day storage period. To note the effect of oxidation upon the pigments of two _Neisseria_ strains in the absence of bacterial cells and substrate, the pigments were extracted with petroleum ether and ethanol and allowed to remain in a cotton-stoppered test tube over a period of seven days. In neither case was any darkening of pigment noticeable and it was concluded that the darkening of pigment was not due to oxidation of pigment by air. The organisms tested in this experiment were two unclassified strains, GP3 and GP8.

The change in colony texture from smooth to rough was reported by Elser and Huntoon (1909), Gordon (1921) and Wilson (1928) and was considered one of the varying characteristics of the gram negative cocci. In the present study, colony texture was considered as a secondary characteristic, inferior to chromogenesis and the identification of the cultures was based primarily upon the colony pigmentation. Variation in colony texture was noted in one instance. N. perflava (2AMS), originally a smooth, glistening, yellow colony displayed a yellow, rough-textured irregular outline after a year on CTA medium. Eventually, the organism reverted to the normal smooth form after successive transfers upon trypticase soy agar and remained in the smooth form. Wilson (1928) had noted changes from smooth to rough among some strains of his organisms, following five successive, rapid transfers on ascitic agar and this smooth to rough mutation remained permanent.

The morphological appearance of all strains of Neisseria in this study conformed to the classical description of the Neisseria - gram negative cocci in pairs or small clumps with adjacent sides slightly flattened. Giant cells were often seen in the microscopic fields but their presence could not be correlated with any physical factors except the physiological activity of the cells. The giant cells appeared more numerous during periods of rapid subculture of the strains rather than in response to specific stimuli in their environment.

B. Biochemical Characteristics

Studies of the biochemical reactions of the cultures centered about their fermentation reactions on five sugars upon which the current classification key is based. Of these five sugars, the early workers referred to fructose as an "unreliable" sugar, although they continued to employ this sugar in their classification schemes. Elser and Huntoon (1909) reported the unstability of fructose when sterilized by intermittent steaming and in the presence of the basal substrate, but their experimental evidence was ignored for fructose continued to be used in the classification of Neisseria species.

Carbohydrate fermentations. Fermentation reactions were determined in CTA medium plus 0.2 per cent of the carbohydrate under test. All carbohydrates were sterilized by Seitz filtration and added aseptically to the tubed basal medium. Inoculation was performed with a drop of washed cell suspension delivered from a pipette. Incubation was at 35 C with readings at 1, 3, and 7 days' incubation.

Catalase test. The presence of catalase was determined by the addition of one ml of a 3 per cent solution of hydrogen peroxide to a 24 hour slant culture of the organism. The rapid evolution of gas was considered evidence for the presence of catalase.

Oxidase test. The presence of oxidase was noted by flooding the surface of a 24 hour plate culture with one ml of a freshly-prepared one per cent aqueous solution of dimethyl-paraphenylene hydrochloride, allowing the dye to remain for 3-5 minutes and decanting the dye. The production of pink coloration on the surface of the colonies was considered a positive test. The biochemical reactions of the cultures of *Neisseria* studied in this investigation are summarized in table 3.

Results. All cultures under study were oxidase positive. With few exceptions, all organisms produced catalase. The interpretation of the catalase test was difficult at times as few bubbles of gas appeared on the surface of the slant. However, when the immediate appearance of gas bubbles was noted upon the addition of the test solution, the test was considered positive.

The limited activity of the *Neisseria* upon carbohydrates was unique. Among the carbohydrates tested for fermentation, a maximum of four carbohydrates were consistently fermented by *N. sicca* and *N. perflava* strains.

Table 3

Summary of Physiological Reactions of Sixty Strains of Non-pathogenic <u>Neisseria</u>

		Glucose	Fructose	Maltose	Sucrose	Mannitol	Galactose	Mannose	Lactose	Cellobiose	Melibiose	Trehalose	Raffinose	Melizitose	Starch	Dextrin	Arabinose	Xylose	Rhamnose	Glycerol	Sorbitol	Oxidase Test	Catalase production
N. catarrhalis	100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	7900	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	8176	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	8193	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	S1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	S2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	S6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	S7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	S8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	S9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
N. sicca	14	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	19	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-
	22	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	24	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	32	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	35	+	+	+	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-	+	-
	36	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	1485	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	7902	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	9913	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	X	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
N. perflava	12	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	876	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	1927	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	7925	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	S3	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	S4	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	2AHS	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	3AHS	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+

Table 3 (continued)

Summary of Physiological Reactions of Sixty Strains of Non-pathogenic *Neisseria*

		Glucose	Fructose	Maltose	Sucrose	Mannitol	Galactose	Mannose	Lactose	Cellobiose	Melibiose	Trehalose	Raffinose	Melizitose	Starch	Dextrin	Arabinose	Xylose	Rhamnose	Glycerol	Sorbitol	Oxidase Test	Catalase production
N. flava	4	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	55	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
N. flavescens	155	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	157	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	9746	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Unclassified	B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	E	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	J	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	L	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	P	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	T	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	U	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	W	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	GP3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	GP4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	GP6	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	GP8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	GP11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	GP13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
	GP14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	GP16	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	Calf	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
	Sheep	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+

Legend: - no reaction
+ acid production or a positive test

These carbohydrates, glucose, fructose, maltose, and sucrose, are used in the classification of the chromogenic Neisseria. The fifth carbohydrate employed in the systematic identification of chromogenic Neisseria, mannitol, was not fermented by any strain of Neisseria. No conclusion could be reached regarding this lack of fermentative ability of organisms, many of which were typed mannitol positive by other investigators.

The inertness of the Neisseria to carbohydrates was exemplified by the action of 21 strains upon the fifteen supplementary carbohydrates tested. One strain, N. sicca (#35), fermented mannose, trehalose and glycerol with acid production. No other strain of Neisseria fermented any of the supplementary carbohydrates used in these tests.

The Neisseria of this study were classified in accordance with the scheme embodied in Bergey's Manual of Determinative Bacteriology. The N. catarrhalis species, non-chromogenic and biochemically inert, presented no problem in classification.

Two strains of N. sicca, a non-chromogenic, biochemically active species, were more a problem. One strain, N. sicca (ATCC 9913), did not ferment fructose or maltose. The other strain, N. sicca (I), fermented glucose only. When typed against polyvalent meningococcus antiserum, both strains were negative by the slide agglutination technique although slight saline sensitivity was noted. On the basis of moderate fermentative ability and non-chromogenesis, these organisms were included with the N. sicca group.

The N. perflava strains were typical and conformed to species specification. One strain, N. perflava (7925), varied in the fermentation of maltose on occasion but was typical in other respects and was included in this group.

No typical N. subflava species were included in this study, since it was impossible to isolate or obtain type cultures of this species.

Three strains of N. flavescens were studied: the type culture strain (9746) and two strains obtained from the original investigator, Dr. Sara Branham. Since the N. flavescens group is a chromogenic, non-fermentative and serologically homogenous group, all strains were identical.

The strains isolated from animal sources presented a taxonomical problem. Although non-fermentative and chromogenic, these cultures were not classified as N. flavescens. Representative strains of this group were studied by Dr. Sara Branham and, in her opinion, these strains were not N. flavescens. She based her conclusions on differences in serological reactions and the differences in pigmentation when contrasted to type strains of N. flavescens. Therefore, for the purposes of this study, the cultures isolated from animal sources were grouped as "unclassified" and studied as a unit.

It should be emphasized that considerable difficulty was experienced during the course of this study in the interpretation of chromogenesis. The present methods of chromogenesis evaluation are uncertain and subject to individual judgment and unless more precise methods for chromogenesis detection are introduced, chromogenesis as a characteristic of taxonomical importance in the genus Neisseria should be discarded.

C. Direct Disaccharide Fermentation

During the investigation of the biochemical reactions of the *Neisseria*, aberrant carbohydrate fermentations were produced by four strains, *N. perflava* (12 and 876) and *N. flava* (4 and 55). On occasion, these strains fermented maltose with acid production but did not ferment glucose. This phenomenon, although uncommon, was reported by Wilson and Smith (1928) for the meningococci and the review by Liebowitz and Hestrin (1945) discussed the literature and the theoretical implications of this "direct" fermentation of disaccharides.

Stock strains of cultures which exhibited fermentation of maltose but not of dextrose were obtained by streak plate isolation from a maltose sugar tube which showed direct maltose fermentation. With the exception of *N. perflava* (876), these stock strains' variants were constant in production of direct maltose fermentation.

Determination of the final pH in glucose and maltose media showed definite acid production in the maltose tube; no acidity or an alkaline reaction in the glucose tubes comparable to the alkaline reactions induced in the sugar-free controls. The fermentation of maltose in the absence of glucose fermentation developed in natural media irrespective of the protein used as substrate. Substitution of meat extract and peptone in lieu of the trypticase did not alter the course of reaction. The use of autoclaved carbohydrates instead of filtered, in any basal medium, resulted in glucose as well as maltose fermentation.

