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Cultural and Biochemical Characteristics of a Leptospire from Frog Kidney

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ABSTRACT

Growth of a leptospire isolated from frog kidney tissue was stimulated by the addition of glucose to a bovine albumin polysorbate 80 medium, did not grow in the absence of polysorbate 80, but did respire on a complex of polysorbate 80 and bovine albumin. Growth was completely inhibited by 2-deoxy-D-glucose in a glucose-free bovine albumin polysorbate 80 medium. A requirement for vitamins B_1 (thiamine) and B_{12} was demonstrated. Oxythiamine and the thiazole fragment of thiamine were nearly equivalent to vitamin B_1 in growth supporting activity while thyamine pyrophosphate, thyamine monophosphate, the pyrimidine fragment of vitamin B_1 and pyrithiamine were much less effective. The isolate was continually propagated at 31.5, 29, 20, 15, and 9 C. Growth could be initiated at 33.5-34 C. but serial subculture failed. Growth was not initiated at 35 u.c. The organism had a cellular but no extracellular tributyrinase.

INTRODUCTION

A leptospire from frog kidney tissue has been isolated and some of its serologic characteristics described.[4,5] For years there has been the question of why leptospires grow optimally at 29 C *in vitro* while parasitizing hosts of higher body temperature. Since the frog kidney is considered to be primitive in nature and the amphibian is a cold-blooded host, the isolation of this leptospire from frog kidney offered an opportunity to study an organism which might have characteristics in keeping with the host from which it was isolated. This organism was studied during a search for leptospires with distinctive upper and lower temperature growth limits. It was desired to find a model which could grow continuously at elevated temperatures (35 C and above) and at low temperatures. The objectives of this study were: (1) to define some specific nutritional requirements and (2) to determine the upper and lower temperature limits for growth of the frog kidney isolate.

MATERIALS AND METHODS

The leptospiral isolate was maintained at room temperature in a bovine albumin-polysorbate 80 medium[8] containing 0.2% agar. The bovine albumin-polysorbate 80 liquid medium was supplemented with D-glucose to a final concentration of 0.36%. Glucose was added to medium as late as eleven days after the start of incubation. Attempts were made to replace the polysorbate 80 in the medium by supplementing the basal medium lacking polysorbate 80 with (1) 0.36% glucose, (2) 1% albumin, or (3) 1% albumin and 0.36% glucose. Concentrations of glucose employed were 1,000 to 3,600 ug/ml. The sugar analogue 2-deoxy-D-glucose was used at a concentration of 900 μg/ml of complete medium. The glucose (20% solution in sterile distilled H_2O) was sterilized by filtration through serum sterilizing pads (Seitz-100 mu porosity). Growth studies were performed in 300 ml baffled nephelo-culture flasks* using 50 and 100 ml

* (JP-520-727-F-514), Bellco Glass, Inc., Vineland, N.J.

of medium. For inoculum, cells in the logarithmic phase of growth were centrifuged, washed twice, and resuspended in 0.005 M phosphate buffer (pH 7.5) to a nephelometer density reading[10] of 15 plus or minus 2. Each flask was inoculated with 10.0 ml of washed cells. Magnetic stirring bars 50 mm in length and 10 mm in diameter were incorporated in the culture flasks as previously described.[10] Soon after the organism from frog kidney (ICFK) tissue was isolated by Dr. S. L. Diesch of the University of Iowa, Institute of Agricultural Medicine liquid cultures were incubated at 35, 29, 15, 13, and 9 C.

Further determinations of the effect of temperature on growth were made at 33.5-34, 32.5, 31.5, 6, and 3 C. Where the temperature is designated, for example 33.5-34, this is meant to convey that the many temperature values recorded for this incubator were within this limit of a half degree. Thermometers immersed in distilled H_2O and graduated in 0.1 degree intervals per 1.0 C (0 to 50 C) were used in the incubators. Incubators were monitored for temperature fluctuations for 1 week prior to experimental use. Temperatures were recorded daily at 8:30 A.M. and 4 P.M.

The thiamine compounds were dissolved in 0.005 M phosphate buffer, and sterilized by filtering through 450 mu HA-type Millipore filters and the solutions stored at 5 and —80 C. Thiamine HCl, oxythiamine, thiamine pyrophosphate, thiamine monophosphate, and pyrithiamine were from Calbiochem, Los Angeles, Calif. 90063. The thiazole and pyrimidine fragments of thiamine were a gift from Dr. W. E. Scott of Hoffman-LaRoche, Inc., Nutley 10, N.J. All compounds were employed at a final medium concentration of 0.16 μg/ml.

