Thiamine Pyrophosphate (Cocarboxylase) as a Growth Factor for *Haemophilus somnus*

MAURICE D. ASMUSSEN AND CLARENCE L. BAUGH*

Department of Biological Sciences, Texas Tech University, Lubbock, Texas 79409

Received 23 January 1981/Accepted 15 April 1981

The effect of a commercially available, chemically defined enrichment (IsoVitaleX; BBL Microbiology Systems, Cockeysville, Md.) on the growth of 10 strains of *Haemophilus somnus* was studied. A 6- to 10-fold increase in growth, as measured turbidimetrically, was observed when IsoVitaleX was added to a basal medium of brain heart infusion broth to a final concentration of 1% (vol/vol). Thiamine pyrophosphate (cocarboxylase), a constituent component of IsoVitaleX, was found to be the only growth-promoting factor, and it could be used as a substitute for IsoVitaleX. An equimolar concentration (2.2 μM) of thiamine monophosphate promoted growth equal to that of thiamine pyrophosphate. Thiamine was nonstimulatory for all 10 strains tested. When alkaline thermal-treated brain heart infusion broth was used as the basal medium, 7 of the 10 strains had an absolute requirement for thiamine monophosphate or thiamine pyrophosphate. The three remaining strains showed minimal growth when thiamine was added to this basal medium; however, excellent growth was observed when thiamine monophosphate or thiamine pyrophosphate was utilized. Factor X (hemin) was found to further enhance the growth when concentrations of 5 to 10 μg/ml were coupled with thiamine pyrophosphate. No increase in growth was observed when factor V (nicotinamide adenine dinucleotide) was coupled with thiamine pyrophosphate. This is the first report of a growth factor requirement for *H. somnus.*

*Haemophilus somnus* is the etiologic agent of several diseases of cattle. This small gram-negative pleomorphic coccobacillus is involved in a variety of syndromes including thromboembolic meningo-encephalitis (1, 9, 11, 16), sudden death due to septicemia, acute respiratory disease, polyarthritis and tendinitis, necrotic laryngitis, polyloid tracheitis (16), and abortions (18). It also has been suggested that *H. somnus* may be involved in the weak calf syndrome (20).

Although this organism has been the subject of considerable study, its appropriate taxonomic position has not been resolved. Bailie (W. E. Bailie, Ph.D. thesis, Kansas State University, Manhattan, Kans., 1969) suggest the name *H. somnus* because it has a base composition of deoxyribonucleic acid similar to that of *Haemophilus influenzae* (*H. somnus*: 37.3 mol% guanine plus cytosine; *H. influenzae*: 38.2 mol% guanine plus cytosine), shares numerous taxonomic characteristics in common with members of that genus, and causes a sleeper syndrome in some clinically affected cattle. However, this name was not validly published, and, in 1973, Bailie et al. (2) concluded that their results did not justify the inclusion of the organism in the genus *Haemophilus* because of its lack of requirement for factors X (hemin) or V (nicotinamide adenine dinucleotide) or both for growth.

From nutritional studies, Kennedy et al. (11) determined that fresh isolates of the microorganism failed to grow on media not containing blood, body fluids, or certain other enrichment substances of animal origin. After continuous culture on blood agar, the organism could be propagated on a number of media that previously had failed to support growth of fresh isolates. These included serum broth and agar, hemoglobin agar (the constituents of which had been autoclaved for 30 min), and beef infusion broth, although growth was never abundant in infusion broth. A 1% yeast hydrolysate agar proved to be superior to blood medium for the propagation of the organism, and growth was equally abundant in cases in which the yeast hydrolysate had been sterilized by Seitz filtration, as after autoclaving for 45 min. As yet, no defined coenzyme-like substance has been identified as a growth factor.

