THE CARBON DIOXIDE REQUIREMENT OF NEISSERIA GONORRHOEAE

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In some microorganisms CO₂ can be replaced successfully as a growth factor by certain dicarboxylic acids which enter the Krebs cycle (Lwoff and Monod, 1946; Ajl and Werkman, 1948). Other microorganisms require CO₂ for growth even when cultivated on rather complex media (Steinman, et al., 1954; Mueller and Hinton, 1941), and the biochemical basis for this need has remained obscure.

In the course of investigations on the glutathione requirement of certain mutants of Neisseria gonorrhoeae, it was observed that this microorganism grew well in the absence of CO₂ provided a small amount of yeast extract was included in the medium. A survey of yeast extract of various laboratories and from clinical cases revealed that the phenomenon was a general one and not restricted to the glutathione-less mutant.

It was reported in a preliminary note (Griffin and Racker, 1955) that the yeast extract can be replaced by a combination of three factors: hypoxanthine, uracil, and oxaloacetate. It is the purpose of this paper to present the details of this investigation.

METHODS AND MATERIALS

Stock strains of N. gonorrhoeae were maintained at 36 C on the medium of Mueller and Hinton (1941) in an atmosphere of CO₂. The medium of Gould (1944) was used for the studies of the nutritional requirements for CO₂ and glutathione. For the quantitative study of growth, the following fluid medium was devised. Basal fluid medium: casamino acids, technical (Difco), 15 g; glucose, 1.5 g; KH₂PO₄, 1.0 g; Na₂HPO₄, 2.8 g; glutathione, 0.02 g; and distilled water, 1000 ml.

The medium (pH 7.4) was autoclaved for 8 min at 10 lb pressure and, after cooling, a sterile solution of MgSO₄·7H₂O was added to give a final concentration of 0.045 mg per cent.

For the nutritional studies, the inoculum was prepared from cells grown on Gould media under CO₂. The bacteria were washed three times in the basal fluid medium, then suspended and the density standardized turbidimetrically. Klett tubes containing 5 ml of basal fluid medium and various growth factors were inoculated with one drop of the washed bacteria delivered from a 0.2 ml pipette, and the tubes were then agitated in a reciprocal shaking device at 188 rpm at 36 C in air. At various intervals, growth was measured turbidimetrically in a Klett-Summerson photometer with a 660 filter. Probably because of clumping, the relation between turbidimetric readings and dry weight, although reproducible, was not directly proportional. A standard curve based on the following values was used: 222 Klett units corresponding to a dry weight of 1.43 mg per ml; 160 Klett units corresponding to a dry weight of 0.95 mg per ml; 112 Klett units corresponding to a dry weight of 0.5 mg per ml.

The sources of the various materials used in this study were as follows: Phosphoenolpyruvic acid, thymidyllic acid, and oxaloacetic acid were obtained from the California Foundation for Biochemical Research; thymine, hypoxanthine, uracil, adenine, guanine, α-ketoglutarate, thymidyllic acid, thymidine, uridin, and uridylic acid, from the Nutritional Biochemicals Corporation; adenylc acid, adenosine diphasphate, adenosine triphosphate, inosinic acid, inosine, inosine diphasphate, and inosine triphosphate, from the Sigma Chemical Co.; glutathione, from Schwarz Laboratories, Inc.; and another sample of thymine from General Biochemicals, Inc.

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RESULTS

Carbon dioxide replacing factors in yeast extract. Strains of *N. gonorrhoeae*, obtained by the procedure described by Gould (1944), grew only when both glutathione and CO₂ were available. In the presence of a crude yeast extract, however, abundant growth was observed, even in the absence of CO₂ (table 1). To measure quantitatively the response of the microorganisms to the factor or factors present in yeast, the fluid basal medium was employed. Turbidimetric measurements in the presence of various amounts of the yeast factor revealed that optimal growth was obtained with small amounts of yeast extract, while larger amounts inhibited growth (figure 1). Proportionality between growth and amount of yeast extract was obtained only in a very narrow range of concentrations. Since assays of concentrated preparations of yeast extract were complicated by the presence of a growth inhibitor, and because no single dicarboxylic acid, amide acid, vitamin, purine or pyrimidine which was tested replaced the yeast extract, fractionation of the extract was attempted. Of several procedures which were tried, alcohol fractionation yielded the best results in the preliminary separation of the factors.