The vitamin content of the albumin was determined through the assistance of the Pentex Corp. of Kankakee, Ill.; the assay being performed by the Wisconsin Alumnae Research Foundation Laboratory, Madison, Wisc.

Tributyrinase measurements of culture supernatant fluid, whole cells, sonicated cell suspension, and supernatant from sonicated cells were performed as previously described.[7]

Oxygen uptake by cell suspensions was determined by the Warburg technique.[80] Cells from log phase cultures were used on the same day of harvest. Whole cultures were harvested at room temperature by centrifuging in 250 ml polyethylene bottles using a 6 place GSA rotor of a Servall centrifuge at a speed of 7,000 rpm (7970 xG) for 20 minutes. The cell suspension was washed twice in 0.005 M phosphate buffer and concentrated so that a 1:10 dilution of this heavy suspension had an optical density of 0.85-0.90 at 400 mμ (Beckman-DU). The bacterial density of the adjusted cell suspension averaged 4.0-4.25 mg/ml. In order to diminish the endogenous respiration the flasks were shaken at 140 oscillations per minute for 60 minutes at 30 C before closing the manometers.

Lipid respiratory substrates were compounded using bovine serum albumin powder (#BV0262, Pentex Corp., Kankakee, Ill.) and sodium oleate (purified S-352, Fisher Scientific Co., Fairlawn, N.J.), oleic acid (#4954, California Biochemicals, Los Angeles, Calif.), and polysorbate 80 (Nutritional Biochemicals, Cleveland, Ohio). The concentration of lipid in these respiratory substrates was arbitrarily selected as 100 μg/ml comparable to the concentration of sodium oleate used in culture medium.[8] The lipid bovine albumin complexes were formulated in such a manner as to be equivalent to .000328 M oleic acid in a reaction volume of 2.0 ml per Warburg flask. The substrates were prepared in the following manner: Sodium oleate (M.W. 305)—100 mg was added to 20 ml of sterile distilled H_2O and this solution added dropwise to a constantly stirred solution of 10% bovine albumin powder in 60 ml of sterile Stuart's phosphate buffer. At final adjustment to 100 ml the solution had a pH of 6.97. When 0.2 ml of this concentrated solution was added to 1.8 ml of Stuart's buffer the pH was 7.37. This resulted in 100 μg/ml of sodium oleate equivalent to 0.328 micromole/ml and a total available concentration of 0.656 micromole/respirometer flask. Oleic acid (M.W. 282.45) was prepared in a 3.28 mM solution in a 10% bovine albumin solution by weighing 92.64 mg of acid and adjusting the volume to 100 ml; the addition of 0.2 ml of this substrate likewise resulting in 0.656 micromole/flask.

Polysorbate 80 (M.W. 1254-22.5% oleate (Davis[8])) was prepared by taking 1.828 g and dissolving in the albumin buffer solution similar to that employed for the sodium oleate and oleic acid.

RESULTS

Stimulatory effect of glucose. Although the leptospire was readily isolated in semisolid bovine albumin-medium,[8] early propagation in liquid medium was erratic. Since this had occurred previously with other leptospiral serotypes, this liquid medium was supplemented with glucose which resulted in stimulation of growth (Fig. 1). Glucose failed to stimulate growth in the basal medium when polysorbate 80 was lacking regardless of alterations of the composition of that medium (Fig. 2). Maximum growth was obtained with concentrations of glucose from 1,000 to 3,600 μg-ml in bovine albumin-polysorbate 80 medium. There was no indication of growth inhibition due to these levels of glucose. The replacement of glucose by 2-deoxy-D-glucose (900 ug/ml) in the bovine albumin-polysorbate 80 medium completely inhibited growth.

Vitamin requirements. The concentrations of vitamins in the powdered bovine albumin were determined (Table 1) prior to studying the requirements for vitamins B_1 and B_{12}. The effect of the deletion of either vitamin B_1 or vitamin B_{12} is presented (Fig. 3). There was minimal growth on a second subculture in the absence of vitamin B_{12}. Without thiamine, the pattern of growth was slightly different. With vitamin B_{12} present and no thiamine added, there was little lag period; however, growth was reduced. On second subculture without vitamin B_1 added there was minimal growth.