The use of IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) as an additive to certain media as a substitute for yeast autolysate.
has been recommended for the isolation and cultivation of nutritionally fastidious microorganisms (14, 19). Recently, it has been advocated as a supplement to Mueller-Hinton agar with hemoglobin in the isolation of Legionella pneumophila (15). Results presented in the current study demonstrate that IsoVitaleX can be used as an alternative to beef serum and yeast extract to promote the growth of H. somnus in a beef infusion broth. The chemicals composing IsoVitaleX were studied compositely and singularly to identify the growth-promoting factors(s).

MATERIALS AND METHODS

Bacterial strains. Strains 1528, 5511, 5512, 8025, 8904, 13285, and 13401 of H. somnus were obtained from L. N. Brown and C. Reggiardo, Texas A & M Diagnostic Laboratory, Amarillo, Tex. H. somnus strains 1441, 1489, and 14557 were obtained from L. H. Lauerman, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, Colo. On receipt, all strains were checked for purity on brain heart infusion (BHI) (Difco Laboratories, Detroit, Mich.) agar supplemented with 0.5% (wt/vol) yeast extract (Difco Laboratories) and 5% (vol/vol) defibrinated sheep blood cells (BHI-YSC agar), and their identity was confirmed by the more typical microbiological characteristics as described by Bailie et al. (2). Strains were subsequently transferred to BHI broth containing 20% (vol/vol) glycerol and stored at −70°C.

Media and enrichment. BHI and alkaline thermal-treated BHI broths were used as the test media. Alkaline thermal-treated BHI broth was prepared by adjusting 500 ml of BHI broth to pH 9 with 10 N NaOH, refluxing at 100°C for 30 min, and adjusting the cooled broth to pH 7.2 with 2.5 N HCl. The pH-adjusted broth was sterilized by routine autoclaving. Alkaline thermal treatment was used to inactivate thiamine or thiamine derivatives endogenous to BHI broth (5).

IsoVitaleX enrichment was rehydrated with 10 ml of sterile glass-distilled water rather than the 10 ml of 10% (wt/vol) dextrose solution supplied by the manufacturer. The enrichment was added to test media to a final concentration of 1.0% (vol/vol) as recommended by the manufacturer.

Reagents. Chemicals utilized for the duplication of the chemical composition of IsoVitaleX were of commercial preparation: thiamine pyrophosphate (cocarboxylase), thiamine HCl, L-glutamine, adenine, guanine HCl, p-aminobenzoic acid, and L-cysteine (Sigma Chemical Co., St. Louis, Mo.); L-cysteine HCl and nicotinamide adenine dinucleotide (NAD) (Nutritional Biochemicals Corp., Cleveland, Ohio); and cyanocobalamin (vitamin B12) (Eastman Kodak Co., Rochester, N. Y.). Other chemicals used for growth-promoting potential that are not constituent components of IsoVitaleX were thiamine monophosphate acid chloride (Sigma Chemical Co.) and hemin. All commercial reagents except L-cysteine were prepared in glass-distilled water, sterilized by membrane filtration (0.45-µm filter; Millipore Corp., Bedford, Mass.), and stored at 4°C. L-Cystine was prepared in 0.1 N HCl, sterilized, and stored as described above. Hemin was prepared from human blood collected in 5-ml samples in Vacutainer tubes containing 7.5 mg of sodium edetate (Becton, Dickinson & Co., Rutherford, N. J.). The blood was pooled and centrifuged at 1,500 × g for 10 min at room temperature, the plasma and Buffy coat were discarded, and the erythrocytes were washed three times with an equal volume of physiological saline. Hemin was then extracted from the washed erythrocytes with saturated NaCl-gla
cic acid solution according to the method of Fisher (6). Fresh solutions of hemin were prepared just before use by dissolving the crystals in a 0.1 M NaHPO4 solution and autoclaving the resulting solution at 121°C for 15 min. All reagent solutions were added to previously sterilized test media to a final concentration of 1.0% (vol/vol).

