NUTRITIONAL STUDIES OF A VIRULENT STRAIN OF 
HAEMOPHILUS DUCREYI

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*Haemophilus ducreyi* has been described as a 
fastidious organism requiring blood for growth 
(Greenblatt, 1953). It has been successfully 
cultivated on clotted blood (Teague and Diebert, 
1920) and on various artificial media enriched 
with blood, (Sanderson and Greenblatt, 1937; 
Beeson and Heyman, 1945; Beeson, 1946; 
Deacon *et al.*, 1956). However, little is known 
concerning the specific nutritional requirements 
of this organism.

In an effort to define the factors necessary for 
the growth of *H. ducreyi* this preliminary in-
vestigation of a representative virulent strain 
was begun.

METHODS

*Culture.* Throughout this investigation, *H. du-
creyi* strain CH-1A was used. This strain was iso-
lated from a chancroidal bubo and was found to 
be virulent when inoculated intradermally on the 
shaved abdomen of rabbits and the skin of 
human volunteers (Deacon *et al.*, 1956). For 
the present experiments, virulent CH-1A cultures 
were maintained in screw-capped tubes on slants 
of nutrient agar, 1.5 per cent, overlaid with 0.7 
ml of sterile rabbit serum and incubated at 33 C. 
Although such cultures were viable for about 
one week, they were transferred every 48 or 72 
hr to keep them actively growing. A few colonies 
usually formed on the sloped surface of the agar 
but growth occurred mainly in the serum por-
tion of the culture. A small loopful of this serum 
was used as inoculum unless otherwise indicated. 
Saline-washed inocula lost viability rapidly and 
could not be used.

*Testing Procedures.* Tests were conducted in 
16- by 125-mm screw-capped tubes and all of the 
compounds under investigation were incorporated 
into 5 ml of a 0.15 per cent semisolid saline agar 
base overlaid with 0.2 ml of sterile rabbit serum. 
This method was selected because the nature of 
*H. ducreyi* to grow colonially or in chains, even in 
liquid media, and to settle out rapidly made im-
possible precise quantitative estimates of growth 
by turbidimetric or cell-counting procedures. Ex-
perience had shown that slight differences in 
growth could be detected easily and reliably in 
the semisolid medium where the organism grew in 
a layer just below the surface of the agar. Control 
tubes of a 0.15 per cent agar-0.85 per cent saline 
base overlaid with 0.2 ml of rabbit serum consist-
ently permitted a slight amount of growth. When 
various nutrients were incorporated into such a 
basal medium, differences in the amounts of 
growth compared with the control were taken 
to be an expression of the growth-promoting, 
inhibitory or inert nature of the substances tested.

*Peptones.* The excellence of nutrient agar, 
1.5 per cent, plus blood or serum as a medium for 
*H. ducreyi* led to an investigation of the ability 
of various peptones to support growth. Accord-
ingly, peptone, proteose-peptone, proteose-
peptone #3, neopeptone, protone, tryptone, and 
tryptose (products of the Difco Laboratories), 
and gelysate, lactalysate, milk protein hydro-
lyzate, myoeate, phytone, thiotone, and tryp-
ti-case from the Baltimore Biological Laborator-
ies were tested at 1.0 per cent concentrations. 
The optimum quantity of peptone was de-
termined by comparing the amount of growth 
that occurred after 24 to 48 hr in saline-agar 
prepared with various concentrations of peptone 
and overlaid with serum.

*Sodium chloride.* Sodium chloride was incor-
porated into a semisolid 1 per cent peptone 
medium so that the final salinity ranged from 
0.2 to 3 per cent. Chlorides of potassium and 
calcium and sodium citrate were also tested to
determine whether other ions could be substituted.

Carbohydrates. Glucose, fructose, sucrose, lactose, maltose and inositol were added in 0.5 per cent concentrations to tubes containing 5 ml of phenol-red broth base plus 0.15 per cent agar and overlaid with 0.2 ml of rabbit serum.

Various nitrogen sources, vitamins and minerals. In an attempt to define the substances in peptone utilized by strain CH-1A, casein digests, various amino acid mixtures, individual amino acids and inorganic nitrogen compounds were studied. Three acid-hydrolyzed caseins (casamino acids; casein amino acids, technical; and caseamino acids, vitamin-free) in which all the nitrogen was "converted to amino acids or other compounds of relative chemical simplicity" (Difco Manual, 1953) and one pancreatic digest of casein (casitone), in which hydrolysis was not complete, were tested in amounts approximating 50 mg of nitrogen per 100 ml of semisolid medium.

The efficacy of a liquid casitone medium enriched with rabbit and other animal sera was also investigated.

