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A New Solid Medium for the Isolation of *Neisseria gonorrhoeae*

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Biological products have been widely used in media intended for the primary isolation of Neisseria gonorrhoeae. *Most of these substances are expensive and, in some places, difficult to procure. Ascitic fluid, which has been extensively used, is becoming increasingly rare. On the other hand, attempts to replace these biological substances by chemical compounds have met with little success, and a satisfactory synthetic medium does not exist. The authors describe the laboratory preparation of a reproducible medium which does not contain any fresh biological substances. It was developed from a routine "chocolate medium" which contained ascitic fluid and heated horse blood. Experiments with sparsely growing fresh laboratory strains showed that the ascitic fluid could be replaced by a combination of yeast and liver autolysates and the horse blood by haemoglobin. This Haemoglobin-Yeast-Liver (HYL) medium proved to be well suited for the primary isolation of* N. gonorrhoeae *and preliminary experiments indicated that it might also be suitable for use as a reference medium in sensitivity determinations. Attempts to use yeast and liver autolysates to replace ascitic fluid in a fermentation medium which did not contain blood or haemoglobin were less successful. Germination on the modified medium was relatively poor and it seems that the addition of a protective agent, such as albumin or starch, will be necessary.*

INTRODUCTION

It is a general experience that *Neisseria gonorrhoeae* does not grow on simpler media such as plain broth-agar plates. This fastidiousness is ascribed partly to toxic substances present in the agar and partly to the need for special growth factors. Several attempts have been made to analyse the growth requirements of *N. gonorrhoeae*, and synthetic media have also been described (Welton & Scherp, 1944; Welton et al., 1944; Gould et al., 1944; Hill, 1948; Hill et al., 1948). These complex media do not support the growth of all strains of *N. gonorrhoeae* on primary isolation from pathological material. A "rich", non-toxic medium would be desirable; usually, this condition is achieved by adding, to a broth-agar base, heated blood (or haemoglobin) and fresh serum (or plasma) or other biological material, for example ascitic or hydrocele fluid. The relevant literature on the study of suitable nutritional media and on the special growth factors required by *N. gonorrhoeae* is very extensive, and excellent reviews have been published (Scherp, 1955; Juhlin, 1963). Therefore, this question will not be discussed in detail in the present paper.

Various supplements and substitutes have been described (Morton & Lebermann, 1944), some of which are chemically defined substances whereas others are prepared from biological sources, as for instance the Bacto-Supplements [3] (Lankford, 1950). However, in some countries the commercially available biological supplements are difficult to procure and apparently the chemical supplements are not always sufficient (Reyn, Bentzon & Møller, 1965).[4]

The first aim of the present work was to build up a "good" non-commercial and reproducible medium which did not contain any fresh biological factors and would support the growth of the vast majority of the gonococcal strains in circulation. Preferably, this medium should be suited both for primary

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[3] See footnote 1 on page 473.

[4] See the article on page 503 of this issue.

isolation and for the evaluation of fermentation of sugars—in the latter case without the addition of haemoglobin. A reference medium suitable for the determination of the sensitivity of *N. gonorrhoea* to antibiotics (especially penicillin) would also be desirable for interlaboratory or international comparisons; this is of interest because the inhibitory concentrations do depend on the medium employed (Reyn, Bentzon & Møller, 1965).[1]

MATERIAL AND METHODS

Gonococcal strains

The gonococci used in these studies comprised 142 fresh strains from the routine work of the Neisseria Department of the Statens Seruminstitut and one old control strain (50700/1955)—from experiments with antibiotics—which had been kept in a freeze-dried condition.

Media

Chocolate medium. This is prepared by the addition of horse blood and ascitic fluid to a broth-agar base consisting of 2.4% of Danish AKI agar in beef-heart broth with 1% of peptone ("Orthana" special), 0.3% of NaCl and 0.2% of $Na_2HPO_4.12H_2O$. A detailed description of the preparation of this medium has been given by Reyn (1965).[2] The finished medium was poured into aluminium or plastic Petri dishes 9 cm in diameter to form plates 4-5 mm thick. Inoculated plates were placed for 20 hours at 36°C in a moist atmosphere containing 8-10% of CO_2 and for a further 20-24 hours without CO_2.