Preparation of inoculum and growth studies. For each experiment, strains were thawed and streaked to BHI-YSC agar and incubated in a candle extinction jar or 5% (vol/vol) CO2-in-air environment at 37°C for 24 h. Isolated colonies were removed with sterile cotton-tipped swabs and suspended in 10 ml of BHI broth enriched with 10% (vol/vol) fetal bovine serum (KC Biologicals, Inc., Lenexa, Kans.) and 0.5% (wt/vol) yeast extract. The cell suspensions were blended in a Vortex mixer (Vortex-Genie, model K-550-G) to disperse clumps of cells, and incubated without shaking for 24 h. The 24 h cultures were centrifuged at 2,500 × g for 10 min at room temperature, washed twice with sterile phosphate-buffered saline (pH 7.0), and standardized to a 0.20 optical density reading at 660 nm on a Bausch and Lomb Spectronic 70 spectrophotometer. The cell suspensions were then diluted 1:100 in phosphate-buffered saline, and 0.1-ml samples of these diluted cell suspensions were inoculated in duplicate into optically standardized screw-capped tubes (16 by 125 mm) containing 10 ml of the appropriate test broth media to give an approximate initial inoculation of 5 × 104 colony-forming units per ml. After 24 to 48 h of incubation without shaking, final optical densities were measured at 660 nm. All broth cultures were incubated in a 5% (vol/vol) CO2-in-air environment at 37°C.

RESULTS

Bacteriological identification. Confirmation of the identity of the 10 strains of H. somnus was by the following: (i) Gram stain reaction was negative and organisms were nonmotile in wet-mount preparation; (ii) colonies were smooth, entire, and pinpoint size, and in areas of heavy confluent growth there was a green-brown discoloration of blood agar; (iii) lemon-yellow pigment was apparent when colonies were picked with a needle from the surface of the agar plate; (iv) no appreciable growth was observed under aerobic conditions; (v) catalase reaction was negative and cytochrome oxidase reaction was positive; (vi) no growth on MacConkey agar; and (vii) growth occurred in the absence of both X
and V factors.

Growth studies. The influence of adding complex enrichment (fetal bovine serum and yeast extract) or defined enrichment (IsoVitaleX) to a basal medium of BHI broth on the growth of H. somnus strain 8025 is shown in Fig. 1. The unsupplemented basal medium did not show significant growth throughout the 30-h growth period. To determine if the minimal growth in the basal medium was due to endogenous nutrients from the initial inoculum, primary and secondary passages were made. Growth in primary and secondary cultures persisted with a minimal growth response, equal to that of the original culture. Maximum growth was obtained for complex enrichment as measured both turbidimetrically (optical density, 0.64) and by colony-forming units per ml (5 x 10^9 colony-forming units ml) in 18 h. The IsoVitaleX-enriched basal medium showed a comparable growth rate; however, final optical density (0.49) was less than that observed for complex enrichment. Increasing the concentration of IsoVitaleX did not significantly increase the final growth.

Although there were some variations in the final optical densities, all 10 strains showed similar growth responses to the two supplements. IsoVitaleX increased growth 6- to 10-fold over that observed in the basal medium. Strains 5511 and 1441 did not grow in the basal medium in the first 24 h, however minimal growth was observed within 48 h.

For convenience, the components of IsoVitaleX were divided into two groups (A and B) and the growth potential of each group was studied (Table 1). Group A, containing vitamin B12, L-glutamine, adenine, guanine HCl, p-aminobenzoic acid, and NAD, was found to be non-stimulatory, and the growth response was similar to that of the basal medium. Group B, containing thiamine pyrophosphate, thiamine, L-cysteine, L-cystine, and ferric nitrate, was found to promote growth equal to that of IsoVitaleX. Further studies of group B chemicals (Table 1) revealed that thiamine pyrophosphate was the primary growth-promoting component of IsoVitaleX.