Related amino acids were tested initially in seven groups:

Group C—L-proline, L-tryptophan, L-tyrosine, DL-phenylalanine.
Group D—L-lysine, L-arginine, L-histidine, DL-ornithine.
Group E—cysteine hydrochloride, DL-methionine, L-cystine.
Group F—L-aspartic acid, L-asparagine, L-glutamic acid, L-glutamine.
Group G—hydroxy-L-proline (tested separately as it is inhibitory to many microorganisms).

A mixture of all seven groups and other mixtures in which a single group at a time was eliminated were also prepared and tested (table 1). All of the above groups and mixtures were tested at a concentration of 50 mg of nitrogen per 100 ml of medium.

Individual amino acids and inorganic nitrogen compounds were tested at concentrations equivalent to 50, 25, 12.5, 5, and 1 mg of nitrogen per 100 ml of medium. The sulfur-containing amino acids of Group E were also tested at 0.5 mg of nitrogen per 100 ml of medium.

A stock mixture of water-soluble vitamins was prepared so that when added to 5 ml of medium the following concentrations were present: biotin, 0.25 μg; thiamin, 1.0 μg; riboflavin, 5.0 μg; ascorbic acid, 1.0 μg; choline chloride, 25.0 μg; folic acid, 25.0 μg; inositol, 250.0 μg; nicotinic acid, 10.0 μg; nicotinamide, 10.0 μg; p-aminobenzoic acid, 25.0 μg; calcium pantothenate, 1.0 μg; and pyridoxine, 1.0 μg.

A trace mineral mixture composed of the following compounds was tested in the concentrations indicated per 5 ml of medium: H3BO3, 3.0 μg; CuSO4·5H2O, 10.0 μg; MoO3 (85%), 2.0 μg; Fe3(SO4)2·(NH4)2·24H2O, 85.0 μg; MnSO4·H2O, 3.0 μg; ZnSO4·7H2O, 40.0 μg; and MgSO4, 500.0 μg.

All components described in this section were prepared in 0.85 per cent saline solution, adjusted where necessary to pH 7.0–7.4 and, except for heat-labile compounds, were autoclaved at 120 C for 15 min. Glutamine and cysteine hydrochloride were sterilized by filtration through sintered glass, whereas the stock vitamin mixture was sterilized by passage through a membrane filter.

Studies on blood. To test the effect of defibrinated rabbit blood, serum and plasma on growth, 2-fold serial dilutions of each of these substances prepared in physiological saline were overlaid in 1-ml amounts on nutrient agar slants and inoculated with a loopful of culture from a blood or serum slant. In an attempt to adapt strain CH-1A to grow in a blood-free medium, tubes showing growth after 48 hr were transferred to fresh tubes of equal dilution and to the next higher dilution. This was repeated until no further increase in adaptation became apparent.

The thermostability of the growth factors in serum was determined by heating aliquots of fresh rabbit serum, diluted 1:5 with distilled water, from 5 to 30 min in a water-bath at 100 C or autoclaving at 120 C. The coagulum was removed by centrifugation and equal parts of the supernatant fluid were incorporated into a double-strength, semisolid peptone agar before inoculation.

To establish whether or not the growth-promoting substances in blood were diffusible, plates containing 15 ml of nutrient agar enriched with 5 per cent defibrinated rabbit blood were overlaid with plain nutrient agar in amounts ranging from 5 to 100 ml, giving thicknesses of approximately 0.8 to 16 mm. The surfaces were
then streaked with the growth from a blood-nutrient agar plate and all plates were incubated in candle-jars.

The effect of $X$ and $V$ factors was determined by a modification of the method of Pickett and Stewart (1953). To preclude the possibility of omitting other essential factors in serum, nutrient agar plates without serum and plates enriched with 0.5 to 5 per cent rabbit serum were streaked heavily with strain CH-IA. Across this inoculum were drawn parallel streaks of catalase-positive Sarcina lutea strain 368 and catalase-negative Streptococcus faecalis strain 40a. The plates were then incubated in a candle-jar for 24 hr and examined for satellite growth. Control plates of trypticase-soy agar inoculated with Haemophilus influenzae and Haemophilus parainfluenzae instead of $H$. ducreyi were run simultaneously.

Serum components. Rabbit serum was dialyzed aseptically against distilled water in cellophane bags or Whatman diffusion shells and the resulting fractions were tested for their ability to support growth. When commercially-prepared serum fractions were used, 5 per cent stock solutions were made up in 0.85 per cent saline, adjusted to pH 7.0 with normal sodium hydroxide and sterilized by Seitz filtration.