HYL medium. This is a Haemoglobin-Yeast-Liver medium prepared by the addition of haemoglobin, yeast autolysate and liver autolysate to the broth-agar base described above. Full details of the preparation of these autolysates and the formula for HYL medium are given by Reyn (1965).[2]

HAP medium. This is a solid fermentation medium (Haemin-Ascites-Placenta medium) introduced by Juhlin in 1963. The preparation is described by Reyn (1965).[2]

Ascitic fluid. The ascitic fluid was withdrawn in a sterile manner, the patient being questioned beforehand to ensure that he had not been treated with antibiotics. The fluid used in the fermentation media and in experiments to determine the sensitivity to antibiotics was pretested and fluid from the same patient was used for long periods.

Evaluation of results in growth experiments

In the preliminary experiments, the bacterial growth was spread by means of a platinum loop on the various plates, and after incubation the amount of growth was compared without any measuring or counting. Later on, bacterial suspensions were prepared, and the germination of the bacteria was judged by plating known quantities of different dilutions followed by colony countings. The principle was to count on plates with as many non-confluent colonies as possible. In addition, the diameters of the colonies were measured, usually those of both the smallest and the largest ones. The size of the colonies depends upon the number of colonies on the plate and upon how thick it is. In the present experiments the number of colonies (per strain) did not vary appreciably and the thickness of the plates was also uniform. Depending upon the growth rates, the measurements were performed by the naked eye after incubation for either 20-24 hours or 40-48 hours.

Sensitivity determination

A plate dilution technique, which has recently been described in detail (Reyn et al., 1963), was used for the determination of the sensitivity of *N. gonorrhoeae* to crystalline penicillin G "Leo". The composition of the medium used was similar to that of the "chocolate" medium described above, but Japanese agar Kobe 1 was used in place of Danish AKI agar, the peptone was omitted, and the quantity of ascitic fluid was reduced to 11%.

RESULTS

Preliminary experiments

Before starting the experiments proper, preliminary experiments were performed in which the previous experience obtained by other workers was largely confirmed. It was found that beef-heart broth-agar base alone supported the growth very poorly, but that colony size and germination were greatly improved when bovine albumin or cow's milk was added to this base. Cohn's fraction IV_4 (contains α- and β-globulin, albumin and trace of γ-globulin) had some beneficial effect which was ascribed to the albumin. Potato starch (Danish Pharmacopoeia, 1948), ion-exchange resins (Amberlite IR-4B, DEAE-cellulose and CM-cellulose), methyl-cellulose, sodium alginate, activated carbon, and cholesterol combined with Tween 80 improved the growth somewhat, but

[1] See the article on page 503 of this issue.

[2] See the article on page 449 of this issue.

not as consistently as did albumin and milk. Saponin or carbowax M 20 (polyethylene glycol) combined with oleic acid, lecithin, α- and β-globulin, and technical albumin did not promote the growth.

Using Gould's synthetic medium (Gould et al., 1944) as a basic medium one strain was found to need haemin or haematin (X factor). The colonies were small on this medium and the germination was poor; however, both colony size and viability were improved by the addition of heated blood.

It was also confirmed that a heated haemoglobin solution could replace the heated horse blood, which was usually employed, and its effect was sometimes even better than that of whole blood. Bacto-Yeast Extract [1] did not promote the growth and some strains were even inhibited.

With a view to a possible automatization of the production of nutrient plates, different brands of agar were examined. A Spanish agar was found to combine a suitable degree of purity and low viscosity with a comparatively high gel strength. The Danish AKI agar was pure enough but the relation between viscosity and gel strength was not ideal for the purpose. The quality of Japanese agar Kobe 1 was intermediate between the Spanish and the Danish products. The toxicity of the different brands was checked by growing strains known to be "delicate" on nutrient plates prepared with the different agars and comparing the amounts of growth. A certain amount of moisture is important for the growth of *N. gonorrhoeae*, and the right degree of solidity of the medium is usually controlled by experience. However, a method of checking gel strength of the agar has been published by Fulthorpe (1951). This method was simplified by Møller (to be published) and used to determine the suitable final concentration of a Spanish agar, which was found to be about 0.65% in our plates.