A molar concentration equal to that of thiamine pyrophosphate in IsoVitaleX (2.2 μM) was

![Graph](http://jcm.asm.org/)

**Fig. 1. Comparative growth of H. somnus strain 8025 in BHI broth with complex enrichment (10% fetal bovine serum and 0.5% yeast extract) (●), defined enrichment (1% IsoVitaleX) (▲), and no enrichment (■).**

**Table 1. The effect of IsoVitaleX and other supplements on the growth of 10 strains of H. somnus.**

<table>
<thead>
<tr>
<th>Addition to brain heart infusion broth (μg/ml)</th>
<th>Optical density* (± standard error)b</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.04 (0.008)</td>
</tr>
<tr>
<td>IsoVitaleX</td>
<td>0.54 (0.014)</td>
</tr>
<tr>
<td>Components of IsoVitaleX</td>
<td></td>
</tr>
<tr>
<td>Group A chemicals</td>
<td>0.06 (0.009)</td>
</tr>
<tr>
<td>Group B chemicals</td>
<td>0.51 (0.012)</td>
</tr>
<tr>
<td>Individual group B chemicals</td>
<td></td>
</tr>
<tr>
<td>Thiamine pyrophosphate (1.0-2.2 μM)</td>
<td>0.51 (0.011)</td>
</tr>
<tr>
<td>Thiamine HCl (0.03-0.09 μM)</td>
<td>0.06 (0.008)</td>
</tr>
<tr>
<td>L-Cysteine (259.0)</td>
<td>0.06 (0.008)</td>
</tr>
<tr>
<td>L-Cystine (11.0)</td>
<td>0.06 (0.008)</td>
</tr>
<tr>
<td>Ferric nitrate (0.2)</td>
<td>0.06 (0.008)</td>
</tr>
<tr>
<td>Thiamine HCl (0.73-2.2 μM)</td>
<td>0.05 (0.012) (7.3-22 μM)</td>
</tr>
<tr>
<td>Thiamine monophosphate (0.83-2.2 μM)</td>
<td>0.50 (0.009)</td>
</tr>
<tr>
<td>Hemin (10.0)</td>
<td>0.04 (0.007)</td>
</tr>
<tr>
<td>Hemin (10.0) + thiamine pyrophosphate (1.0)</td>
<td>0.61 (0.008)</td>
</tr>
<tr>
<td>NAD (10.0)</td>
<td>0.05 (0.006)</td>
</tr>
<tr>
<td>NAD (10.0) + thiamine pyrophosphate (1.0)</td>
<td>0.50 (0.009)</td>
</tr>
</tbody>
</table>

a Optical density measured at 660 nm after 24 h of incubation.  
b Data represent the mean of each strain tested in duplicate (n = 20).  
*Composite of (in μg/ml) vitamin B12 (0.1), L-glutamine (100.0), adenine (10.0), guanine HCl (0.3), p-aminobenzoic acid (0.13), and NAD (2.5).
studied for thiamine and thiamine monophosphate (Table 1). It was found that thiamine was nonstimulatory at an equimolar concentration and a 10-fold molar concentration (22 μM). However, in contrast to thiamine, thiamine monophosphate promoted growth similar to that of thiamine pyrophosphate.

Although hemin (factor X) and NAD (factor V) are not required for the growth of _H. somnus_, these growth factors were studied for growth-promoting potential (Table 1). Neither hemin nor NAD were stimulatory without thiamine pyrophosphate. The addition of 5 to 10 μg of hemin per ml to BHI broth containing 1 μg of thiamine pyrophosphate per ml markedly enhanced growth, and strains 13401, 5512, and 13285 showed growth equal to or better than that obtained when the basal medium was supplemented with fetal bovine serum and yeast extract. Concentrations of 1 to 40 μg/ml of NAD were coupled with thiamine pyrophosphate, and no enhancement in growth over that produced by thiamine pyrophosphate was observed.