Throughout this portion of the investigation various overlaying substances were added to tubes containing 0.5 per cent casitone-0.15 per cent agar prepared in physiological saline. Tubes of this medium with an overlay of 0.2 ml of whole sterile rabbit serum were used as controls.

### RESULTS

**Ability of peptone to support growth.** Peptone at 0.5 to 1.0 per cent levels supported optimal growth of $H$. ducreyi. Concentrations of 2 per cent and above resulted in decreased growth, whereas 5 per cent peptone was completely inhibitory. The organism grew more heavily on peptone, proteose-peptone, proteose-peptone #3, tryptone and tryptose than on the other peptones and could be transferred indefinitely on these media.

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**TABLE 1**

*Growth of Haemophilus ducreyi on media containing amino acid mixtures with and without the addition of serum and vitamins and minerals*

<table>
<thead>
<tr>
<th>Groups of Related Amino Acids†</th>
<th>Mixtures of Amino Acid Groups</th>
<th>Casitone Control</th>
<th>Agar Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A B C D E F G</td>
<td>I AB CD EFG</td>
<td>II AB CD EF</td>
<td>III AB CD E</td>
</tr>
<tr>
<td>With 0.2 ml rabbit serum overlay</td>
<td>+ 0 0 + 0 ++ ±</td>
<td>0 0 0 0 ++ ±</td>
<td>± ++</td>
</tr>
<tr>
<td>Without serum</td>
<td>0 0 0 0 0 0 0</td>
<td>0 0 0 0 0 0 0</td>
<td>0 0</td>
</tr>
<tr>
<td>With 0.2 ml rabbit serum overlay plus vitamins and minerals</td>
<td>+ 0 0 + 0 ++ ±</td>
<td>0 0 0 0 ++ ±</td>
<td>± ++</td>
</tr>
<tr>
<td>Without serum; vitamins and minerals added</td>
<td>0 0 0 0 0 0 0</td>
<td>0 0 0 0 0 0 0</td>
<td>0 0</td>
</tr>
</tbody>
</table>

* All amino acid mixtures tested at concentrations of 50 mg of nitrogen per 100 ml of semisolid medium.

† The components of each group are:

- A. Glycine, DL-$\alpha$-alanine, $\beta$-alanine, DL-serine, DL-threonine.
- C. L-Proline, L-tryptophan, L-tyrosine, DL-phenylalanine.
- D. L-Lysine, L-arginine, L-histidine, DL-ornithine.
- E. Cysteine hydrochloride, DL-methionine, L-cysteine.
- F. L-Aspartic acid, L-asparagine, L-glutamic acid, L-glutamine.
- G. Hydroxy-L-proline.
Necessity for sodium chloride in the medium.
Sodium chloride was necessary in the medium in amounts between 0.8 and 1 per cent, very little or no growth being obtained above or below this range. Neither potassium nor calcium could be substituted for sodium, nor could citrate replace the chloride.

Inability to demonstrate sugar utilization. No increase in growth nor clear-cut evidence of utilization could be demonstrated when any of the sugars tested were added to the phenol-red fermentation medium.

Utilization of various nitrogen sources, vitamins, and minerals. Strain CH-1A grew more profusely on casitone, the pancreatic digest of casein, than on acid-hydrolyzed caseins or on any of the simpler nitrogen sources. It was found that *H. ducreyi* also grew well in liquid casitone cultures consisting of 0.25 to 3 per cent casitone dissolved in 0.85 per cent saline and enriched with 5 to 15 per cent serum. Bovine and porcine sera could be used in this medium, but the organism grew heaviest and smoothest with rabbit or human serum.

The results obtained with the various mixtures and groups of amino acids are given in table 1. The occurrence of growth after the removal of group E suggested that one or more of the sulfur-containing amino acids was inhibitory. Although groups B and C, by themselves, seemed inhibitory, this effect was inapparent when they were combined with growth-supporting amino acid mixtures.