Main experiments

1. *Media for primary isolation.* In the Difco manual,[1] Bacto-Supplement B[2] is described as "a sterile yeast concentrate", which is "processed to preserve both the thermolabile and the thermostable growth accessory factors of fresh yeast, including glutamine, coenzyme (v factor), cocarboxylase and other growth factors for the most exacting strains of *Neisseria gonorrhoeae* and *H. influenzae*. It also contains the haematin or x factor required by *H. influenzae*." Hence, it was thought that fresh brewer's yeast would be a suitable source for the preparation of a supplement.

An autolysate was produced from fresh brewer's yeast as described by Reyn (1965),[3] and in preliminary experiments several batches were tested in final concentrations varying from about 0.25% to 2%. Generally, the optimal effect was obtained with a 1% final concentration.

In the first experiment, 4 media were prepared containing beef-heart broth-agar "enriched" with either 1% of Bacto-Supplement C, 1% of yeast autolysate, 22% of ascitic fluid (routine medium), or water. All media contained 6.7% of heated horse blood. Eight gonococcal strains were used: the control strain 50700 and 7 fresh strains, 6 of which had shown comparatively poor growth. These were seeded thinly on routine medium plates which were incubated for 16-18 hours at 36 °C in a moist atmosphere containing 8-10% of CO_2. A suitable amount of the resulting growth was suspended in 0.9% saline to a density known to yield about 10^8 colonies per ml when plated immediately afterwards on the routine medium. The suspensions were diluted in saline (4×10^6 or 2×10^6) and 0.05 ml of each was plated on the various media. Only one suspension was made and plated at a time. The inoculated plates were incubated as described under *Material and methods*. The colonies were counted and their size estimated by naked eye.

On the average, the results of the colony countings were similar whatever the medium, but the colonies were larger on the plates containing ascitic fluid or Bacto-Supplement C[4] than on the other two media; the medium containing yeast autolysate showed slightly larger colonies than that without any enrichment at all. In several other experiments, yeast autolysate had a marked beneficial effect, but the effect varied from strain to strain and it was evident that yeast autolysate alone was not good enough. It was then decided to combine the yeast autolysate with a liver autolysate prepared from pork liver. Six gonococcal strains (50700 and 5 fresh

[1] See *Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures*, 9th ed., Detroit, Mich., Difco Laboratories, 1953, pp. 270, 273.

[2] Bacto-Supplement C, which was used in the present investigation, is the same as Bacto-Supplement B but without glucose; it is dispensed in a freeze-dried state.

[3] See the article on p. 449 of this issue.

[4] In the article by Reyn, Bentzon & Møller on page 503 of this issue, Lankford's chemical supplement (Lankford, 1950) is compared with Bacto-Supplement C, yeast and liver autolysates, and ascitic fluid.

COMPARISON OF THE GROWTH CHARACTERISTICS OF *N. GONORRHOEAE* ON FIVE DIFFERENT MEDIA

Strain [a]	Medium [b]										Average colony count	Dilution factor	Average density of suspension per ml
	1		2		3		4		5				
	A	B	A	B	A	B	A	B	A	B			
50700 [c]	285	1.75	265	1.5	278	2.5	232	2.25	255	2.25	263	4×10^6	1.1×10^9
93300 [c]	91	1.25	78	0.75	126	1.25	77	1.25	87	1.25	92	4×10^6	3.7×10^8
89972 [d]	52	1.0	(8) [e]	0.25	43	1.25	47	1.125	29	0.625	43	2×10^6	8.6×10^7
89973 [d]	68	0.75	85	0.25	(4) [e]	1.5	71	0.75	73	0.75	74	2×10^6	1.8×10^8
90866 [d]	125	0.75	114	0.125	119	1.625	132	0.75	129	0.75	124	2×10^6	2.5×10^8
91675 [d]	206	0 75	215	0 185	181	1.625	173	0.625	171	1.125	189	2×10^6	3.8×10^8
Average of mean colony diameters (mm)		1.0		0.5		1.6		1.1		1.1			

[a] Strain 50700 is a control strain; the remaining strains are fresh strains.

[b] The five media used consisted of routine medium modified in the following ways:

1. Made up with ascitic fluid (16 %) and horse blood (6.7 %).
2. Made up with water (16 %) and horse blood (6.7 %).
3. Containing about 1 % of yeast autolysate and 1 % of liver autolysate and made up with water (16 %) and horse blood (6.7 %)
4. Containing about 1 % of yeast autolysate and made up with water (16 %) and horse blood (6.7 %).
5. Containing about 1 % of liver autolysate and made up with water (16 %) and horse blood (6.7 %).