Chemical determination of the amount of carbohydrates utilized during these fermentations were made by the method of Steinhaus and Isbell. With the method of Steinhoff[1], glucose and maltose were

[1] Browne, C.A., and Zerban, F.W., *Physical and Chemical Methods of Sugar Analysis* (London: Wiley & Sons, Inc., 1941), p. 1005.

determined concurrently. The method of Isbell[2] determined the total reducing power of all carbohydrates, with factors applied for the determination of the amount of specific carbohydrate under analysis. All maltose broths, at the time of analysis, showed a negative chemical test for reducing sugars by the Benedict and Barfoed tests. The results of chemical determination of residual sugars, although in experimental error, showed greater quantitative utilization of maltose than glucose.

More conclusive evidence of direct enzymatic utilization of maltose was obtained by the Thunberg technique—which measured the dehydrogenase activity of the organisms in the presence of specific substrate with methylene blue as indicator. The technique employed was as follows: Bacteria were grown on trypticase soy slants for 24 hours, washed from the slants with physiological saline and centrifuged. They were resuspended in saline, centrifuged and again separated. The cells were finally suspended in a suitable volume of saline and aerated for 30 minutes. For determination of the methylene blue reduction times, Thunberg tubes with hollow stoppers were used. Into the main tube was pipetted 2 ml of phosphate buffer (salt solution B, Chapter III), 2 ml of M/50 substrate and 1 ml of 1/10,000 methylene blue solution. One ml of aerated cells (70–80 per cent light absorption on Fisher electrophotometer, AC model 425 B blue filter) was introduced into the hollow stopper. A series of tubes prepared in this manner were evacuated with a vacuum pump for three minutes and immersed in a water bath at 35 C. After two minutes, the tubes were inverted to mix the solutions and the time for the blue color in each tube to disappear was noted. With each

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[2] Ibid., p. 844.

series, a control tube with no substrate was included, also a control tube with a boiled specimen of the bacterial suspension. Neither of the control tubes ever changed color during the course of the experiments. Oxidation times of a 20 per cent suspension of N. flava (4) in the presence of specific carbohydrate substrates are shown in table 4.

Table 4

Comparison of Reduction Times of Methylene Blue by N. flava (#4) in the Presence of Various Carbohydrates and Related Compounds.

Substrate	Decolorization time in minutes
Glucose	36
Fructose	exceeded 64
Maltose	9
Sucrose	exceeded 64
Mannitol	exceeded 64
Endogenous control	exceeded 64
Boiled control	exceeded 64

It appeared that the oxidation time of glucose was significantly prolonged in comparison to the oxidation time for maltose. Similar experiments performed with organisms N. perflava (12) and N. flava (55) in presence of glucose or maltose substrates showed comparable results, a more rapid attack of maltose.

Oxidation rates of maltose and glucose were obtained by measuring light absorption at varied time intervals of Thunberg tubes which contained glucose or maltose as substrate. The results of a typical experiment are shown in table 5 and diagrammed in figure 1.

Table 5

Reduction Times of Methylene Blue by N. *flava* (#4) in Presence of Glucose and Maltose

Substrate	Per Cent Light Absorption Time in Minutes									
	0	1	3	5	7	9	12	20	23	32
Maltose	47	46	46	32	16	14	13	8	8	8
Glucose	41	39	39	39	39	39	39	38	38	36
Endogenous control	44	44	44	44	44	44	44	44	44	44

The rapid rate of maltose oxidation in comparison with glucose oxidation by the N. *flava* strain is evident from table 5 and figure 1. Similar results were obtained when oxidation rates of maltose and glucose were determined with N. *perflava* (12) and N. *flava* (55). Although it was realized that the Thunberg method specifically measured the dehydrogenating ability of the bacterial suspensions in the presence of methylene blue, it was considered that similar oxidation rates prevailed in the basal medium and that maltose was oxidized and fermented more rapidly and with acid production whereas glucose was utilized slowly, if at all, with little observable acid production.

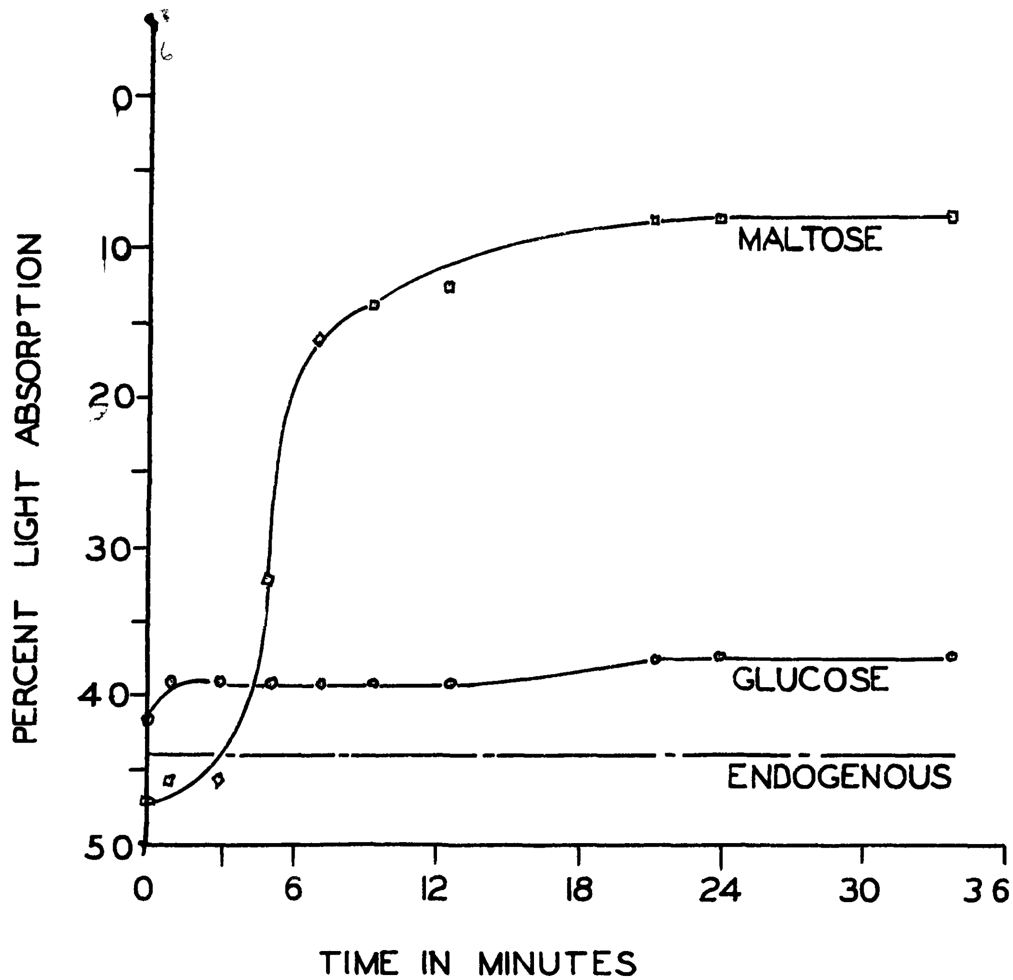

Figure 1. Oxidation rates of glucose and maltose by E. flava (#4), with methylene blue as E_h indicator.

CHAPTER III

VITAMIN REQUIREMENTS OF THE GENUS *NEISSERIA*

Ordal and Busch (1946) reported a biotin requirement for 24 strains of *N. sicca*. No other report has been found regarding the vitamin requirements of the gram negative cocci of the normal nasopharynx.

Preparation of inoculum. The organisms used as inocula for the experiments in this and the following sections were grown through two serial subcultures on trypticase soy slants. The inoculum was prepared as follows: Growth on the slant surface was removed with 5 ml of physiological saline, the cells centrifuged and the supernatant liquid discarded. The packed cells were washed with 5 ml of saline, centrifuged again and the supernatant liquid discarded. The packed cells were suspended for the third time with 5 ml saline and one loop (about 4 mm diameter) of the washed cell suspension served as inoculum for 5 ml of the test medium.

Preparation of test medium. Strains of *Neisseria* were tested for growth on medium of the following composition:

Casein hydrolysate, salt-free, vitamin-free[1]	0.5 g
Glucose, c.p.	0.1 g
Cysteine	2.0 mgm
Tryptophane	50.0 mgm
Salt solution[2]	2.0 ml
Biotin	4.0 micrograms
Thiamin hydrochloride	1000.0 micrograms
Calcium d-pantothenate	1000.0 micrograms
Pyridoxine hydrochloride	1000.0 micrograms
Niacin	1000.0 micrograms
Distilled water to	100.0 ml

pH 7.5

[1] Casein hydrolysate obtained from National Dairy Research Laboratories, Baltimore, Maryland. Designated as Lot #6.

[2] Salt solution contained disodium phosphate, 5 g; potassium chloride, 0.09 g; sodium dichloride, 3.0 g; magnesium sulfate, 0.06 g; water to 100 ml.