Table 2 summarizes the results with the individual amino acids and gives the concentrations at which the most growth occurred. Group F of

TABLE 2

Growth of *Haemophilus ducreyi* on decreasing concentrations of various nitrogen sources

<table>
<thead>
<tr>
<th>Nitrogen Sources</th>
<th>Mg of Nitrogen per 100 Ml of Medium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td>Glycine</td>
<td>0</td>
</tr>
<tr>
<td>DL-α-Alanine</td>
<td>0</td>
</tr>
<tr>
<td>DL-β-Alanine</td>
<td>±</td>
</tr>
<tr>
<td>DL-Serine</td>
<td>0</td>
</tr>
<tr>
<td>DL-Threonine</td>
<td>±</td>
</tr>
<tr>
<td>DL-Valine</td>
<td>0</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>0</td>
</tr>
<tr>
<td>DL-Isoleucine</td>
<td>0</td>
</tr>
<tr>
<td>DL-Norleucine</td>
<td>0</td>
</tr>
<tr>
<td>L-Proline</td>
<td>0</td>
</tr>
<tr>
<td>L-Tryptophan</td>
<td>0</td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>0</td>
</tr>
<tr>
<td>DL-Phenylalanine</td>
<td>0</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>0</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>0</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>+</td>
</tr>
<tr>
<td>DL-Ornithine</td>
<td>0</td>
</tr>
<tr>
<td>Cystine HCl</td>
<td>0</td>
</tr>
<tr>
<td>DL-Methionine</td>
<td>0</td>
</tr>
<tr>
<td>L-Cystine</td>
<td>0</td>
</tr>
<tr>
<td>L-Aspartic acid</td>
<td>++</td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>±</td>
</tr>
<tr>
<td>L-Asparagine</td>
<td>0</td>
</tr>
<tr>
<td>L-Glutamine</td>
<td>0</td>
</tr>
<tr>
<td>Hydroxy-L-proline</td>
<td>±</td>
</tr>
<tr>
<td>Ammonium nitrate</td>
<td>+</td>
</tr>
<tr>
<td>Ammonium chloride</td>
<td>±</td>
</tr>
<tr>
<td>Sodium nitrate</td>
<td>trace</td>
</tr>
<tr>
<td>Saline-agar control</td>
<td></td>
</tr>
<tr>
<td>Casitone control</td>
<td>++++</td>
</tr>
</tbody>
</table>

* An overlay of 0.2 ml of rabbit serum was added to all tubes.
the amino acid mixtures appeared to support growth best, the most stimulatory single amino acid in this group being aspartic acid.

An increment in growth was also noted with various concentrations of ammonium nitrate, 25 mg of nitrogen per 100 ml of medium being optimal. Tests with ammonium chloride and sodium nitrate indicated that nitrogen was more readily available in the ammonium than in the nitrate form.

The addition of mixtures of vitamins and minerals to any of these nitrogen sources did not enhance growth, nor could these compounds substitute for the factors in serum which are vital to *H. ducreyi*. Although time did not permit the testing of individual vitamins or mineral salts other than biotin, concentrations of 0.125 and 0.250 µg of biotin per 5 ml of medium enhanced growth when added to mixtures of all of the stimulatory amino acids. No enhancement occurred with a richer medium such as casitone; nor did biotin stimulate growth on vitamin-free casamino acids.

The nature of the growth factors in blood. Whole blood appeared to support growth better than either serum or plasma. The organism could be maintained on nutrient agar slants overlaid with whole blood diluted 1:128, whereas serum supported transferable growth up to a dilution of 1:32. Plasma gave the poorest growth of all, which was transferable only up to a dilution of 1:4. It was possible, however, by serial transfers to tubes of equal dilution and a 2-fold higher dilution, to obtain cultures which could be maintained over many passages in as high a dilution of blood as 1:1024. It was not possible to obtain a culture which would grow in the absence of blood.

The supernatant fluid from the coagulum of boiled diluted serum, when combined with peptone, supported good transferable growth. However, the organism grew slightly or not at all in a similar medium made with autoclaved serum supernatant.

When blood agar plates were overlaid with various thicknesses of nutrient agar, the essential factors in blood diffused through 12 mm of agar in sufficient quantities to support good growth. A few minute colonies could still be obtained on a 16-mm thick overlay.

No requirement for X and V factors could be established for *H. ducreyi*. Satellite growth of *H. ducreyi* was not induced by *S. faecalis*, strain 40a or *S. lutea*, strain 36b on the serum-free medium, nor was there any enhancement of growth in the vicinity of the streaks in the presence of increasing amounts of serum. Typical satellite growth was demonstrated on the control plates using *H. influenzae*.

When one part of rabbit serum was dialyzed against five parts of sterile distilled water, both the serum residue and the dialyzate were found to contain factors necessary for the growth of *H. ducreyi*. However, neither fraction was able to support growth alone. Enough of these factors were present in 0.1 ml of residue and 0.4 ml of the dialyzate to permit slight growth but, by doubling the amounts of each, growth was greatly increased. The dialyzable factors were found to be stable after 30 min of boiling but were destroyed when autoclaved for 15 min.