The dilutions were made in broth and readings were taken after 40 hours' incubation.
The two sets of figures shown for each medium are:
Column A: number of colonies;
Column B: mean colony diameters in mm.

[c] Normal growth. [d] Sparse growth. [e] Contaminated.

strains) were grown on 5 media, the composition of which is given in the accompanying table. Four of the fresh strains had shown comparatively poor growth on the routine medium. Bacterial suspensions were prepared in 25% ascitic fluid broth; they were diluted as described above and immediately afterwards 0.1 ml of each was plated on the 5 different media.

The table shows that the average colony counts for the individual strains ranged from 8.6×10^7 to 1.1×10^9 per ml. None of the five media showed any systematic deviation from the average colony counts.

The average of the colony diameters (measured after about 40 hours' incubation) varied between 0.125 mm and 3 mm; as was expected, strains 50700 and 93300 showed the largest colonies. The average values for the different media were highest for medium 3, which contained both liver and yeast autolysate, and the lowest values were observed on medium 2, which was made up with water only; surprisingly, medium 5 gave colonies just as large as those on media 1 and 4. In a similar experiment, the routine medium (containing 22% of ascitic fluid) was compared with a medium of similar composition to medium 3 but containing 1% of haemoglobin instead of 6.7% of heated blood and Spanish agar instead of Danish AKI agar (HYL medium). Five fresh strains and the control strain were employed. The viable counts were similar for the two media and the colonies were equal in size.

The two types of medium were also compared in the routine laboratory, in which primary isolation of gonococcal strains was performed. Charcoal swabs infected with discharge were received in the laboratory after having been transported in tubes with solid Stuart's medium [1] (Stuart et al., 1954; Reyn et al., 1960). The infected swabs were used to inoculate two plates prepared with the chocolate medium containing 22% of ascitic fluid and with the HYL medium, alternating between the two media to be inoculated first. Two series of 137 plates were inoculated and the same 42 positive cultures were

[1] The preparation of this medium is described in the article by Reyn on page 449 of this issue.

isolated on each medium. The amount of growth and the size of the colonies were very similar on the two media.

2. *Fermentation experiments.* 78 recently isolated gonococcal strains were inoculated on plates prepared with the HAP medium and with a medium prepared in a similar way except that the ascitic fluid was replaced by yeast autolysate and liver autolysate, both in a final concentration of 1%. Neither of the media contained haemoglobin. The inoculation was performed with a bent 6-mm platinum needle. Six strains were grown on each plate, and care was taken to make the inocula alike on the two media. Generally, the amount of growth was larger on the HAP medium than on the medium containing yeast and liver. The colour change from red to yellow (indicator: phenol red) was more distinct on the latter, this difference presumably being due to the buffering effect of the ascitic fluid in the HAP medium.

In another experiment using much smaller inocula, colony countings were performed on the same two media using six gonococcal strains (five fresh strains plus 50700/1955). Suspensions containing about 10^8 organisms were diluted 2×10^5 in saline, and 0.1 ml and 0.05 ml of these dilutions were plated. On the original HAP medium, germination was poorer than that usually seen on chocolate ascitic agar, and no growth at all was obtained on the HAP medium in which the ascitic fluid was replaced by yeast and liver autolysates.

3. *Determination of the sensitivity to penicillin.* Preliminary comparative experiments showed that the HYL medium resulted in about the same inhibitory values as those found with the routine medium. The results of further experiments are presented in a separate paper (Reyn, Bentzon & Møller, 1965).[1]

DISCUSSION

One of the difficulties in devising the ideal gonococcus medium is that the growth requirements and resistance to toxic substances vary from one gonococcal strain to another (Lankford et al., 1943; Lankford & Snell, 1943; Gould, 1944; Lankford & Skaggs, 1946; Lankford, 1950).

Whole blood, plasma, serum and haemoglobin have a detoxifying effect, which is presumably due to adsorption of inhibitors, such as fatty acids (Ley & Mueller, 1946), on large molecules or to the formation of complexes (Lwoff, 1947). A similar effect can also be obtained by the addition of proteins, especially albumin (Bang & Østergaard, personal communication, 1950; Mullaney, 1956), starch (Gould et al., 1944) or powdered charcoal (Stuart et al., 1954). The various growth factors contained in the blood, etc. are also very important.