The medium was prepared as follows: The casein hydrolysate, glucose, cysteine and tryptophane were dissolved in less than the total volume of water. The salt solution was prepared separately and added to the casein hydrolysate mixture. The medium was made up to volume with allowance for the volumes of vitamins to be added later, adjusted to the desired degree of acidity by means of a Beckman pH meter and sterilised by filtration through Pyrex sintered glass filters. The medium at this point was called the test medium. Sterile solutions of the vitamins were added aseptically to the test medium and the complete medium dispensed into sterile, aluminum-capped test tubes in 5 ml amounts.

Preparation of vitamin solutions. Sterile solutions of vitamins (except biotin) were prepared by solution of the vitamins in distilled water at concentrations of 1000 micrograms per ml, sterilisation by sintered glass filtration and storage of the concentrated vitamin solutions in cotton-stoppered tubes at refrigerator temperature. Biotin was dissolved in a 25 per cent ethanol solution at a final concentration of 10 micrograms per ml, sterilised by sintered glass filtration and stored with the other vitamins.

At this point, a preliminary experiment was undertaken to determine the growth response of strains of *Neisseria* to the complete medium. A single loop inoculation of a series of organisms into the complete test medium containing the five vitamins resulted in the appearance of heavy flocculation following incubation for 48 hours. Slides prepared from the heavy floc revealed the presence of microorganisms among the precipitate and the possibility of phosphate precipitation at the elevated pH of the medium appeared feasible. Accordingly, the salt solution was modified and a standard salt solution for the remainder of the experiments was adopted. The new solution was composed of separate salt solution A[1] and salt solution B[2], two ml of each solution added to 96 ml of basal medium prior to the pH adjustment. The final pH of the medium was adjusted to approximately 7.35, to eliminate the phosphate flocculation at a more elevated pH.

Composition of basal medium. The composition of the basal medium employed in the remainder of the experiments of this investigation was as follows:

Casein hydrolysate, salt-free, vitamin-free	0.5 g
Glucose, c.p.	0.1 g
Cysteine	2.0 mgm
Tryptophane	50.0 mgm
Salt solution A[1]	2.0 ml
Salt solution B[2]	2.0 ml
Distilled water to	100.0 ml
pH 7.35	

The basal medium was prepared as outlined in the section on Preparation of test medium.

The complete test medium of subsequent experiments consisted of the basal medium as noted above with the addition of biotin (free acid), 4 ug; thiamin hydrochloride, calcium d-pantothenate, pyridoxine hydrochloride and niacin, each in 1000 microgram amounts.

Preliminary experiments with the complete test medium resulted in growth of the test organisms and no precipitation of the medium after incubation for 48 hours.

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1 Contained $FeCl_2.4H_2O$, 0.01 g; $MnCl_2.4H_2O$, 0.05 g; $MgSO_4.7H_2O$, 0.5 g; NaCl, 5.0 g; water to 100 ml.

2 Contained KH_2PO_4 (Soerensen's), 1 g; K_2HPO_4 (anhydrous), 3 g; water to 100 ml.

Results. To determine the essentiality of a single vitamin, a series of six basal media were prepared, one medium containing all 5 vitamins, the remaining media each deficient in a single vitamin and inoculated with a series of ten test organisms. The results of this experiment are given in table 6.

Table 6

Growth Response of 10 Strains of Neisseria to the Complete Test Medium Containing 5 Vitamins and to Complete Test Medium with Individual Vitamin Omissions

Complete test medium + vitamins eliminated individually	Cultures Tested									
	22	55	77	547	876	1927	2020	7902	7925	2AMS
No omission	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
Less thiamin	3+	3+	3+	3+	3+	3+	2+	3+	3+	3+
Less calcium d-pantothenate	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
Less niacin	3+	3+	3+	3+	3+	3+	2+	2+	2+	2+
Less pyridoxine hydrochloride	2+	3+	3+	2+	3+	3+	3+	3+	3+	3+
Less biotin	-	-	-	-	-	-	-	-	-	-

Legend: - = no growth
2+ = fair growth
3+ = average growth

The data presented in table 6 indicated that the organisms were unable to grow in the absence of biotin. When this vitamin was present, good growth resulted. In addition, the results indicate that the cultures were able to grow through four serial transfers when each vitamin, other than biotin was eliminated.

To determine the essentiality of biotin in the absence of the other vitamins of the B complex, ten strains of *Neisseria* were tested in seven different media, each of the five media contained a single, different vitamin, the sixth medium contained no vitamins and the seventh medium contained all five vitamins under test. To obtain a more accurate estimation of growth response, a Fisher electrophotometer, AC model with 425 B blue filter, was used to measure turbidity of the medium after the fifth serial transfer. The results of this experiment are given in table 7.

Table 7

Growth Response of 10 Strains of *Neisseria* to the Basal Medium Containing Biotin, Niacin, Pantothenate, Pyridoxine and Thiamin Individually and in Combination

Media	Per cent Light Absorption*									
	Culture Number									
	55	100	876	1485	1927	2020	7900	7902	7925	2AM5
Basal	00	43**	4	5	00	4	34	00	00	00
Basal + 5 vitamins	16	80	4	30	10	25	56	18	40	36
Basal + calcium d-pantothenate	0	43	2	5	0	0	40	0	0	0
Basal + pyridoxine hydrochloride	0	45	0	4	0	0	36	0	0	0
Basal + thiamin hydrochloride	0	50	4	0	0	0	40	0	0	0
Basal + niacin	0	48	3	0	0	0	37	0	0	0
Basal + biotin	17	76	12	30	10	27	46	18	22	40

* Measured with Fisher electrophotometer, AC model, 425 B blue filter.

** Readings of fifth serial transfer with 48 hour incubation periods between transfers.

All organisms tested in this experiment grew well in the presence of biotin. Growth of any single strain was not materially enhanced by the presence of other vitamins of the B complex. Two strains of _N. catarrhalis_ (100 and 7900) grew profusely in the absence of any of the vitamins tested in this experiment but the total amount of growth was increased materially by the addition of biotin; no other vitamins under test were able to effect a similar growth stimulation. Since these organisms showed good growth in the absence of any vitamins but increased growth by the addition of biotin, they were regarded as "biotin-stimulated." The growth of all _N. catarrhalis_ strains was not stimulated by the presence of biotin but no cultures of other species responded to biotin in similar manner.

To determine if additional growth stimulation of _Neisseria_ were obtainable by the addition of other growth factors, an experiment was performed employing the five vitamins of previous experiments, riboflavin and folic acid. The vitamin concentrations in the complete basal medium were as follows: biotin, riboflavin and folic acid, 0.01 micrograms each per ml of basal medium; calcium D-pantothenate, pyridoxine hydrochloride, thiamin hydrochloride and niacin, one microgram each per ml of basal medium. The riboflavin and folic acid were aqueous solutions, sterilized and stored as noted for the other vitamins. The media which were inoculated consisted of a basal medium devoid of vitamins, basal medium plus biotin, basal medium plus all seven vitamins and trypticase soy broth. The trypticase soy broth was included for comparative purposes. _Neisseria_ grew more luxuriantly upon this media than upon any other natural medium. The results of this experiment, after four serial transfers, are given in table 8.

Table 8

Growth Response of 10 Strains of <u>Neisseria</u> in a Basal Medium plus Biotin, A Complete Medium Plus Riboflavin and Folic Acid, and in a Natural Medium

Media	Per cent Light Absorption									
	Culture Number									
	55	100	876	1485	7900	7925	8176	8193	9746	2AMS
Basal alone	4	47	6	11	72	10	4	5	6	2
Basal + biotin	40	90	28	24	67*	34	16	14	6	16
Basal + 7 vitamins	43	90	30	23	74*	33	13	8	6	19
Trypticase soy broth	40	77	43	37	61*	44	60	12	8	35

* growth granular, readings inaccurate

The results of this experiment indicated that neither riboflavin nor folic acid rendered any growth stimulus to the organisms tested. The slight differences between readings were not significant. Of significance, however, were readings obtained by growth of organisms in trypticase soy broth. In comparison with trypticase soy broth, the growth in partially synthetic media appeared less than optimum and this deficiency was not remediable either by the addition of one per cent starch, 0.01 per cent yeast extract, para-aminobenzoic acid or i-inositol. It must be concluded, that either the conditions of the experiment were not optimum or a vital nutritive substance, irreplaceable by the vitamins noted above, was absent from the medium.

During the investigation on minimum vitamin requirements of <u>Neisseria</u>, a series of organisms under test did not respond to the presence of biotin in the basal medium but grew well in a basal medium which contained five vitamins of the B complex. To determine the minimum vitamin requirements of these organisms, six media were prepared, one medium containing all five vitamins, biotin, pyridoxine, niacin, pantothenate and thiamin, the remaining media each deficient in a single vitamin. The results of this experiment after inoculation and 5 serial transfers are recorded in table 9.

Table 9

Growth Response of 11 Unclassified Strains of Neisseria in a Complete Test Medium with Individual Omissions of Biotin, Pantothenate, Pyridoxine, Niacin and Thiamin.