Oxythiamine, thiamine pyrophosphate, thiamine monophosphate, pyrithiamine, and the thiazole and pyrimidine fragments of vitamin B_1. The effect on growth of various forms of thiamine, pyrithiamine, and the two fragments of the vitamin are shown (Fig. 4 and 5). The growth stimulating activity of thiamine HCl remained unchanged, whether it was autoclaved in medium or added aseptically. Oxythiamine stimulated growth almost as much as thiamine HCl while the phosphorylated forms of thiamine were only approximately 50% as active as the hydrochloride. Pyrithiamine had little if any B_1 growth stimulating activity but was not seriously inhibitory as shown by the fact that 0.16 μg/ml of thiamine HCl stimulated growth in the presence of an equivalent amount of pyrithiamine (Fig. 5). The thiazole fragment of the vitamin was the active portion of the molecule when compared to the pyrimidine portion. The cellular yield in the absence of added thiamine (first subculture) was 45 mg dry weight per liter of culture medium, while in medium supplemented with thiamine HCl, thiaminmononitrate, or oxythiamine it was 165, 165, and 160 mg respectively.

TABLE 1. *Vitamin content of bovine albumin employed in polysorbate 80 bovine albumin medium.*

Vitamins	Micrograms/ Gram of albumin	Micrograms/ ml of medium
Biotin	0.001	.00001
Folic acid	0.038	.00038
Riboflavin	0.14	.0014
Viitamin B_6	0.306	.003
Niacin	0.33	.0033
PABA	1.0 (at most)	.01
Pantothenic acid	1.42	.0142
Inositol	154.000	1.54
Thiamine	0.1	.001
B_{12}	0.0171	0.000171milligammas

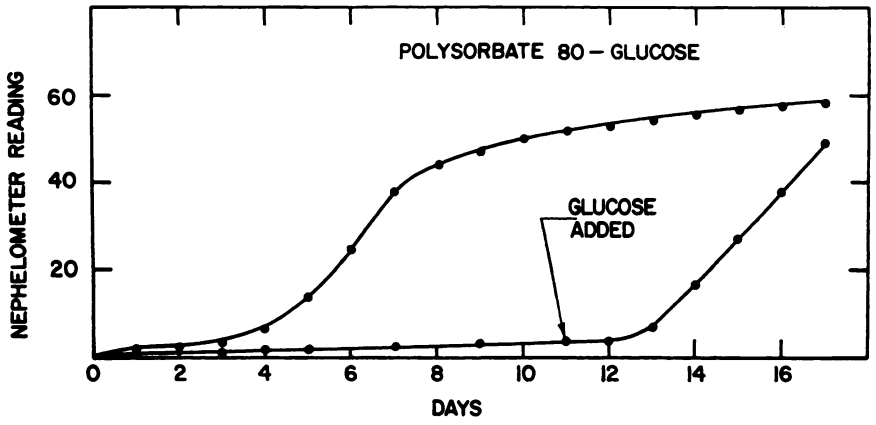

FIGURE 1. *The growth-stimulating effect of glucose on a leptospire (ICFK) isolated from the kidney of a frog.*

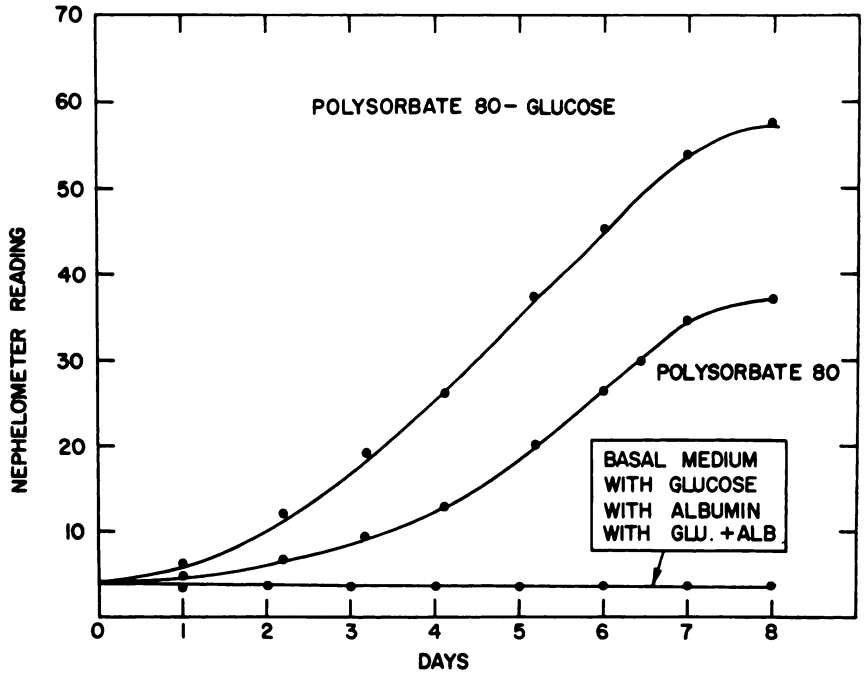

FIGURE 2. *Effect of polysorbate-80 and glucose upon growth of leptospire isolate ICFK.*