The choice of medium for routine purpose depends upon the local circumstances. If blood, ascitic fluid, etc. are readily available, it is natural to use these products as they are. In other cases, it is more practical to use the commercially available dehydrated complete media or dehydrated media bases. Recently, however, it has become more difficult to procure fresh human or animal biological products. Several therapeutic substances are now prepared from serum or plasma, and ascitic fluid is becoming rare because the patients are treated with diuretics instead of being relieved of their ascitic fluid by puncture. It has also become more difficult to control the content of antibiotics in the ascitic fluid.

In the present paper a medium has been described in which no fresh biological substances or commercially available enrichments are used. The broth-agar base is prepared from a peptone-free beef-heart broth; it is enriched with yeast and liver autolysates, both in a final concentration of about 1%, and with haemoglobin in a final concentration of about 1%. No attempts were made to devise a synthetic medium or to analyse all the relevant factors, as for example, the significance of various peptones or amino acids (Hill et al., 1948, 1949; Juhlin, 1963) for the growth of *N. gonorrhoeae.*

Several previous investigators (for example, Carpenter, 1943; Carpenter et al., 1949) have used haemoglobin instead of blood, and fresh yeast and liver extracts have also previously been recommended as substitutes for serum and plasma (Lankford et al., 1943). Alture-Werber (1944) found that extracts prepared from fresh liver which had been dried at low temperature were just as good as fresh liver preparations.

One prominent feature of the present experiments was the beneficial effect of certain proteins. This was demonstrated most clearly in the fermentation experiments; in fermentation media, the addition of haemoglobin will disturb the readings, and in the present experiments it was therefore omitted. But, as was to be expected, germination was poor on the modified HAP medium in which the ascitic fluid was

[1] See the article on page 503 of this issue.

replaced by a mixture of yeast and liver autolysates. In order to prepare a good fermentation medium without haemoglobin or ascitic fluid, it will be necessary to add a protecting substance as, for example, albumin or starch. If not, heavy inoculation will be required. However, further experimentation is needed.[1]

In the present experiments, the growth-promoting effect of the combined yeast and liver autolysates was quite as good as that of ascitic fluid in a concentration of about 20% or of Bacto-Supplement C in a concentration of 1%. This applied to media prepared both with horse blood and with haemoglobin. However, in a later experimental series (Reyn, Bentzon & Møller, 1965) [2] it was found that the use of horse blood resulted in a higher number of colonies than did the use of 0.9% haemoglobin. Yet, the difference was very small and from a practical point of view the new medium is regarded as being satisfactory for the primary isolation of *N. gonorrhoeae* from pathological material.

[1] Later experiments have shown that some batches of albumin are more protective than others and that some contain glucose.

[2] See the article on page 503 of this issue.

RÉSUMÉ

Il est connu que la culture de *Neisseria gonorrhoeae* sur milieu artificiel est très difficile, probablement en raison de la présence de substances toxiques dans la gélose et des exigences en facteurs de croissance du gonocoque. Des produits biologiques ont été utilisés dans une large mesure dans les milieux destinés à l'isolement primaire de l'agent de la blennorragie, le liquide d'ascite en particulier. Or ce dernier produit devient rare et cher, et l'on a cherché à mettre au point un milieu qui ne contiendrait pas de substance biologique fraîche.

Les auteurs décrivent le milieu qu'ils ont mis au point. Le point de départ est une gélose au sang, contenant 20% de liquide d'ascite et 6,7% de sang de cheval. L'expérience a montré que l'on pouvait remplacer le liquide d'ascite par une combinaison d'autolysats de levure et de foie (1% de chaque) et le sang de cheval par de l'hémoglobine (1%). Ce milieu à l'hémoglobine-levure-foie (*Haemoglobin-Yeast-Liver* " *HYL* ") convient à l'isolement primaire du gonocoque, et les premiers essais indiquent qu'il pourrait être utilisé comme milieu de référence pour la détermination de la sensibilité du gonocoque aux antibiotiques. La suppression de l'hémoglobine ou du sang, qui a été tentée, n'a pas conduit à de bons résultats. Il faut reconnaître que, sur le milieu en question, la croissance du gonocoque a été plutôt faible, et il semble que l'addition d'un agent protecteur, tel que l'albumine ou l'amidon serait nécessaire.

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