Media	Per cent Light Absorption										
	Culture Number										
	B	J	K	36	E	F	X	L	GP6	Calf	Sheep
Complete- Basal + five vitamins	38	32	32	50	15	17	43	25	36	0	0
Complete less biotin	2	0	0	0	24	13	0	3	2	0	0
Complete less calcium d-pantothenate	32	22	17	30	35	14	38	32	36	0	0
Complete less pyridoxine hydrochloride	38	24	26	40	23	18	37	25	36	0	0
Complete less niacin	0	0	0	0	0	2	3	2	0	0	0
Complete less thiamin hydrochloride	35	28	30	29	31	22	41	2	36	0	0

Lack of growth in the complete medium less niacin indicated that biotin was not the sole essential vitamin requirement among the Neisseria. The strains X, GP6, B, J, K, and 36 required niacin in addition to biotin for normal growth. Strains of E and F required niacin only for continued growth in partially-defined medium. Strain L required biotin, niacin and thiamin and the two strains designated as Calf and Sheep required other factors or combination of factors for normal growth. Subsequent experiments indicated that growth of Sheep and Calf strains could not be stimulated by the addition of riboflavin, folic acid, i-inositol, para-aminobenzoic acid or indole-3 acetic acid to the complete medium presented in the above experiment.

A compilation of the minimum vitamin requirements of all strains of Neisseria investigated in this study and their growth response to these vitamin requirements is presented in table 10. The vitamin requirements were established by the methods reported earlier in this chapter.

The majority of Neisseria investigated had positive vitamin requirements; the vitamin requirement centered about a need for biotin. The individual growth response to biotin was not always uniform nor was the requirement for biotin concisely met by the presence of this vitamin in all instances. Among the organisms which grew feebly on natural medium, as well as in the complete medium, a period of acclimitization appeared necessary before the organism responded to the presence of biotin. This response was noted by increased light absorption through the sequence of serial transfers and was considered to be a selection of biological mutants in a medium adequate but not optimum for their growth. In few instances, upon prolonged incubation, pigmentation among the chromogenic strains was noted and was considered evidence for growth approaching the optimum.

Among the established species, the biotin requirement was most evident as the sole vitamin requirement of N. perflava, N. flava and N. flavescens. The N. catarrhalis strains showed a maximum vitamin requirement of biotin, although this vitamin was unnecessary in some instances. The strains of N. sicca required niacin in addition to biotin although Ordal & Busch (1946) reported a minimum requirement of biotin for this species.

Table 10

Minimal Vitamin Requirements and Growth Response of Sixty Strains of Non-pathogenic *Neisseria* Through Five Transfers in a Casein Hydrolysate Medium

Species and Strain Designation		Minimal Vitamin Requirements	Per cent Light Absorption — Serial Transfers				
			1st	2nd	3rd	4th	5th
N. catarrhalis	100	none	57	55	62	63	63
		biotin stimulation	80	80	72	76	80
	7900	none	64	54	64	60	57
		biotin stimulation	56	60	62	69	66
	8176	none	42	32	38	31	30
	8193	none	32	24	28	28	45
		biotin stimulation	52	43	61	54	55
	S1	none	59	55	59	59	55
	S2	biotin	10	15	18	24	26
	S6	none	22	42	44	50	49
	S7	biotin	10	55	23	35	35
	S8	biotin	33	22	5	5	15
	S9	biotin	22	10	17	28	27
N. sicca	14	biotin	13	15	15	20	30
	19	biotin	7	13	10	24	25
	22	biotin	28	22	30	48	40
	24	biotin	25	17	14	25	14
	32	biotin	28	16	20	14	14
	35	biotin + niacin	4	10	8	26	25
	36	biotin + niacin	50	35	45	55	50
	1485	biotin	30	26	25	26	30
	7902	biotin	25	12	18	27	26
	9913	biotin	32	20	22	24	25
	X	biotin + niacin	48	47	54	63	63
N. perflava	12	biotin	33	9	12	25	26
	876	biotin	28	33	32	36	36
	1927	biotin	46	37	26	36	36
	7925	biotin	23	23	27	36	28
	S3	biotin	14	10	13	12	24
	S4	biotin	18	18	33	27	33
	2AMS	biotin	29	30	33	38	35
	3AMS	biotin	30	36	30	38	36
N. flava	4	biotin	50	65	62	65	66
	55	biotin	40	44	36	50	53
N. flavescens	155	biotin	21	24	27	20	18
	157	biotin	20	22	23	18	21
	9746	biotin	16	17	16	23	20

Table 10 (continued)

Species and Strain Designation		Minimal Vitamin Requirements	Per cent Light Absorption				
			Serial Transfers				
			1st	2nd	3rd	4th	5th
Unclassified	B	biotin + niacin	16	18	28	30	30
	C	none	62	45	50	50	54
	E	niacin	22	18	22	20	32
	F	niacin	20	20	17	24	30
	J	biotin + niacin	5	17	13	28	25
	K	biotin + niacin	21	28	25	34	31
	L	biotin + niacin + thiamin	22	30	15	36	30
	N	biotin + niacin	14	28	15	26	24
	O	biotin + niacin + thiamin + pantothenate	16	11	15	12	15
	P	biotin + niacin	10	16	8	10	12
	R	biotin	17	8	10	17	9
	S	biotin + niacin	13	22	13	28	26
	T	biotin + niacin	17	10	10	14	14
	U	biotin + niacin	13	18	27	36	34
	V	biotin + niacin	11	16	18	32	28
	W	biotin + niacin	5	3	7	10	10
	GP3	none	35	39	42	50	45
	GP4	none	35	28	36	38	35
	GP6	biotin + niacin	36	65	64	60	62
	GP8	biotin	15	7	8	6	8
	GP11	biotin + niacin	18	33	62	41	52
	GP13	biotin + niacin	20	24	26	28	30
	GP14	biotin + niacin + thiamin + pyridoxine + pantothenate	24	30	23	27	25
	GP16	none	58	60	59	65	69
	Calf	no minimum requirements known					
	Sheep	no minimum requirements known					

Among the unclassified strains, however, the vitamin requirements for growth were numerous and diverse. Biotin, niacin and mixtures of these two vitamins were required for most strains. A few cultures required no vitamins and a few cultures needed a moiety of vitamins for adequate growth. Two strains did not respond to any vitamin mixtures.

The response of four strains of *Neisseria* to graded amounts of biotin in the basal medium is shown in table 11.

Table 11

Growth Response of Five Strains of _Neisseria_ to Graded Amounts of Biotin in a Casein Hydrolysate Medium

Media	Per cent Light Absorption Culture Number				
	1876	7900	2AMS	7925	9746
Basal, no vitamin	0	36	0	0	0
Basal + 10^{-8}ug/ml biotin	0	45	0	0	0
Basal + 10^{-7}ug/ml biotin	0	47	0	0	0
Basal + 10^{-6}ug/ml biotin	0	46	10	15	16
Basal + 10^{-5}ug/ml biotin	40	47	16	15	15
Basal + 10^{-4}ug/ml biotin	47	44	20	28	15
Basal + 10^{-3}ug/ml biotin	45	45	20	29	15

Curves drawn with these data are shown in figure 2. The organism, 7900, a _N. catarrhalis_, biotin-stimulated strain, showed a relatively constant response to all additive increments of biotin, irrespective of total concentration. The response of _N. catarrhalis_ (1876) was detected at a concentration of 10^{-5} ug biotin per ml of basal medium whereas the _N. perflava_ strains (7925 and 2AMS) and the _N. flavescens_ strain (9746) showed growth at a biotin concentration of 10^{-6} ug per ml. The growth response to increasing concentrations of biotin was not linear.