Fractionation of yeast extract. A 10 per cent aqueous solution of yeast extract was treated with four volumes of ethyl alcohol, the precipitate centrifuged off and dissolved in water to one-fifth the original volume. The supernatant solution was concentrated in vacuo at 45°C to one-fifth of the original volume and then centrifuged for 30 min at 15,000 rpm in a refrigerated centrifuge. The supernatant solution and the solution of the first alcohol insoluble precipitate were tested separately and in combination for CO₂-replacing activity. As is shown in table 2, neither the precipitate nor the supernatant fluid initiated the growth of *N. gonorrhoeae* in air. However, the combined fractions reconstituted the activity of the original yeast extract. The above procedure of purification eliminated most of the growth inhibitor present in the crude yeast extract.

Replacement of yeast fractions with known growth factors. From the above data it became apparent that at least two factors in yeast extract were involved in the replacement requirements for CO₂. It became necessary therefore to retest the various known vitamins and growth factors in the presence of each of the two yeast

**TABLE 1**

*Effects of carbon dioxide and yeast extract on growth of *Neisseria gonorrhoeae*.*

<table>
<thead>
<tr>
<th>Additions*</th>
<th>Growth</th>
<th>Air</th>
<th>CO₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Glutathione 2 mg per cent</td>
<td>−</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Glutathione + 1.0 ml 10 per cent yeast extract</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

* 20 ml Gould basal agar medium minus glutathione.

**Figure 1.** The effect of yeast extract on growth, in air, of *Neisseria gonorrhoeae*. Liquid-shake cultures. 48 hr incubation at 30°C.

**TABLE 2**

*The CO₂-replacement activity of alcohol fractions from 10% aqueous yeast extract (Difco).*

<table>
<thead>
<tr>
<th>Additions to 5.0 ml Medium</th>
<th>Amount (ml)</th>
<th>Klett Units*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Centrifuged</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitate</td>
<td>0.05</td>
<td>0</td>
</tr>
<tr>
<td>Supernatant fluid</td>
<td>0.05</td>
<td>0</td>
</tr>
<tr>
<td>Precipitate + supernatant fluid</td>
<td>0.05 of each</td>
<td>222</td>
</tr>
<tr>
<td>Original yeast extract</td>
<td>0.3</td>
<td>216</td>
</tr>
</tbody>
</table>

* Turbidity of liquid shake cultures in basal medium in air after 21 hr.
fractions. In this manner it was found that a combination of hypoxanthine, uracil, and oxaloacetate permitted growth of *N. gonorrhoeae* in an air atmosphere, although the growth was less abundant than that obtained with the crude yeast factors.

Requirement and specificity of replacement factors. The need for the three factors for growth of *N. gonorrhoeae* in air is shown in table 3. The requirement for uracil and oxaloacetate was obscured if too large an inoculum was used. The washed suspension of the bacteria used for inoculation was routinely prepared to give a turbidimetric reading of no more than 20 units in the Klett-Summerson photometer; with larger inocula only the requirement for hypoxanthine was demonstrable. Turbidimetric readings were proportional to the concentration of hypoxanthine (figure 2). In the case of uracil and oxaloacetate, however, quantitative assays were difficult to reproduce and therefore not reliable.

In view of the possible usefulness of *N. gonorrhoeae* as a sensitive assay for hypoxanthine in the presence of other purines, the specificity of the need for the replacement factors was explored. None of the purine derivatives which were tested replaced hypoxanthine. The following compounds gave no growth response: guanine, adenine, adenosine mono-, di-, and triphosphate, xanthine, inosine, inosine mono- and di-, and triphosphate, adenosine, and guanosine.

