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# A Chemically Defined Protein-free Liquid Medium for the Cultivation of Some Species of *Neisseria**

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*Chemically defined, protein-free, liquid culture media are needed for research with pathogenic bacteria and for the preparation of vaccines from them. Variations in growth or yield of organisms should be due only to differences in strain, and the medium itself should have little or no antigenic properties or toxicity. The medium should maintain the integrity of the bacterial species fully and it is particularly important that the complete antigenic complement of the organism should remain unaltered. The medium should be inexpensive and simple to prepare. Such a medium for the cultivation of some species of* Neisseria, *known to be fastidious, is described.*

*In this new medium all the criteria are satisfied, and its freedom from sensitizing proteins makes it of particular value for use in man. Results of safety tests of vaccines prepared from* Neisseria gonorrhoeae *and* N. meningitidis *grown in this medium were encouraging. It should also be useful in studies on the physiological characteristics of the individual species.*

The need of a chemically defined medium for research with pathogenic species of *Neisseria*, and for vaccine production, is obvious. Although such studies have been in progress for many years, success has been limited to date. Frantz (1942) developed a medium for the growth of meningococcus in liquid culture which was later modified by Watson & Scherp (1958) by the addition of an undefined casamino acid component. In the present paper we describe an entirely chemically defined medium which was designed to aid our studies with *Neisseria*. It has been designated *Neisseria* chemically defined medium (NCDM).

## MATERIAL AND METHODS

### *Method of preparation of NCDM*

*Solution 1.* Medium 199 (Morgan, Morton & Parker, 1950) without sodium bicarbonate.[2] An 11.0-g quantity is dissolved in 1000 ml of double-distilled water and autoclaved at 15 lbf/in² (1.05 kgf/cm²) for 15 minutes.

*Solution 2.* The following compounds are dissolved in 100 ml of double-distilled water and sterilized by membrane filtration (pore size 0.20 μ)[3]:

| | |
|---|---|
| Cocarboxylase (thiamine pyrophosphate) | 0.001 g |
| L-glutamine | 0.500 g |
| Dextrose | 20.00 g |

*Solution 3.* 0.10 g of ferric chloride is dissolved in 100 ml of double-distilled water and this solution is sterilized by membrane filtration as for solution 2.

For every 100 ml of solution 1, 2.0 ml of solution 2 and 2.0 ml of solution 3 are added. The pH is adjusted to 7.4 with 1.4% sodium bicarbonate just prior to inoculation.

### *Growth response of test organisms in various media*

Three media were used in this study; heart infusion broth (Difco), modified Frantz medium (Watson & Scherp, 1958) and NCDM. The following species of *Neisseria* were studied: *N. gonorrhoeae*, *N. meningitidis*, *N. catarrhalis*, and *N. flavescens*. A strain of *Mima polymorpha* var. *oxidans* was also studied.

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[2] Obtained in powder form from Grand Island Biological Company, Grand Island, N.Y., USA.

[3] Sartorius Corp, Göttingen, Federal Republic of Germany.

## PROCEDURES AND RESULTS

### *Growth response of test organisms in various media*

Suspensions of the cultures were made by washing the 18-hour growth from Columbia blood agar plates [1] (Ellner et al., 1966) with Hanks' balanced salt solution, and counts were made. Aliquots of 100 ml of experimental media in 250-ml Erlenmeyer flasks were then inoculated to contain a final population of $10^6$ organisms per ml. Counts were performed at 4-hour, 7-hour, and 24-hour intervals.

All population counts were performed on a Coulter counter (model B) fitted with a 30-$\mu$ aperture tube. The counts were performed using the following instrument settings: 1/AC—0.707, 1/AMP—1/4, preamplifier or matching switch, 32L, lower threshold—5, upper threshold—100. The diluent was particle-free Hanks' balanced salt solution. The background counts were negligible. Counts were made on culture dilutions of 1 : 200 and the values recorded represent the average of three instrument counts in each case. The cultures were incubated on a Gyrotory rotary shaker [2] set for 150 rev/min in an incubator set at 37°C.

### *Growth of* N. gonorrhoeae *in NCDM*

In our preliminary experiments we found that *N. gonorrhoeae* grew well in tissue culture medium 199 following incubation in an atmosphere of 10% carbon dioxide in air provided the medium contained 10% calf serum. The next logical step, then, was to determine whether the addition of Lankford's (Lankford & Snell, 1946) supplement together with an increased concentration of ferric ions could be substituted for the calf serum. This was found to be so and further experimentation soon established the optimal concentrations required for rapid growth.

