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A STUDY ON THE GROWTH REQUIREMENTS OF *NEISSERIA GONORRHOEAE* AND ITS CLINICAL APPLICATION†

Routine methods for the isolation of *Neisseria gonorrhoeae* employ various complex media in conjunction with devices designed to supply an atmosphere of approximately 10 per cent carbon dioxide. Recent investigations[6,7] of the CO_2 requirement of *N. gonorrhoeae* have shown that various strains of this microorganism can be grown on a Casamino-salts-glucose agar medium in the absence of CO_2, provided an extract of yeast is added. The apparent effect of yeast extract in eliminating the requirement for CO_2 could be duplicated by a combination of hypoxanthine, uracil, and oxaloacetate; however, growth in air with this combination proved inferior to the growth with yeast extract alone.

An investigation of the nature of the additional factors in yeast extract was undertaken. This report presents evidence of the necessity for tryptophan and biotin in the metabolism of *N. gonorrhoeae* and the role of oxaloacetic acid as a source of CO_2. The recognition of the importance of CO_2 and the stimulatory effect of yeast extract has made possible the development of a simplified medium and a new technique for the isolation of *N. gonorrhoeae* from clinical specimens.

MATERIALS AND METHODS

Stock cultures grown on Mueller-Hinton medium[18] were maintained in an atmosphere of CO_2 (candle-jar method). The inoculum consisted of a loopful (5 sq. mm.) of an 0.9 per cent saline suspension of cells obtained after 18 hours of incubation. The density of these suspensions was standardized by means of a Klett-Summerson photometer with a #66 filter.

The agar surface was streaked in the conventional manner used to obtain the greatest number of isolated colonies. The amount of growth was recorded arbitrarily as zero to four plus on the basis of the number and size of the colonies at the most

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isolated portion of the agar surface. The medium of Gould,[5] modified only by the addition of 2.0 mg. per cent of glutathione, was employed for most of the growth factor determinations and this medium is hereafter referred to in the text as "basal agar." All cultures were incubated at 37°.

The following components (in grams) comprised the medium devised for the clinical studies: technical casamino acids, 15*; starch, 1.5; glucose, 1.5; KH_2PO_4, 1; $Na_2HPO_4 \cdot H_2O$, 2.8; $MgSO_4 \cdot 7H_2O$, 0.45; yeast extract, 7.5*; agar, 17, and distilled water, 1,000 ml. The medium was adjusted to pH 7.4 with 10 per cent Na_2CO_3, autoclaved for 10 minutes at 10 pounds pressure (113°), cooled quickly and, to avoid reheating, immediately poured into glass or plastic petri dishes. After solidification, each plate was hermetically sealed with a rubber petri dish seal and then stored at 3-5°. In the preparation and use of this medium it should be noted that, for optimal growth, both reheating the sterile medium and the addition of larger amounts of yeast extract are to be avoided.

EXPERIMENTAL

Preliminary studies using direct transfers of microorganisms from stock cultures showed that both old and recently isolated strains of *N. gonorrhoeae* grew under CO_2 on basal agar. Growth did not occur in air unless the medium contained a supplement of hypoxanthine, uracil, and oxaloacetate. However, this supplemented agar supported only slight growth for the same time period (24 hours) in which moderately heavy growth was obtained under CO_2; at the end of 42 hours, heavy growth also was obtained in air.

It seemed likely that, when the organism is grown in air, the decomposition of oxaloacetic acid in the alkaline medium produces the CO_2 necessary for growth. In general, one of the effects of CO_2 on the growth of bacteria appears to be concerned with a reduction in the lag period of growth. Therefore, increasing concentrations of oxaloacetic acid might be expected to cause a corresponding reduction in the lag phase of growth in *N. gonorrhoeae*. Oxaloacetic acid was cautiously neutralized with Na_2CO_3, the solution sterilized by filtration through a Swinny filter (Becton-Dickinson Company), and employed in concentrations varying from 5 to 80 mg. per 20 ml. of basal agar. From Table 1 it may be seen that when the sole addition to the basal medium is oxaloacetic acid, there is no observable growth in air. However, in the presence of hypoxanthine and uracil, which promote growth in air, the addition of oxaloacetate increases the amount of growth. The amounts of hypoxanthine and uracil employed were those found previously[7] to be optimal for the growth of *N. gonorrhoeae*. The addition of 0.05 gm. of yeast extract (previously sterilized at 113° for 10 minutes) to

* Generously supplied by Difco Laboratories, Detroit, Michigan.

the completely supplemented basal agar not only caused a significant reduction in the lag period but also increased the amount of growth in air.