Uridin, uridylic acid, thymine, thymidine, and orotic acid did not replace the requirement for uracil. One commercial preparation of thymine (General Biochemicals, Inc.) was active. However, paper chromatography with N-butanol-10 per cent urea as solvent (Carter, 1950) revealed a second component with an *Rf* value (0.39) corresponding to that of a uracil control. Another commercial preparation of thymine (Nutritional Biochemicals Corporation) gave a single component in the chromatogram and did not replace uracil in the growth experiment. A commercial preparation of calcium thymidylate exerted a slight growth promoting activity in place of uracil. Although chromatograms of this material failed to reveal the presence of uracil, trace contamination could not be ruled out.

Oxaloacetate could be replaced by α-ketoglutarate. With other members of the Krebs cycle, less reproducible growth responses were obtained.

**TABLE 3**  
Effect of inoculum size of washed cells on growth response of Neisseria gonorrhoeae to hypoxanthine, uracil, and oxaloacetate acid

<table>
<thead>
<tr>
<th>Additions per ml Liquid Basal Medium</th>
<th>Klett Units of Inoculum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>48-hr growth response in Klett units</td>
</tr>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Hypoxanthine 2 µg</td>
<td>0</td>
</tr>
<tr>
<td>Hypoxanthine + oxaloacetate acid 200 µg</td>
<td>0</td>
</tr>
<tr>
<td>Hypoxanthine + uracil 3 µg</td>
<td>0</td>
</tr>
<tr>
<td>Hypoxanthine + uracil + oxaloacetate acid</td>
<td>110</td>
</tr>
<tr>
<td>Uracil + oxaloacetate</td>
<td>0</td>
</tr>
<tr>
<td>Yeast extract (Difco)</td>
<td>220</td>
</tr>
</tbody>
</table>

**Figure 2. Assay of hypoxanthine with Neisseria gonorrhoeae.** Basal liquid medium plus uracil (3 µg per ml) and oxaloacetate acid (300 µg per ml).

Response of various strains to CO₂ replacement factors. Since most of the nutritional investigations described above were carried out with glutathione-less mutants of *N. gonorrhoeae*, a survey was undertaken to evaluate the more general applicability of the nutritional findings.
Of particular interest was the behavior of strains obtained from clinical cases. Colonies of *N. gonorrhoeae* freshly isolated on Peizer medium (Peizer and Steffen, 1942) were transferred to Mueller-Hinton agar and then to Gould agar medium containing hypoxanthine, uracil, and oxaloacetate in the presence and absence of CO₂. Growth was observed in all of these secondary cultures in the presence of CO₂ and about 80 per cent also grew in the absence of supplemental CO₂. When the Gould agar medium containing hypoxanthine, uracil, and oxaloacetate was employed for the primary isolation of *N. gonorrhoeae* from 15 male patients, all strains grew well in the presence of CO₂, but only two grew in air. This was true even in the presence of yeast extract.

**DISCUSSION**

The requirement for CO₂ by microorganisms such as *Brucella abortus*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *T. pallidum* Strain 69 is of particular interest with regard to the possible relationship of this requirement to the synthesis of nucleic acid constituents. The problem has been a vexing one since in certain instances the major products of C¹⁴O₂ fixation have been determined and yet these same materials did not satisfy the requirement for supplemental CO₂. Pappenheimer and Hottle (1940) reported that adenyl acid replaced the requirement for CO₂ in a strain of *A. hemolyticus*. Gitterman and Knight (1952) have shown that oxaloacetic acid would replace the CO₂ requirement in *Penicillium chrysogenum*; however the rate of breakdown of oxaloacetic acid indicated that growth probably was still in response to CO₂ released through the rapid decomposition of the acid. Tuttle and Scherp (1952) found that under certain conditions either a mixture of guanine, uracil, and cytosine or yeast extract replaced the supplemental CO₂ required by a strain of *N. meningitidis*. Steinman, et al. (1954) have shown that even in the presence of amino acids, purines, and pyrimidines, *T. pallidum* Strain 69 still maintains a specific requirement for supplemental CO₂. An analogous situation exists in the case of several strains of *B. abortus*. The major products of CO₂-fixation in the nucleic acids of this microorganism occurs in the pyrimidines and fixation of C¹⁴O₂ into purines appears to be quantitatively insignificant. Moreover, when given a choice of pyrimidine precursors, these microorganisms preferentially continue to synthesize pyrimidines from CO₂ (Newton, *et al.*, 1954; Newton and Wilson, 1954; Newton, *et al.*, 1955).