An inoculum of 1.0 ml of seed culture ($10^8$ cells/ml) was added to the culture medium to give a final concentration of $10^6$ cells/ml. A mixture of sterile 10% carbon dioxide in air was bubbled into the flask for 30 seconds. It was then sealed with a sterile rubber stopper and incubated at 37°C on the rotary shaker for 18 to 24 hours after which there was no further growth. During this time the pH of the medium dropped to 5.0.

By subculturing from NCDM to conventional media, such as GC agar base (Difco) and Columbia blood agar, we were able to demonstrate that the gonococcus had maintained its typical morphological, biochemical and microbiological characteristics. Morphologically, the organisms were typical Gram-negative diplococci, the colonies were oxidase-positive, and glucose was the only carbohydrate fermented with the production of acid. When phase I colonies (Kellogg et al., 1963) of the gonococcus were inoculated into NCDM, subsequent subculture onto GC agar base yielded only phase I colonies.

### *Growth of other* Neisseria

The cell population counts obtained with the organisms tested over a 24-hour period are shown in Table 1. Better growth of *N. gonorrhoeae* was obtained in NCDM than in the other two media studied. A higher yield of *N. meningitidis* was also observed in NCDM than in the Frantz medium, which had originally been designed for studying the meningococcus. Fig. 1 and 2 are graphical representations of the comparative yields of *N. gonorrhoeae* and *N. meningitidis*, respectively. The meningococcus grew better in the heart infusion broth.

TABLE 1

CELL NUMBERS [a] AFTER GROWTH FOR 24 HOURS IN VARIOUS MEDIA

| Organism | NCDM | HIB [b] | Frantz |
|---|---|---|---|
| *Neisseria gonorrhoeae* | 3.50 | NG [c] | NG [c] |
| *N. meningitidis* | 1.81 | 6.68 | 1.51 |
| *N. catarrhalis* | 2.55 | 2.61 | 2.27 |
| *N. flavescens* | 3.41 | 2.01 | 3.63 |
| *Mima polymorpha* | 2.02 | 4.25 | 5.49 |

[a] Values given in the table represent cell numbers $\times 10^{-8}$. The populations listed are averages of three counts by Coulter counter (Model B).

[b] Heart infusion broth.

[c] NG = no growth.

### *Production of experimental lots of vaccines*

Vaccines in 5-litre quantities were prepared from strains of *N. gonorrhoeae* and *N. meningitidis*. Three lots were made for each species using NCDM as the culture medium. The flasks were incubated on the shaker at 37°C for 24 hours and population counts were made. In all cases, the count was in the range 2.23–3.15 $\times$ $10^8$ cells/ml. The bacteria were killed

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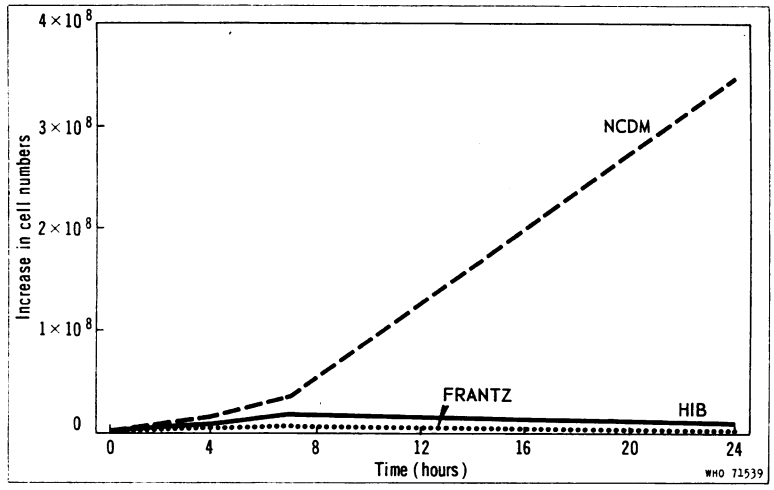
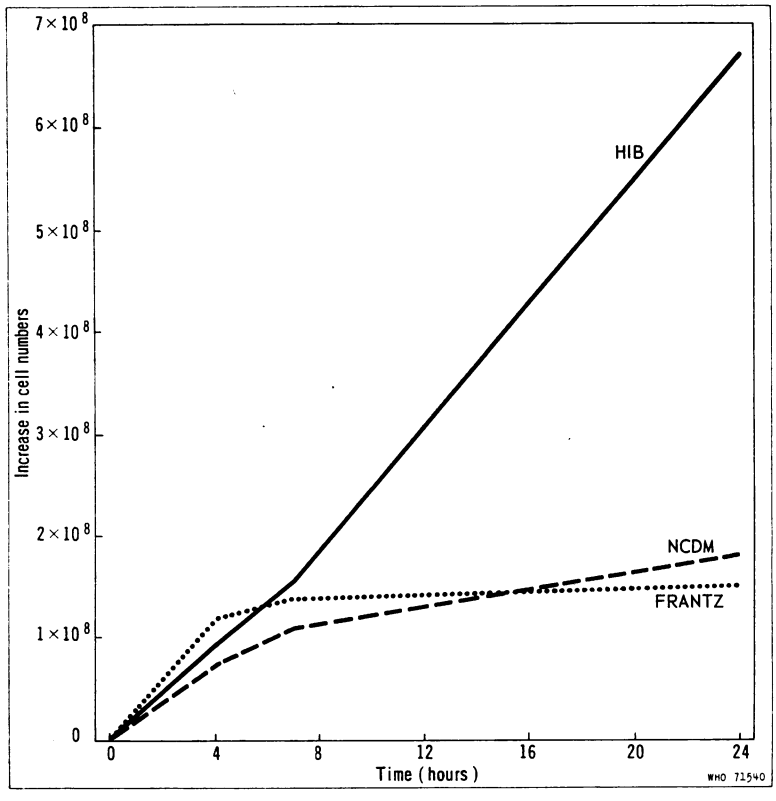
[2] New Brunswick Scientific Company, New Brunswick, N.J., USA.