If the function of oxaloacetate is to supply small amounts of CO_2 which is lost when the plate is exposed to air, then growth should occur within 24 hours in sealed basal agar plates containing only oxaloacetic acid but should not occur in plates containing only hypoxanthine and uracil. Furthermore, if the free CO_2 were to be absorbed by NaOH (in sealed Spray anaerobic

TABLE 1. EFFECTS OF OXALOACETATE AND YEAST EXTRACT ON GROWTH OF *Neisseria gonorrhoeae*

	Air		*Carbon dioxide*	
	Hours of incubation			
Basal agar	*24*	*42*	*24*	*42*
20 ml. Supplemented with:				
oxaloacetic acid (5,10, 20,40,80 mg.)	—	—	2+	3+
hypoxanthine (70 μg.) and uracil (80 μg.)	—	1+	2+	3+
hypoxanthine (70 μg.) uracil (80 μg.) oxaloacetic acid (5 mg.)	—	3+	2+	3+
hypoxanthine (70 μg.) uracil (80 μg.) oxaloacetic acid (5 mg.) yeast extract (0.05 gm.)	3+	4+	3+	4+
Yeast extract only (0.05 gm.)	3+	4+	3+	4+

Density of suspension = 25 Klett units.

petri dishes), growth would not be expected in the presence of either oxaloacetic acid alone or combined with hypoxanthine and uracil. The data presented in Table 2 are in accord with this hypothesis.

The importance of the total concentration of CO_2 on the growth of *N. gonorrhoeae* can be inferred from the data in Table 2 since in the presence of oxaloacetate growth occurred sooner in hermetically sealed petri dishes (12 mm. deep) than in sealed Spray dishes (50 mm. deep) containing water instead of NaOH. This would be expected if an effect of oxaloacetate is to contribute to a suitable concentration of CO_2. The marked effect of yeast extract demonstrated in Table 2 also was noted when even lighter inocula were used, further indicating the need for factor(s) in addition to

hypoxanthine and uracil. In view of the suggested relationship of biotin to CO_2 fixation and oxaloacetic acid synthesis, and because of the presence of biotin in yeast extract, this material was tested for its effect on growth. In these experiments, vitamin-free casamino acids (Difco) replaced the usual technical casamino acid component. The addition of 10 μg. of biotin to basal

TABLE 2. GROWTH OF *Neisseria gonorrhoeae* IN HERMETICALY SEALED AND UNSEALED DISHES IN PRESENCE AND ABSENCE OF NaOH

	Standard petri dishes				*Sealed Spray-anaerobic dishes*			
	not sealed		*sealed*		*over NaOH*	*over H_2O*		
Basal agar (20 ml.)	*18 hrs.*	*22 hrs.*	*18 hrs.*	*22 hrs.*	*48 hrs.*	*18 hrs.*	*22 hrs.*	*48 hrs.*
oxaloacetic acid 5 mg.)	—	—	—	+(s)**	—	—	—	±
hypoxanthine (70 μg.) uracil (80 μg.)	—	—	—	—	—	—	—	—
hypoxanthine (70 μg.) uracil (80 μg.) oxaloacetic acid (5 mg.)	—	+(s)	+	2+	—	—	—	+
hypoxanthine (70 μg.) uracil (80 μg.) oxaloacetic acid (5 mg.) yeast extract (0.05 gm.)	2+	3+	3+	4+	—	2+	3+	4+
Basal agar plus 0.05 gm. yeast extract*	3+							

Density of suspension = 19 Klett units.
* Control plate maintained in the presence of 10 per cent CO_2.
** Slight growth.

agar medium containing hypoxanthine, uracil, and oxaloacetate reduced the lag period noted in air (Table 3).