The growth response of five strains of _Neisseria_ to varied concentrations of niacin in the presence of 0.01 ug biotin per ml of media was determined and is recorded in table 12. As four of the organisms tested required biotin in addition to niacin for growth, the basal medium was fortified with a constant amount of biotin in addition to the varying increments of niacin under test. For this experiment, it was concluded that moderate stimulation of the cultures became apparent at

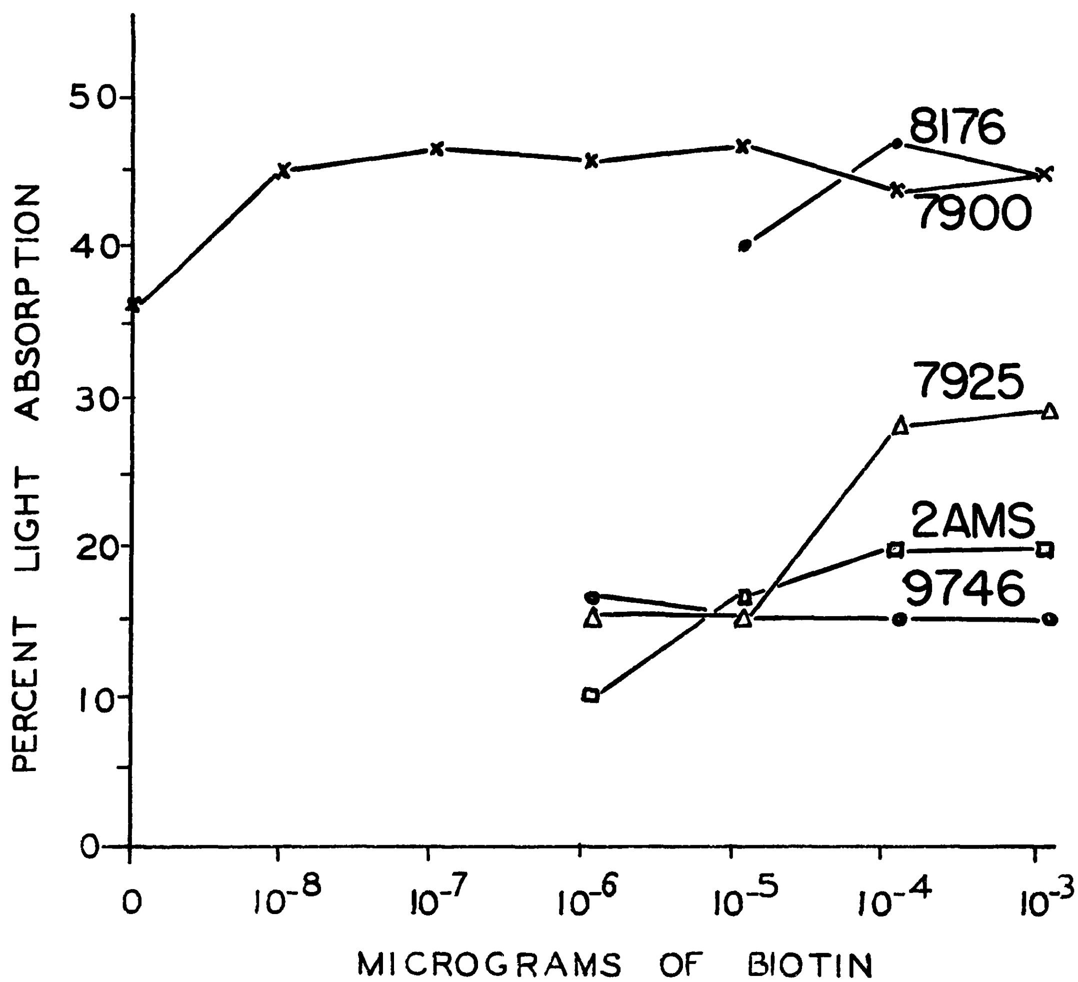


Figure 2. Response of five strains of *Neisseria* to graded amounts of biotin in a casein hydrolysate medium.

Table 12

Growth Response of Five Strains of _Neisseria_ to Graded Amounts of Niacin in the Presence of Biotin in a Casein Hydrolysate Medium

Media	Per cent Light Absorption Culture Number				
	J	K	X	OP6	E
Basal, no niacin	0	0	0	0	0
Basal + 10^{-4}ug niacin/ml	5	5	10	2	2
Basal + 10^{-3}ug niacin/ml	6	8	9	4	10
Basal + 10^{-2}ug niacin/ml	11	12	40	10	15
Basal + 10^{-1}ug niacin/ml	20	22	38	20	15

niacin concentrations of 10^{-2} to 10^{-3} ug niacin per ml of basal medium in the presence of biotin. The unclassified strain, E, which had a minimal vitamin requirement of niacin only , demonstrated maximum growth with a niacin concentration of 10^{-2} micrograms of niacin. Curves drawn with these data are shown in figure 3.

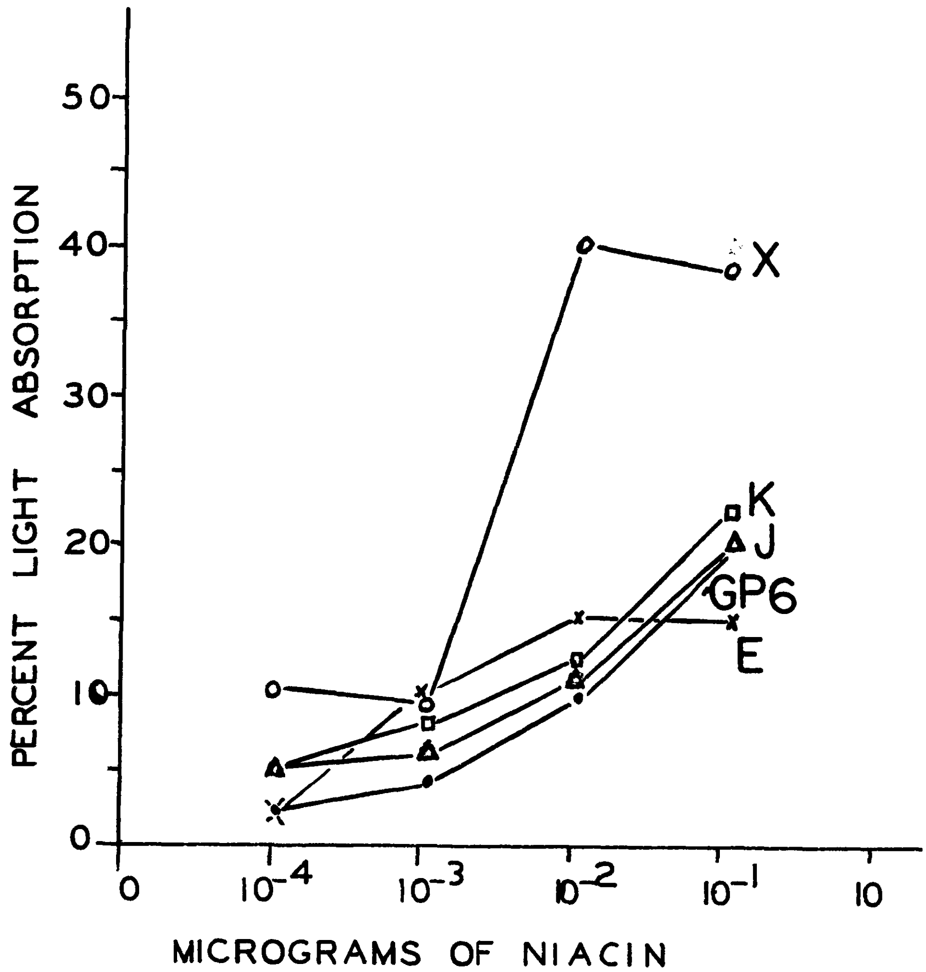


Figure 3. Response of five strains of *Neisseria* to graded amounts of niacin in the presence of biotin in a casein hydrolysate medium.

CHAPTER IV

AMINO ACID REQUIREMENTS OF THE *NEISSERIA*

The amino acid requirements of the *Neisseria* of the normal nasopharynx have been unreported except for the reference of Ordal & Busch (1946). Reporting on *N. sicca*, these authors noted:

> "..... the hydrolysed casein was successfully replaced with a mixture of 19 amino acids (the amino acids of casein hydrolysate with the exception of hydroxyproline). Several of the strains were then cultured on a series of media, each of the media lacking one of the 19 amino acids. The omission of any one amino acid did not materially affect the growth of these organisms."

Investigation on the closely related organisms *N. gonorrhoeae* and *N. meningitidis* have been more numerous and detailed. Gould, Kane and Mueller (1943) reported slight and erratic growth of *N. gonorrhoeae* strains when grown on a mixture of three amino acids, improved growth when a mixture of amino acids present in casein (excluding cystine and tryptophane) were added to the original mixture and good growth when an acid-hydrolysate of casein was added to the original mixture. Weltch, Stokinger and Carpenter (1944) recorded the growth of *N. gonorrhoeae* in a mixture of eight amino acids plus indole-3-acetic acid. Growth of additional strains could be induced by the addition of glutamine and choline to the basal medium. Frantz (1941) reported growth of *N. meningitidis* in a mixture of eight amino acids. By the addition of ammonium chloride to the medium, all but two amino acids could be deleted and the final medium contained ammonium chloride, glutamic acid, salts and glucose. Grossowicz (1945) criticised the medium of Frantz as inadequate and noted the growth of meningococci only under strictly controlled conditions. Grossowicz, in turn, recommended a more adequate medium which contained sodium glutamate as the sole source of nitrogen.

Other investigators have recommended media for the growth of *Neisseria*, among whom were Lankford, Scott, Cox and Cooke (1943), Gould (1944) and Landy and Gerstung (1945) but the amino acid mixtures suggested by these investigators were enriched by the addition of casein hydrolysate and the media were considered as partially defined or "semi-synthetic."

To determine whether the *Neisseria* could grow in a synthetic medium, the casein hydrolysate used in the experiments described in Chapter III was omitted and amino acids were substituted.

Inocula for these experiments were prepared in the manner employed in the investigations on vitamin requirements.