FIG. 1
GROWTH OF *N. GONORRHOEAE* IN VARIOUS MEDIA [a]




[a] *Neisseria* chemically defined medium, modified Frantz medium and heart infusion broth.

FIG. 2
GROWTH OF *N. MENINGITIDIS* IN VARIOUS MEDIA [a]




[a] *Neisseria* chemically defined medium, modified Frantz medium and heart infusion broth.

TABLE 2
SAFETY TESTS ON EXPERIMENTAL NEISSERIAL VACCINES

| Vaccine | Lot No. | Final cell count per ml ($\times 10^{-8}$) | Pyrogen test | Toxicity [b] | |
|---|---|---|---|---|---|
| | | | | Mice | Guinea-pigs |
| *N. gonorrhoeae* | GC-L | 2.23 | 0.40 | 0/50 | 0/5 |
| | GC-HK | 2.60 | 0.50 | 0/50 | 0/5 |
| | GC-100 | 2.56 | 0.30 | 0/50 | 0/5 |
| *N. meningitidis* | MC-1 | 2.99 | 0.28 | 0/50 | 0/5 |
| | MC-1L | 3.00 | 0.60 | 0/50 | 0/5 |
| | MC-3 | 3.15 | 0.25 | 0/50 | 0/5 |
| NCDM (medium) | 7 | — | −0.02 | 0/50 | 0/5 |

[a] The values given are averages of three bacterial counts made on the Coulter counter.
[b] Results refer to number of deaths per number of animals tested.

by the addition of thiomersal (1 : 10 000). All vaccines were then subjected to a series of tests for sterility, toxicity in mice and guinea-pigs, and for pyrogens following procedures used routinely at the Laboratory of Hygiene, Ottawa.

In Table 2, the results of a typical series of safety tests are given. The pyrogen test values shown were the average temperature rises in three rabbits inoculated intravenously with 0.5 ml of the test material per kg of body-weight. For toxicity tests, 50 mice were inoculated intraperitoneally, each with 1.0 ml, and 5 guinea-pigs were inoculated with 5.0 ml, also intraperitoneally. In both instances the test animals were observed for 7 days following the injection. There were no deaths and none of the animals demonstrated distress or loss in weight.

## DISCUSSION

Certain criteria were established which the medium had to satisfy before it could be considered acceptable. They were as follows:

(1) It should be chemically defined so that variation in yield or growth characteristics could be due only to differences in the strain itself.

(2) It should be free of protein so that when used for vaccines the medium itself would have little or no antigenicity, thereby reducing to a minimum the possibility of hypersensitive reactions in the vaccinated.

(3) It should be non-toxic and non-pyrogenic.

(4) It should maintain the integrity of the bacterial species after growth—namely, its antigenic structure and morphological and biochemical characteristics. It is of particular importance in the production of a vaccine from a species, for which the immunizing antigen or antigens have not been isolated or established, that the complete antigenic complement be maintained unaltered.

(5) Finally, it should be simple to prepare and low in cost.

These criteria have been satisfied with NCDM. Its freedom from sensitizing proteins makes it of particular interest for use in man. The safety tests on the vaccine lots of *N. gonorrhoeae* and *N. meningitidis* gave encouraging results.