Unusually high concentrations of thiamine, thiamine monophosphate, and thiamine pyrophosphate were added to BHI broth to determine their effects on the growth of _H. somnus_ strain 8025. Thiamine, at a concentration of 50 μg/ml, completely inhibited the minimal growth observed in BHI broth. In contrast, the phosphorylated derivatives produced excellent growth at the same concentration. To determine if thiamine pyrophosphate could overcome the inhibitory effect of thiamine, increasing concentrations of thiamine pyrophosphate were added to broth cultures containing 100 μg of thiamine per ml. Thiamine pyrophosphate overcame the inhibitory effect of 100 μg of thiamine per ml; however, a 12-fold increase (0.1 μg/ml) over the control (0.008 μg/ml) was required to establish maximum growth.

The growth response of each strain of _H. somnus_ in a supplemented and nonsupplemented basal medium of alkaline thermal-treated BHI broth was studied. No growth was observed in the nonsupplemented basal medium for any isolate, and only minimal growth was observed for strains 13401, 5512, and 13285 after 48 h of incubation when thiamine was added. If the phosphorylated thiamines were added, all 10 strains showed excellent growth in 24 h with a response equal to that of untreated BHI broth enriched with thiamine pyrophosphate.

**Microscopic observations.** Although _H. somnus_ grew primarily as single cells with few filaments in a BHI broth enriched with fetal bovine serum and yeast extract, a notable change in cell morphology was observed when it was grown in BHI broth enriched with IsoVitaleX or thiamine pyrophosphate (Fig. 2). These cultures grew with extensive filamentation and, frequently, chains of 8 to 15 bacilli were observed. This change in cell morphology led to difficulties in obtaining accurate, reproducible cell counts from broth cultures supplemented with these two enrichments. Filamentation persisted with the addition of yeast extract, but beef serum allowed for single cell growth to occur. Furthermore, the component(s) of beef serum that allows for single cell morphology to occur was found to be heat stable, as determined by routine autoclaving.

**DISCUSSION**

Using IsoVitaleX as an alternative to fetal bovine serum and yeast extract in the growth of _H. somnus_ not only markedly enhanced the growth of the bacterium over that observed in a basal medium of brain heart infusion broth, it also provided specific information as to a growth requirement for this microorganism. However, because of the presence of trace amounts of thiamine-phosphorylated thiamines in the basal medium, it was necessary to thermally inactivate these thiamines under alkaline conditions to demonstrate an absolute requirement for thia-

![Fig. 2. Cell morphology of _H. somnus_ grown in BHI broth with 10% fetal bovine serum and 0.5% yeast extract (A) and 1% IsoVitaleX (B). Magnification, ×800.](http://jcm.asm.org/Downloaded from http://jcm.asm.org/ on May 7, 2021 by guest)
mine, thiamine monophosphate, or thiamine pyrophosphate. This is the first report of a growth factor requirement for *H. somnus*.

There are rare reports of organisms having an absolute requirement or preferentially utilizing the coenzyme thiamine pyrophosphate over that of the vitamin thiamine. Lankford and Skaggs (12) found occasional strains of *Neisseria gonorrhoeae* that would not grow on semisolid medium adequately supplemented with thiamine and L-glutamine. It was only when a thermostable factor present in yeast, blood, or tissue extracts was added to the medium that they were able to successfully cultivate the gonococci. Thiamine pyrophosphate was found to substitute completely for these natural materials. The seven strains of *H. somnus* that displayed an absolute requirement for thiamine monophosphate or pyrophosphate and could not utilize the coenzyme precursor thiamine resembled the etiological agent, *Haemophilus piscium*, of “ulcer disease” in salmonoid fishes (7, 8). The three strains of *H. somnus* that efficiently used the phosphorylated thiamine, but in which free thiamine had only limited growth-promoting activity, showed growth characteristics similar to that of *Pasteurella haemolytica* as described by Wessman (21). However, in contrast to *H. somnus* that would not grow efficiently with higher concentrations of thiamine, Wessman found that *P. haemolytica* could be sufficiently stimulated to grow with unusually high levels of thiamine. *H. somnus* strain 8025 was completely inhibited at higher concentrations of thiamine. A more than 10-fold increase in thiamine pyrophosphate over that of control was required to establish maximum growth in the presence of 100 μg of thiamine per ml suggests that the inhibition was competitive.