Media were prepared by solution of the amino acids under test in distilled water with gentle heat, addition of glucose, salt solutions A and B, the mixture made up to volume, acidity adjusted and the medium sterilised by filtration through Pyrex glass filters. Calculated quantities of biotin and niacin were added aseptically and the complete medium dispensed aseptically in aluminum-capped test tubes in 5 ml amounts.

Preliminary experiments indicated that neither ammonium chloride nor ammonium nitrate were adequate nitrogen sources for the cultivation of *Neisseria*. Initial experiments also indicated that casein hydrolysate, in concentrations from 0.2 per cent to 0.5 per cent fulfilled the nitrogen requirements of the *Neisseria* under study.

To determine whether *Neisseria* would grow in a simple synthetic medium, the casein hydrolysate used previously was replaced by the amino acids recommended by Frantz (1941) for the cultivation of the meningococci. The composition of the medium is given in table 13.

Table 13

Composition of a Chemically Defined Medium for Cultivation of Neisseria (after Frantz)

Glucose, c.p....	0.2 g	Niacin...............	100.0 micrograms
Salt solution A.	2.0 ml	d-Glutamic acid......	0.13 g
Salt solution B.	2.0 ml	l-Cystine	0.0022 g
Biotin..........	0.1 microgram	Distilled water to...	100.0 ml

pH 7.35

Among the thirteen organisms tested for growth in this medium, three strains - 1485, 7925 and 2AMS - grew poorly on the first transfer and no strains grew through a second transfer in the above medium after four days' incubation.

The chemically defined medium of Grossowicz (1945) reproduced in table 14, with added biotin and niacin, was tested for ability to support growth of six Neisseria strains.

Table 14

Composition of a Chemically Defined Medium for Cultivation of Neisseria (after Grossowicz)

Sodium chloride..........	5.0 g	Sodium thiosulfate.......	2.0 g
Disodium phosphate:12 H_2O	2.5 g	Magnesium sulfate: 7 H_2O	0.3 g
Monopotassium phosphate..	0.35 g	Calcium chloride.4 H_2O...	0.1 g
Ammonium chloride........	0.3 g	Thiamine hydrochloride...	0.001 g
Ferrous sulfate..........	0.001 g	Biotin...................	1.0 microgram
Manganous sulfate........	0.001 g	Niacin...................	1.0 mgm
Sodium glutamate.........	1.0 g	Glucose, c.p.............	2.0 g

Water to 1000.0 ml

Only one strain, N. catarrhalis (7900), grew through four serial transfers in this medium. The growth of this strain, though consistent, was poor in comparison to the growth of the organism in a casein hydrolysate medium.

To determine if the *Neisseria* strains of this study were capable of consistent growth in a synthetic medium, a complex chemically-defined medium of the composition reproduced in table 15 was made. In addition to biotin and niacin, the medium also contained thiamin as Grossowicz (1945) had noted increased metabolism of *N. intracellularis* in the presence of this vitamin. Supplementary nitrogenous compounds were added to the amino acid mixture to stimulate or support the growth of the *Neisseria* strains. Of the supplementary nitrogen compounds added, yeast nucleic acid alone was defined but not chemically synthesised.

To note the effect of elimination of the amino acids and the supplementary nitrogenous compounds, media of simpler composition were made by the elimination of two or more nitrogen sources from the complete medium. The composition of the simpler synthetic media and the growth responses of *Neisseria* to strains in the complex synthetic medium of table 15 and in the simpler media are presented in table 16.

Table 15

Composition of a Completely Synthetic Medium for the Growth of *Neisseria*

Biotin	1 microgram	1+ Lysine	60 mgm
Niacin	100 microgram	dl Valine	80 mgm
Thiamin	100 microgram	dl alpha Alanine	18 mgm
Glucose, c.p.	0.2 g	Glycine	4 mgm
Salt solution A	2.0 ml	dl Leucine	48 mgm
Salt solution B	2.0 ml	dl Isoleucine	48 mgm
1+Glutamic acid	200.0 mgm	dl Serine	60 mgm
1+Glutamine	2.0 mgm	dl Threonine	40 mgm
Glutathione	2.0 mgm	1 Hydroxyproline	2 mgm
dl Aspartic acid	40.0 mgm	dl Methionine	36 mgm
Asparagine	40.0 mgm	dl beta Phenylalanine	40 mgm
dl Tryptophane	22.0 mgm	1- Tyrosine	66 mgm
1+ Histidine	24.0 mgm	Yeast nucleic acid	15 mgm
1- Proline	80.0 mgm	Para-aminobenzoic acid	5 mgm
1 + Cystine	3.0 mgm	Adenylic acid	10 mgm
1 + Arginine.HCl	56.0 mgm	Choline	3 mgm

Distilled water to 1000.0 ml

Table 16

Growth Response of Five Strains of Neisseria in a Completely Synthetic Medium and in the Synthetic Medium with Successive Deletion of Nitrogen Sources

Media Composition	Per cent Light Absorption ** Culture Number				
	1485	2485	F	9746	7900
1. Complete medium	21	21	24	0	66
2. As above less para-aminobenzoic acid, adenylic acid and choline	16	14	23	0	68
3. As above less nucleic acid	18	14	19	0	61
4. As above less tyrosine and beta alanine	11	7	23	0	72
5. As above less isoleucine and hydroxyproline	22	7	18	0	68
6. As above less valine and proline	22	10	26	0	65
7. As above less serine and threonine	12	6	10	0	-*
8. As above less leucine and methionine	10	15	10	0	-
9. As above less histidine	14	14	5	0	-
10. As above less aspartic acid and asparagine	17	13	15	0	-
11. As above less arginine	13	10	20	0	-
12. As above less cysteine and lysine	15	4	10	0	-

* not determined

** fourth serial transfer; 4 days' incubation between transfers; reading with Fisher electrophotometer AC model, 425 B blue filter.

The results in table 16 indicated that all of the Neisseria strains (except N. flavescens 9746) in this experiment grew well in the complete, complex medium. Simplification of the complete medium by elimination of nitrogenous substances resulted in diminished growth although the lesser growth could not be attributed to the elimination of any specific compound.

Similar experiments on the elimination of amino acids from complex synthetic medium, with varied amino acid composition and content, indicated that a few Neisseria strains were capable of growth in a medium with few nitrogenous sources. Among all the classified strains of this study, the majority were unable to grow in a medium containing all amino acids, either in proportion found in casein hydrolysate or in the proportions indicated in table 15.

N. flava (55), N. perflava (1927) N. catarrhalis (8176) and N. sicca (X) were grown in a synthetic medium with these nitrogenous sources: 1+ glutamic acid, 100 mg per cent; glutamine, 15 mg per cent; 1+arginine, 100 mg per cent; dl aspartic acid, 75 mg per cent; dl valine, 100 mg per cent; 1+ lysine, 100 mg per cent; asparagine, 100 mg per cent; and glutathione, 1.5 mg per cent. After four serial transfers with four day incubation periods, N. flava (55) grew to a 16 per cent light absorption; N. perflava (1927) and N. catarrhalis (8176) grew to a 15 per cent light absorption. These growth responses, although slight, were one-half the density of growth obtained with casein hydrolysate as the nitrogen source and demonstrated growth of Neisseria species in a chemically defined medium, although the amount of growth was far inferior to growth obtained on the best natural medium.

Growth of N. flava (55) could be enhanced to a 25 per cent light absorption by cultivation in a synthetic medium containing all amino acids found in casein hydrolysate. The concentration of the individual amino acids must be increased threefold over the reported concentrations of these acids in casein hydrolysate.

The synthetic medium recommended by Welton, Stokinger and Carpenter (1944) for the growth of N. gonorrhoeae was among a series of 45 amino acid mixtures incorporated into a chemically defined media, none of which supported growth of the thirteen Neisseria test species through four serial transfers. Carry-over of nutrilites in the inoculum often supported the growth of test species through one transfer but rarely two transfers.

Under the conditions of the tests noted above, very few strains of *Neisseria* were capable of growth in a completely synthetic medium. The addition of purine and pyrimidine bases and energy sources including lactate, pyruvate and acetate to amino acid mixtures as reported in casein hydrolysate resulted in no response of the test organisms and it is the opinion of the investigator that, with few exceptions, the requirements of the non-pathogenic *Neisseria* are more detailed than of the pathogenic *Neisseria*.

CHAPTER V

NUTRITIONAL SUBSTITUTES FOR BIOTIN IN GROWTH OF *NEISSERIA*

Recent investigations have indicated that biotin is utilized in the nitrogen metabolism of microorganisms. Allison, Hoover and Burke (1933) reported an absence of respiration by *Rhizobium* in the presence of coenzyme R unless available ammonia was present; aspartic acid, in the presence of available ammonia, increased respiration by *Rhizobium*. Burk, Winzler and du Vigneaud (1941) confirmed these observations and noted, in addition, a greater increase in respiration rate than growth rate. Later, Winzler, Burk and du Vigneaud (1944) called this increase in respiration and growth rate, upon the addition of biotin to biotin-deficient cells, the "biotin effect." The biotin effect was examined more intensely by other investigators and specific functions for biotin were reported.