Welton *et al.*[21] have described the use of indoleacetic acid as a component of a defined medium for the growth of *N. gonorrhoeae*. Since tryptophan is destroyed in large part by acid hydrolysis, it seemed possible that the yeast stimulation noted in basal agar containing casamino acids prepared by acid hydrolysis might also reflect a requirement for tryptophan. This substance was found to stimulate growth and, furthermore, complemented the effect of biotin (Table 3).

The presence of still another stimulating factor(s) is evident since heavier growth occurred in all cases when yeast extract was added to vitamin-free casamino acid agar alone or together with optimal amounts of hypoxanthine, uracil, oxaloacetic acid, tryptophan, and biotin. The effect of yeast extract employed alone could not be accounted for solely on the

TABLE 3. REDUCTION OF GROWTH LAG PERIOD IN *Neisseria gonorrhoeae* BY BIOTIN AND TRYPTOPHAN

Vitamin-free casamino acid agar plus vitamin mix, hypoxanthine, uracil, oxaloacetate†*	*Growth in air hours*	
additions/20 ml. agar	*24*	*42*
none	—	+(s)*
biotin (10 μg.)	+	2+
L-tryptophan (0.6 mg.)	+	2+
biotin (10 μg.) L-tryptophan (0.6 mg.)	3+	4+
biotin (10 μg.) L-tryptophan (0.6 mg.) yeast extract (0.05 gm.)	4+	
yeast extract (0.05 gm.)	4+	

Density of suspension = 17 Klett units.

* Vitamin mix (in mg.): pyridoxine HCl, 3.0; thiamine HCl, 4.5; riboflavin, 2.0; nicotinic acid, 26; water, 10 ml., pH 6.9. Autoclaved at 113° for 10 minutes. Employed at 0.01 ml./20 ml. agar.

** Slight growth.

† Additions per 20 ml. agar: hypoxanthine, 70 μg., uracil, 80 μg., and oxaloacetate, 5 mg.

basis of the content of biotin and tryptophan. The concentration of yeast extract used throughout these experiments is of paramount importance since too much yeast extract decreases or prevents growth presumably because of the presence of an inhibitory substance.

In another series of experiments the growth of *N. gonorrhoeae* (stock strains) from very dilute inocula (density of 5 Klett units) on hermetically sealed, basal agar plates supplemented with yeast extract was found to be superior to that obtained with Mueller-Hinton agar under CO_2. On the basis of these observations a possible clinical application was suggested.

A comparative clinical evaluation was made of the growth and isolation of *N. gonorrhoeae* from materials obtained from both male and female patients. Growth in 24 hours on the yeast-agar medium (see methods) by this technique was compared with that obtained using the standard chocolate agar-CO_2 method. Data from 83 unselected, consecutive cases are presented in Table 4. Without exception, the new method duplicated the results obtained with the standard technique; in many instances it yielded more abundant growth and in three cases a positive finding was obtained, whereas negative cultures resulted through the use of the standard technique.

TABLE 4. COMPARISON OF STANDARD METHODS WITH PROPOSED AGAR-TECHNIQUE IN THE CLINICAL ISOLATION OF *N. gonorrhoeae*

	*Number of cases**	
Readings	*Chocolate agar carbon dioxide*	*Yeast-agar hermetically sealed*
Negative	48	45
Positive	35	38

* Nineteen from female patients with the following distribution: positive, 3; negative, 16.

DISCUSSION

It is generally accepted that essentially all microorganisms require a certain minimal concentration of CO_2 before reproduction occurs, the required level of CO_2 varying with the microorganism. This is particularly evident among certain members of the genera *Brucella* and *Neisseria*. Various reports (Walker,[20] Winslow *et al.*,[22] Gladstone *et al.*,[4] Tuttle and Scherp[19]) have demonstrated a relationship between CO_2 concentration and the lag phase of growth, and the concept is that cell division occurs only after a certain critical level of CO_2 concentration has been reached. The attainment of such concentrations is determined by factors regulating the rate of release of small amounts of CO_2 from substances present in the medium, combination of CO_2 with constituents in the medium, loss through diffusion, and the amount produced by the respiration of the microorganism.