The data are not adequately explained by simply suggesting that *H. somnus* cannot phosphorylate thiamine efficiently. Thiamine monophosphate is not an intermediate in the conversion of thiamine to thiamine pyrophosphate in other systems. In bakers’ yeast (3) and in rat liver (13), the monophosphate is first dephosphorylated to thiamine, and thiamine pyrophosphate is formed by direct pyrophosphorylation of thiamine with the aid of adenosine triphosphate. Considering that *H. somnus* requires thiamine monophosphate or thiamine pyrophosphate for efficient growth, the free thiamine formed when thiamine monophosphate was provided as the source of the vitamin should result in limited growth. It may be reasonable to suggest that the phosphorylated forms of thiamine are transported more readily into the cell or that the response to the monophosphate is a reflection of its direct conversion to the diphosphate. Additional studies are needed to resolve the physiological mechanisms of thiamine incorporation and activation in *H. somnus*.

The filamentous nature of *H. somnus* in brain heart infusion broth supplemented with IsoVitaleX suggests that this characteristic may be a response to the more limited nutritional quality of this more specifically defined medium. Studies that have dealt with the growth of two different species of *Lactobacilli* in media with suboptimum concentrations of vitamin B₆ (10) or B₁₂ (4) indicate that such deficiencies led to abnormal cell morphology or to the formation of long filamentous cell forms respectively. A deficiency in B₆ or B₁₂ cannot be advocated in the abnormal cell morphology observed with *H. somnus* because adding yeast extract as a source of B₆ or increasing the level of B₁₂ above that already present in IsoVitaleX would not reverse the filamentous cell forms. It was only when fetal bovine serum was added to the test medium containing IsoVitaleX that we were able to observe single bacillary forms. Apparently a metabolic characteristic of serum allows for single cell growth to occur in the *H. somnus* bacterium. The specific characteristic of beef serum was not resolved.

Although no attempt was made to adapt *H. somnus* to an assay method for thiamine pyrophosphate, the sensitivity to concentrations as low as 0.001 μg/ml suggest such a possibility. The obvious disadvantage of such a test organism is that the growth response is similar for thiamine monophosphate and thiamine pyrophosphate.

It is interesting to note that the requirement for thiamine may account in part for the clinical features observed when cattle are infected with *H. somnus*. The differential diagnosis of infectious thromboembolic meningocencephalitis (causative agent; *H. somnus*), polioencephalomalacia, hypovitaminosis A, lead poisoning, and listerial meningocencephalitis in cattle is often difficult to establish because of similarities in neurological symptoms (17). Of particular interest is that of polioencephalomalacia; cattle affected by this disease are in a state of thiamine depletion as a result of changes in symbiotic ruminal bacteria. The thiamine depletion results in a number of neurological symptoms due to the highly sensitive nature of the central nervous system to depressed levels of thiamine. Undoubtedly, the majority of the neurological symptoms caused by *H. somnus* result from direct infection of the brain. However, the persistence of neurological signs in cases in which the respiratory and skeletal systems are the sites
of infection (16), may be accounted for by the exhaustion of thiamine by the H. somnus bacterium. The growth response to phosphorylated thiamine does not resolve the present uncertainties of the taxonomic position of H. somnus. Although members of the genus Haemophilus have been classified only in terms of their requirement for factors X and V (22), it appears that H. somnus should be classified as a Haemophilus incertae sedis, based on its fastidious nutritional requirements and moles percent of guanine plus cytosine.

ACKNOWLEDGMENT

This work was supported in part by a grant from Texas Vet Labs, Inc., San Angelo, Texas.

LITERATURE CITED