*N. gonorrhoeae* has been regarded as a fastidious species which has generally proved to be difficult to isolate and to grow in bulk quantities. Since it grew well in NCDM, we fully expected that other species, somewhat less fastidious, would also grow in this medium and this was shown to be so.

In our experience, the lowest count that would potentiate maximal growth in NCDM in 24 hours was $10^4$ organisms per ml for the gonococcus and $10^3$ organisms per ml for the meningococcus.

To the best of our knowledge, NCDM is the only entirely chemically defined protein-free medium described for the growth of *Neisseria* in which the various strains can be produced in quantity. NCDM could thus be used as a basis for studying the actual metabolic requirements of some of these organisms.

While all of the typical characteristics of the *Neisseria* are maintained intact in the medium, as described above, changes in morphological characteristics may be brought about by omitting or altering the concentrations of some of the ingredients. It was observed, for example, that when different

concentrations of ferric chloride were incorporated into NCDM, enlargement of the capsular layer of the gonococcus occurred as the concentration of ferric ions decreased. This was noted by indirect fluorescent antibody preparations. The significance of this is not clear, but the possibility of using studies of this nature for comparing virulent and avirulent strains may be of help in defining pathogenicity in terms of biochemical differences. Some authorities have, in fact, stressed the need and importance of investigations into the basic biochemistry of the neisserial pathogens. NCDM could readily serve as a base medium for such studies by virtue of its defined nature.

## ACKNOWLEDGEMENTS

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## RÉSUMÉ

On a besoin de milieux de culture liquides, chimiquement définis et exempts de protéines pour les recherches sur les espèces pathogènes de *Neisseria* et pour la production de vaccins à partir de ces espèces pathogènes. Les tentatives précédentes de mise au point d'un milieu liquide convenable n'ont obtenu que des succès partiels. Les auteurs décrivent un milieu, qu'ils appellent « milieu chimiquement défini pour les *Neisseria* » (MCDN), qui satisfait aux cinq critères d'acceptabilité: 1) être chimiquement défini de façon que les variations du rendement ou des caractères de la croissance ne soient dues qu'à des différences de la souche elle-même; 2) être exempt de protéines de manière à ne posséder pratiquement pas de pouvoir antigénique quand on l'utilise pour la fabrication de vaccin; 3) être atoxique et apyrogène; 4) conserver l'intégrité de la souche en culture, c'est-à-dire ses caractères antigéniques, morphologiques et biochimiques; et 5) être simple à préparer et peu coûteux.

Le MCDN est préparé à partir de trois solutions: 1) dissoudre 11 g de milieu 199 de Morgan, Morton & Parker (1950) sans bicarbonate de sodium dans 1000 ml d'eau bidistillée et mettre à l'autoclave à 1,05 kgf/cm² pendant 15 minutes; 2) dissoudre 0,001 g de cocarboxylase (pyrophosphate de thiamine), 0,5 g de L-glutamine, et 20 g de dextrose dans 100 ml d'eau bidistillée et stériliser en filtrant sur membrane; 3) dissoudre 0,1 g de chlorure ferrique dans 100 ml d'eau bidistillée et stériliser par filtration sur membrane. Dans chaque fraction de 100 ml de solution 1, ajouter 2 ml de solution 2 et 2 ml de solution 3. Ajuster le pH à 7,4 par addition de 1,4% de bicarbonate de sodium juste avant l'ensemencement.

On a comparé la croissance de quatre espèces de *Neisseria* et d'une souche de *Mima polymorpha* var. *oxidans* dans trois milieux: MCDN, milieu de Frantz et milieu à l'infusion de cœur. *N. gonorrhoeae*, qui est considéré comme une espèce exigeante, s'est mieux développé dans le MCDN que dans les deux autres milieux, alors que *N. meningitidis* a donné un meilleur rendement en MCDN qu'en milieu de Frantz, mais a eu une croissance meilleure dans le bouillon à l'infusion de cœur.

On a préparé des vaccins à partir de souches de *N. gonorrhoeae*, qui se sont révélées généralement difficiles à isoler et à cultiver en grande quantité; on prévoyait que d'autres espèces de *Neisseria* croîtraient aussi en MCDN et on l'a effectivement observé.

Le MCDN, en raison de sa nature définie, pourrait aussi servir de milieu pour l'étude des besoins métaboliques et de la biochimie fondamentale des espèces de *Neisseria*. On a déjà noté que l'on pouvait provoquer certains changements des caractères morphologiques en n'incorporant pas certains des ingrédients ou en changeant leurs concentrations.

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