In the present study, the need for added CO₂ in several strains of *N. gonorrhoeae* has been obviated by a combination of uracil, oxaloacetic acid, and hypoxanthine. That these microorganisms retained their ability to utilize CO₂ was apparent since, when the replacement factors were omitted, growth occurred only in a CO₂ atmosphere. In contrast, in most cases, primary isolates from patients with gonorrhea could not be obtained without carbon dioxide.

The requirement of *N. gonorrhoeae* for a high concentration of CO₂ is a complex one. Oxaloacetic acid, hypoxanthine, and uracil permit growth in air but the response is not quite equal to growth in a medium supplied with a crude yeast extract. Thus, a further search for CO₂ replacement factors appears justified. Several compounds known to be involved in CO₂ fixation reactions (Ochoa, 1951; Utter and Wood, 1951) have been tried with no effect. Citrullin seemed to stimulate growth slightly in the presence of the three replacement factors, but the mixture was still inferior to the crude yeast extract.

The term "replacement factor" which has been used for the compounds which permitted growth in air is not meant to convey the impression that these compounds necessarily substitute for CO₂ because they represent end products of CO₂ fixation reactions. Although this mechanism may be likely for oxaloacetate, it is not necessarily true for hypoxanthine. A possible explanation of a requirement for hypoxanthine is offered by the work of Utter and Kurahashi (1954 a, 1954 b) and Utter, *et al.* (1954) on the participation of inosine diphosphate in CO₂-fixation to phosphoenolpyruvate. If hypoxanthine is required in *N. gonorrhoeae* specifically for a CO₂ fixation reaction, it may permit growth in air by catalyzing a more efficient utilization of CO₂ at low concentrations.

To explore this possibility, experiments were performed with extracts obtained by sonic vibration of washed cells of *N. gonorrhoeae*. The assistance of Mrs. L. Peizer and Drs. Steffen, Widelock, and Pollock of the Department of Health, City of New York, in obtaining these strains is gratefully acknowledged.

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The fixation of C\(^4\)O\(_2\) was measured with either oxaloacetate or phosphoenolpyruvate as substrate. The experimental conditions were essentially as described by Utter and Kurahashi (1954a). It was found in preliminary studies that CO\(_2\)-fixation was stimulated by the hypoxanthine derivative inosinic acid and close to 50 per cent of the total counts incorporated were found in oxaloacetic acid. The adenine derivative, adenylc acid, was not only inactive but actually inhibitory. The addition of a yeast extract (which contained the CO\(_2\)-replacing factors) to this cell-free system resulted in a very striking stimulation of C\(^4\)O\(_2\)-fixation. However, in this case the counts were not found in oxaloacetate.

Thus, in some respects the metabolic behavior of the cell-free extract appears to resemble the response of the intact cells during growth. It remains to be demonstrated, however, whether the CO\(_2\)-fixation reaction studied in vitro is indeed a limiting factor in the growth of \textit{N. gonorrhoeae}.

**SUMMARY**

Growth of several strains of \textit{Neisseria gonorrhoeae} has been obtained in air on an agar medium and in a fluid medium containing casamino acids and salts in the presence of small amounts of yeast extract. Larger amounts of yeast extract are inhibitory. Yeast extract can be replaced by a mixture of hypoxanthine, uracil, and oxaloacetate. The requirement for hypoxanthine is specific. Growth of \textit{N. gonorrhoeae} in shake cultures is proportional to the concentration of this substance and can be used as a quantitative assay in the presence of other purines.

**REFERENCES**