The work of Eagle and Steinman (1943) with the Reiter strain of *Treponema pallidum* suggested that bovine albumin might effectively replace the serum residue fraction. This was found to occur also in our studies. As little as 0.1 ml of 5 per cent bovine albumin Fraction V (Armour), when combined with rabbit serum dialyzate, supported growth. However, neither crystalline bovine albumin (Armour) nor soluble egg albumin (Difco) could be substituted for Fraction V.

Unlike the findings of Eagle and Steinman, neither glucose nor sodium acetate could replace the essential dialyzable factors in rabbit serum. Negative results were also obtained when the amino acids found by chromatographic means to be present in the dialyzate were substituted. However, undiluted Sims’ serum ultrafiltrate and supplement B (Difco), diluted 1:8, could be used in place of the dialyzate. Furthermore, supplement B was found to support growth when combined with crystalline bovine albumin or egg albumin.

**DISCUSSION**

In the presence of a small amount of rabbit serum, the nutritional requirements of *H. ducreyi* were satisfied by any one of a number of peptones prepared in a physiological sodium chloride solution. The need for sodium chloride at this concentration seemed to be specific as other salts failed to permit growth. Because no increase in the amount of growth occurred in the presence of glucose or other sugars, it is likely that this organism utilized as its carbon source the more complex organic substances
occurring naturally in peptones or serum or possibly, atmospheric carbon dioxide.

It was found that several inorganic nitrogen compounds, single amino acids, amino acid mixtures, and acid-hydrolyzed caseins supported growth to a limited extent, but \textit{H. ducreyi} grew much better in media containing partially hydrolyzed proteins such as peptones.

The work of Beeson (1946) has shown that either serum or large quantities of washed erythrocytes can supply the nutritional factors in blood essential for \textit{H. ducreyi}, that these factors are not identical, and that whole blood is more effective than either of these components. The present experiments give additional evidence of the superiority of whole blood over serum or plasma in supporting the growth of \textit{H. ducreyi}. The fact that erythrocytes seem to play a role in enhancing and maintaining growth has been noted frequently in the laboratory, for \textit{H. ducreyi} invariably grows better and survives longer on whole-blood enriched media than in cultures containing serum. Although red blood cells have been shown to contain a small proportion of the essential growth factors, it is possible that their ability to absorb toxic metabolic products may account for the superiority of whole blood over serum. The contention of Greenblatt and Sanders (1944) and Beeson that factors in blood other than X and V are responsible for the growth of \textit{H. ducreyi} is further supported by the negative results obtained with the satellite test of Pickett and Stewart.

Although Beeson first demonstrated the stability of the essential factors in serum to boiling but not to autoclaving, our experiments show that after the serum proteins were coagulated by heat and removed by centrifugation, the growth-supporting elements were found to have remained in the supernatant. This indicates that whole serum proteins are not essential and that the ability of bovine albumin fraction V to replace the serum residue after dialysis is probably not due to the albumin but rather to one or more substances carried by it. The ability of the factors in blood to diffuse through agar gives additional evidence that these factors are not whole proteins, but rather compounds of relatively small molecular size.

The results also suggest that an impurity in bovine albumin fraction V, not present in crystalline bovine albumin or in egg albumin but essential for the growth of \textit{H. ducreyi}, is absent from the serum dialyze and is supplied by supplement B.

\textbf{ACKNOWLEDGMENT}

The authors wish to thank Dr. Lucille K. Georg, Mycology Unit, Communicable Disease Center, for her advice concerning certain of the nutritional experiments.

\textbf{SUMMARY}

Any one of several peptones dissolved in physiological saline to which a small amount of rabbit serum was added satisfied the growth requirements of a virulent strain of \textit{Haemophilus ducreyi}. This organism seemed to be able to utilize to a very limited extent a number of amino acids, inorganic nitrogen compounds and acid-hydrolyzed caseins, but grew much better in media containing partially hydrolyzed proteins as peptones. A mixture of vitamins and minerals did not appear to enhance growth, but biotin alone had a stimulatory effect in some media.

Although \textit{H. ducreyi} could be adapted to grow in the presence of minute quantities of blood, it would not grow in a medium from which blood in some form was entirely eliminated. Whole blood was superior to serum or plasma in supporting growth and prolonging viability, but this may have been due in part to the ability of erythrocytes to absorb toxic metabolic products.

The essential factors in serum were stable at 100°C, were diffusible through agar, and could not be identified as X and V factors. They were found in the residue and dialyze of dialyzed rabbit serum, neither fraction being able to support growth alone. Commercially prepared bovine albumin could be used to replace the serum residue and “Sims’ serum ultrafiltrate” or “supplement B” could be substituted for the dialyze.

\textbf{REFERENCES}