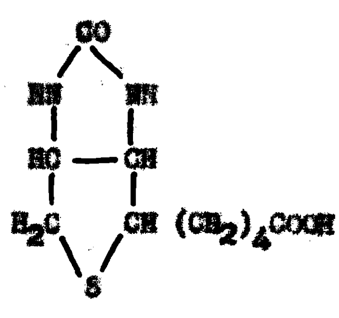
Koser, Wright and Dorfman (1942) noted that aspartic acid stimulated the growth of *Torula cremoris* in the absence of biotin and demonstrated that biotin was concerned in the synthesis of aspartic acid. Stokes, Larson and Gunness (1947) also reported that biotin is needed in the synthesis of aspartic acid by *Streptococcus fecalis*, *Lactobacillus arabinosus* and *Lactobacillus casei*.

Another function of biotin was suggested by Lichstein and Umbreit (1947a). Biotin was required for the decarboxylation of aspartic, malic and oxalacetic acids, yielding pyruvic acid and carbon dioxide as the end products. Lardy, Potter and Elvehjem (1947) provided supporting evidence for this theory when they demonstrated aspartic acid synthesis from pyruvate and bicarbonate in the presence of biotin. Oxalacetate, a condensation product between pyruvic acid and carbon dioxide, promoted growth of *L. arabinosus* on a medium deficient in biotin and aspartic acid.

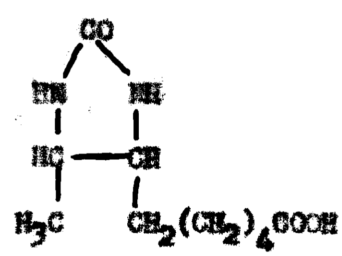
Ochoa, Mehler and Kornberg (1947) suggested that biotin was concerned in the synthesis of dicarboxylic acids by carbon dioxide fixation; this possible function was suggested also by Williams, Broquist and Snell (1947). The synthesised acid was thought to be oleic acid.

Lichstein and Umbreit (1947b) reported that aspartic acid, serine and threonine deaminases were activated by biotin.

Of considerable interest to the course of the present investigation were statements in literature indicating the possible replacement of biotin by other compounds. Stokes and Gunness (1947), in a report of compounds related to biotin, noted no "biotin-activity" of the compound desthiobiotin in experiments performed with *Rhizobium trifolii*, *L. casei* and *L. arabinosus*. *S. cerevisiae*, and other yeast cells grew well in a medium containing desthiobiotin but lacking biotin. These investigators reported the conversion of desthiobiotin to biotin by the yeast cells and the subsequent utilisation of the synthesised biotin. The formulas of these compounds appear below.



Biotin



Desthiobiotin

A. Biotin Replacement by Desthiobiotin

To determine the response of Neisseria to desthiobiotin, three basal media were prepared, as detailed in Chapter III, with casein hydrolysate as the nitrogen source. To the first basal medium, biotin was added. The second and third lots of basal medium received desthiobiotin. One hour after the tubes were inoculated, 0.05 micrograms of biotin was added to the third tube of each serial transfer. The results of this experiment, after the fourth serial transfer, are recorded in table 17.

Table 17

Growth Response of Four Strains of Neisseria to Desthiobiotin Replacement of Biotin

Media	Per cent Light Absorption			
	Culture Number			
	1485	7900	2ANB	X
Basal + 0.05 ug biotin/ml	16	70	18	65
Basal + desthiobiotin 0.05 ug/ml	21	68	15	72
Basal + desthiobiotin 0.50 ug/ml Delayed biotin addition	22	70	20	70

The results of the experiment disclosed that desthiobiotin could replace biotin as a growth factor for Neisseria. No inhibition of growth was demonstrable by the addition of desthiobiotin in either the 0.05 or the 0.50 microgram concentrations. In the case of the tubes receiving biotin in addition to desthiobiotin, the activity of the cultures was not repressed, either by the increased concentration of the growth factors or by suppression of biotin activity by the desthiobiotin. Thus, the Neisseria as well as yeast can utilize desthiobiotin in place of biotin.

B. Non-replacement of Biotin by Oleic Acid

Williams and Fieger (1946) had reported oleic acid as a growth stimulant for *L. casei*; oleic acid, emulsified in ethanol, supported growth better than biotin and no synthesis of biotin from oleic acid could be demonstrated. Williams, Broquist and Snell (1947) reported better growth of lactic acid bacteria in Tween 80 (sorbitan esterified with oleic acid) than in oleic acid because of the lessened toxicity of the former. To assess the value of these compounds as possible biotin-substitutes for the *Neisseria*, a series of basal media containing these compounds in the recommended concentrations were prepared and inoculated. The concentrations of the compounds and the results of the experiment are given in table 18.

Table 18

Response of Four Strains of *Neisseria* to Oleic Acid and Tween 80 as a Replacement for Biotin, in Casein Hydrolysate Medium

Media	Per cent Light Absorption			
	Culture Number			
	55	1485	2AMS	X
Basal + oleic acid, 0.1 ug/ml	0	0	0	0
Basal + oleic acid, 1 ug/ml	0	0	0	8
Basal + Tween 80, 1 mg/ml + oleic acid, 5 ug/ml	0	0	0	0
Basal + Tween 80, 0.1 mg/ml	0	0	0	8
Basal + Tween 80, 0.5 mg/ml	0	0	0	9

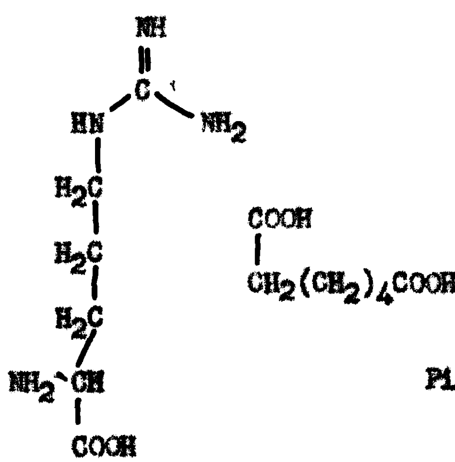
From the adverse results of this experiment, it was concluded that neither oleic acid nor Tween 80 could replace biotin. The growth of *N. sicca* (X) indicated the non-replacement value of oleic acid and Tween 80 for biotin but did demonstrate the non-toxidity of these compounds for the *Neisseria*.

G. Non-replacement of Biotin by Pimelic Acid and Urea

At this point, it was desirable to determine if pimelic acid could replace biotin in the nutrition of the *Neisseria*. Because of differences in numbers of carbon atoms and configuration, additional compounds were added to the pimelic acid medium in order to approximate the molecular weight and structural formula of desthiobiotin. As diagrammed below,

Desthiobiotin — 2-carbon chain — pimelic acid

pimelic acid, urea and a two carbon compound might possibly form a substitute for desthiobiotin. The two carbon compounds tested in this experiment were acetic acid, ethanolamine ($NH_2CH_2CH_2OH$) and ethylamine ($CH_3CH_2NH_2$). In addition, a mixture of arginine and pimelic acid were tested, the spatial configuration of arginine supplementing the configuration of pimelic acid.



Arginine — Pimelic acid

Basal media containing mixtures of these compounds were inoculated and carried through four serial transfers. The concentrations of the compounds employed and the results of the experiment are recorded in table 19.

Table 19

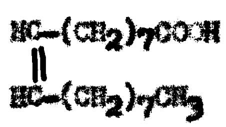
Growth Response of Four Strains of Neisseria to Pimelic Acid, Arginine, Urea, Acetic Acid, Ethylamine and Ethanolamine in Combinations as a Replacement for Biotin in Casein Hydrolysate Medium.

Media	Per cent Light Absorption			
	Culture Number			
	55	1485	2AM5	X
1. Basal medium + pimelic acid, 0.1 ug/ml	0	0	0	0
2. Basal medium + pimelic acid, 0.1 ug/ml + arginine, 0.5 ug/ml	0	0	0	3
3. Basal medium + pimelic acid, 0.1 ug/ml + urea, 1 ug/ml + acetic acid, 1 ug/ml	0	0	0	3
4. Basal medium + pimelic acid, 0.1 ug/ml + urea, 1 ug/ml + ethylamine, 2 ug/ml	0	0	0	0
5. Basal medium + pimelic acid, 0.1 ug/ml + urea, 1 ug/ml + ethanolamine, 2 ug/ml	0	0	0	0

The negative results showed conclusively that biotin or desthiobiotin could not be replaced either by pimelic acid or pimelic acid in conjunction with the compounds under test.

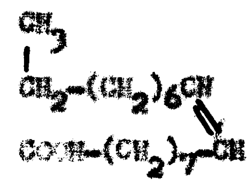
D. Biotin Substitution by Oleic Acid Plus Urea

The possibility of replacement of desthiobiotin by a mixture of oleic acid and urea was considered feasible for the following reason. Oleic acid, as diagrammed below, is a cis-acid and the proximity of the methyl group to the carboxyl group renders the methyl group slightly acidic. As such, the acidic methyl group may react with a basic amide group of urea, forming the greater component of desthiobiotin, in the following fashion.