Some controversy exists as to the actual replacement of CO_2 by known growth factors. Ajl and Werkman[1] reported that certain components of the citric acid cycle met the CO_2 requirement of *Escherichia coli* whereas Lwoff and Monod[10, 11] contended that CO_2 per se is essential. Support for the latter view has been provided by Gutterman and Knight,[8] who suggest that oxaloacetic acid satisfies the CO_2 requirement of *Penicillium chrysogenum*

through decomposition and the slow release of CO_2, and by Tuttle and Scherp,[19] who described various combinations which partially replace the requirement for supplemental CO_2 but failed to eliminate this need completely. Furthermore, Newton *et al.*[14,16] and Newton and Wilson[15] showed that the addition to the medium of some of the products of $C^{14}O_2$ fixation (pyrimidines) in *Brucella abortis* did not completely satisfy the CO_2 requirement of this organism. The persistent requirement by *Treponema pallidum,* strain 69, for CO_2 is another example of the phenomenon (Steinman *et al.*[18]).

In the present report the effect of oxaloacetate on the growth of *N. gonorrhoeae* has been shown to be related to the requirement of this organism for CO_2. These data lend additional support to the proposal that CO_2 is needed for the growth of this microorganism and that no growth will occur under conditions where CO_2 is efficiently and rapidly removed.

In a variety of bacteria, biotin has been shown to be necessary in the decarboxylation of oxaloacetic acid to pyruvate and CO_2 (Lardy *et al.*,[8] Shive and Rogers[17]). Biotin has also been implicated in purine biosynthesis.[12] The participation of biotin in CO_2 fixation by certain microorganisms has been described by Lardy *et al.*[9] and Blanchard *et al.*[2] The latter authors suggested that, in their experiments, biotin does not function directly as a cofactor in the carboxylation reactions of *Lactobacillus arabinosus* but may be a component in those reactions leading to the synthesis of enzymes which control the fixation of CO_2.

Against this background, the demonstration in the present report of the absolute need for CO_2 by *N. gonorrhoeae* and the finding of the stimulatory effect of biotin suggests an interrelationship between these substances. This problem currently is under investigation. The further stimulation of growth by yeast extract added to basal agar supplemented with hypoxanthine, uracil, oxaloacetate, biotin, and tryptophan reflects the participation of still another factor(s), the nature of which is also under study.

The recognition of the need for suitable levels of CO_2 as such in the growth of *N. gonorrhoeae* and the ability of yeast extract to substitute for these factors suggested the use of a technique for the isolation of *N. gonorrhoeae* from clinical specimens which employs a medium hermetically sealed in petri dishes. This new technique possesses the following advantages: it allows more growth in a shorter period of time compared with some of the media in standard use, it is convenient to prepare, economical, stable on storage, and, because of the rubber seal, can be stored for prolonged periods without contamination or loss of moisture. Furthermore, the method eliminates the need for blood or serum, as well as the use of cumbersome CO_2

jar devices. In view of these advantages, the use of this technique in the isolation of *N. gonorrhoeae* from clinical materials is proposed.

SUMMARY

1. It has been demonstrated that a rôle of oxaloacetate in the growth of *N. gonorrhoeae* in air on basal agar supplemented with hypoxanthine and uracil is to supply CO_2. Furthermore, under these conditions the absolute need for CO_2 has been shown.

2. Using a light inoculum and a supplemented vitamin-free casamino acid agar-medium, the need for biotin and tryptophan was established. An additional factor(s) in yeast, which stimulates the growth of this microorganism, was also demonstrated.

3. A simplified agar medium used in combination with hermetically sealed petri dishes for the isolation of *N. gonorrhoeae* from clinical specimens is described and the advantages of such a technique are discussed.

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