Oleic acid

urea



Oleic acid

To test this possibility, a series of basal media containing varied amounts of oleic acid and urea was prepared and inoculated. The results of four serial transfers with four day incubation periods are given in table 20.

Table 20

Growth Response of Four Strains of *Neisseria* to Oleic Acid and Urea, in Varying Amounts, as a Substitute for Biotin in Basal Medium

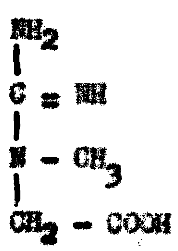
Media	Per cent Light Absorption Culture Number			
	55	1485	2AMB	157
Basal + oleic acid, 10 ug/ml	0	0	0	0
Basal + urea, 2 ug/ml	0	0	0	0
Basal + oleic acid, 10 ug/ml + urea, 2 ug/ml	0	0	3*	0
Basal + oleic acid, 15 ug/ml + urea, 2 ug/ml	0	0	4*	0
Basal + oleic acid, 15 ug/ml + urea, 4 ug/ml	0	0	5*	0

* Clumps of growth

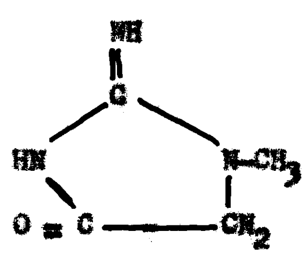
The results of table 20 did not appear significant for the percentage of light absorption indicated only feeble growth. Visual examination of the tubes, however, revealed the bacterial growth, not as uniform turbidity, but as small spherules of compact growth, about three mm in diameter. The mass of growth which appeared in the tubes would, by visual inspection, be considered a three plus growth for *Neisseria* and the oleic acid-urea mixture was considered a substitute for biotin in the nutrition of *N. perflava* (2AMS). The oleic acid - urea mixture was unable to support growth of strains *N. flava* (55), *N. sicca* (1485) or *N. flavescens* (157).

E. Biotin Substitution by Oleic Acid and Oleic Acid Plus Creatine.

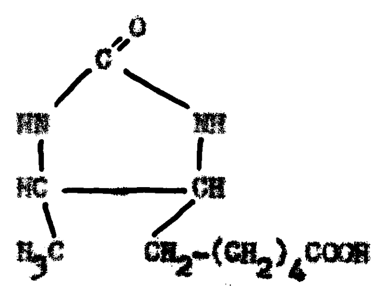
During the survey of simple mixtures of compounds which would function as a substitute for biotin in the nutrition of the *Neisseria*, the similarity of the structural formulae of creatine and desthiobiotin, and the formation of creatine anhydride, creatinine, by the elimination of a molecule of water, was noted.



Creatine



Creatinine



Desthiobiotin

To determine the possible nutritional value of a mixture of creatine and oleic acid for *Neisseria*, a series of basal media containing creatine, and oleic acid, individually, and in mixture, was prepared and inoculated. These concentrations of the compounds and results of the fourth serial transfer with four day incubation periods are given in table 21.

Table 21

Growth Response of Four Strains of Neisseria to Creatine and Oleic Acid, Individually and in Mixture, as a Substitute for Biotin in Basal Medium.

Media	Per cent Light Absorption Culture Number			
	55	1485	155	157
Basal + oleic acid, 10 ug/ml	0	0	20	0
Basal + creatine, 2 ug/ml	0	0	0	0
Basal + oleic acid, 10 ug/ml + creatine, 2 ug/ml	0	0	25	10

In contrast to the granular growth of N. perflava (2AMS) reported in table 20, the N. flavescens strains (155 and 157) grew with uniform turbidity in their respective media. N. flavescens (155) grew well in an oleic acid medium whereas creatine in addition to oleic acid was essential for the growth of N. flavescens (157). The essentiality of the creatine in addition to oleic acid in the nutrition of N. flavescens (157) appeared peculiar and unexpected for these two strains were identical morphologically, biochemically and, according to Dr. Sara Branham, serologically.

An observation on the physiological state of the cultures should be noted here. The N. flavescens strains (155 and 157) when used in the above experiment were growing well—almost luxuriantly, upon trypticase soy slants prior to preparation as inoculum. In the past, variation of growth response of N. flavescens in test media was noted to be dependent, in some degree, upon the amount of growth present upon the natural medium prior to inoculum preparation.

Under the conditions of the experiment noted above, oleic acid was considered to be a substitute for biotin in the nutrition of N. flavescens (155) and oleic acid plus creatine, a substitute for biotin in the nutrition of N. flavescens (157).

SUMMARY AND CONCLUSIONS

A group of *Neisseria* cultures isolated from human and animal sources were classified in accordance with the scheme of classification outlined in *Bergey's Manual of Determinative Bacteriology*. Identification of the organisms upon morphological and physiological bases permitted separation of strains of human origin into established species although two strains did not conform precisely with the designated biochemical reactions. Strains from animal sources differed from type species of *Neisseria* in the type of pigmentation primarily and were relegated to an "unclassified" division - a division encompassing all strains which exhibited a peculiar yellow pigment, were non-fermentative and serologically distinct from *Neisseria flavescens*.

Pigmentation as a primary key to classification of *Neisseria* was difficult to assess - the interpretation of the reaction dependent upon the investigator and the conduct of the test. Chromogenesis of organisms upon Loeffler's serum slants resulted in caramel colored cells in contrast to grey, yellow or pink colored cellular mass observed in washed suspensions of *Neisseria*. A complete absence of green pigmentation was noted. Absorption of pigments upon inert material revealed the presence of yellow and pink pigments in separate, isolated regions. Isolation of the yellow pigment from a mass of cells resulted in no alteration of pigment intensity, no observable physical change in pigment hue.

The colonial morphology of the *Neisseria* was varied - the majority of the strains produced a smooth colony irrespective of taxonomical position. The colonial morphology was considered a minor characteristic and a variation in the physical texture of colonies of *N. perflava* (2AMS) from smooth to granular and reversion to smooth was noted. The degree of difference displayed among colony morphology of various strains was much less than differences in chromogenesis.

Biochemically, among twenty carbohydrate and related compounds tested, a maximum of seven compounds was fermented by a strain of *Neisseria sicca*. With this exception, the other strains of *Neisseria* showed a limited biochemical activity and fermented a maximum of four of the carbohydrate and related compounds tested. Some strains of *Neisseria* which fermented mannitol in the past failed to ferment this carbohydrate during these experiments. The fermentation of maltose with no parallel fermentation of glucose was noted and this abnormality considered evidence for direct maltose fermentation without previous hydrolysis of the disaccharide to glucose. Dehydrogenase activity of resting cells indicated an active oxidative action of certain *Neisseria* strains upon maltose and supported the theory of direct fermentation of disaccharides.

The nutritional requirements of *Neisseria* of the normal nasopharynx were complex. Among the vitamins tried, biotin was most commonly required for growth. Members of all species required this vitamin for growth - the strains of *N. perflava*, *N. flavescens* and *N. flava* presented a homogenous requirement for this vitamin. *N. catarrhalis* included strains which required no vitamins for growth, required biotin for growth or grew in the absence of but were stimulated by biotin. *N. sicca* included biotin dependent strains and three strains which required niacin in addition. The unclassified *Neisseria* of animal origin displayed a range of vitamin requirements. In few instances, no vitamins were required for growth, in others, five vitamins were needed for adequate growth.

Response to biotin was noted at a biotin concentration of 10^{-6} micrograms per ml; niacin response detected at a 10^{-4} microgram level with maximum response obtained at a 10^{-1} to 10^{-2} microgram level. A non-linear response to increments of these vitamins was noted; to biotin over a range of 10^{-8} to 10^{-3} ug per ml and to niacin, over a range of 10^{-4} to 10^{-1} ug per ml.

Among 34 classified strains of Neisseria tested, continued growth of only seven strains was obtained in a chemically defined medium. Five of the seven strains demonstrated fair and continued growth in a simple, chemically defined medium but better growth in a synthetic medium which contained the majority of known amino acids. Additions of purine and pyrimidine bases, energy sources (lactate, acetate and pyruvate), and other growth-promoting compounds were not able to stimulate continued growth of the strains which did not grow in the chemically defined medium.

A biotin inhibitor, desthiobiotin, was shown to fulfill the biotin requirements of a series of test strains. No inhibition of biotin action in the presence of desthiobiotin was noted. Successful replacement of biotin by an oleic acid - urea mixture was demonstrated by growth of N. perflava (2AMS) in a basal medium devoid of biotin but containing oleic acid and urea. This replacement was not permitted by pimelic acid, oleic acid or Tween 80. Replacement of biotin by oleic acid was demonstrated by growth of N. flavescens (155) in a basal medium containing oleic acid; replacement of biotin by oleic acid plus creatine, by growth of N. flavescens (157) in a basal medium containing oleic acid plus creatine.

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