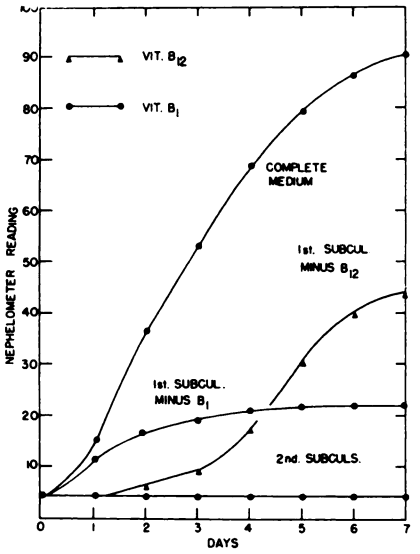

FIGURE 3. *Growth of leptospire ICFK in complete bovine albumin polysorbate-80 glucose medium, and first and second subcultures in media supplemented with vitamin B_{12} or thiamine (B_1) but not both.*

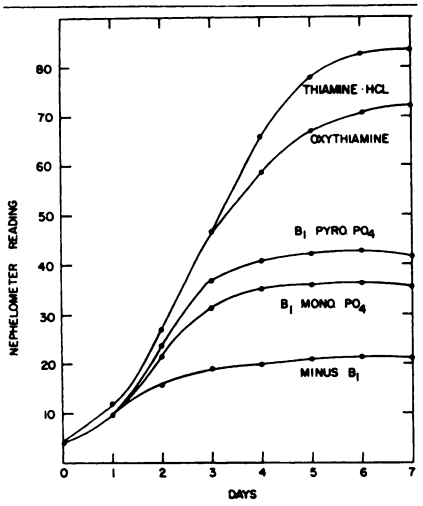

FIGURE 4. *Growth of leptospire ICFK in bovine albumin polysorbate-80 glucose medium supplemented with various forms of thiamine.*

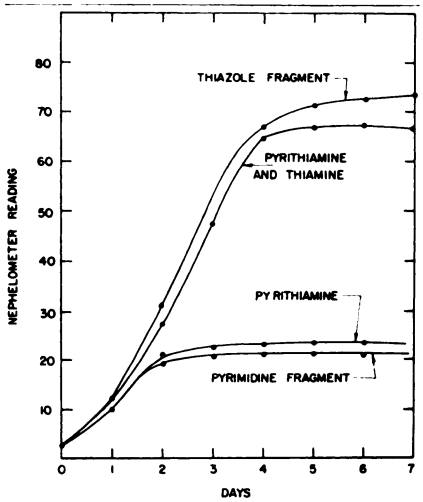

FIGURE 5. *Growth of leptospire ICFK in bovine albumin polysorbate-80 glucose medium with the thiazole fragment of thiamine, pyrithiamine and thiamine, pyrithiamine, and the pyrimidine fragment of thiamine.*

Effect of temperature upon growth. The influence of temperature on growth of the frog-kidney isolate is shown in Fig. 6 and 7. Growth was initiated at 33.5-34 C but serial subculture failed. The cells appeared stranded, filamentous, and 2 to 3 times longer than normal, when examined in the dark field. Growth at 33.5-34 C was only 40% of the growth at 29 C. Cells grown at 33.5-34 C resumed normal growth when subsequently incubated at 29 C. At 31.5 C there was a 30% reduction in growth compared to growth at 29 C. The total growth of the second subculture at 31.5 C equaled the first subculture and continuous propagation was possible. The optimum temperature for growth was 29 C. Excellent growth was obtained at 20 C. At 15 and 13C growth was continuous and undiminished. The inoculum cells for the 13 C culture were taken from log phase cells of the 15 C culture.. The first subculture at 9 C exhibited a lag period. Final growth at 9 C approached the total growth obtained at 13 and 15 C. Upon second subculture at 9 C, there was a diminished lag period. When log phase cells from the 9 C culture were transferred to 6 C, growth could not be measured by the nephelometer during the first 48 hours. At the end of 96 hours, the nephelometer reading had changed from 2 to 12 and terminal growth finally equaled a nephelometer reading of 66. Continuous propagation took place at 10 C with inoculum cells that were grown at 10, 15, or 29 C. Inoculum cells grown at 29 C when immediately placed in media at 6 and 3 C did not grow. These cultures at the two lowest temperatures had cells with full motility and after 10 days of non-growth could be restored to full growth when incubated at 29 C. The same was true of identical 6 and 3 C cultures after an extended incubation period of 31 days. When total growth (Fig. 7) was plotted for 4, 7, and 15 days the deleterious effect of temperatures above 29 C was obvious.

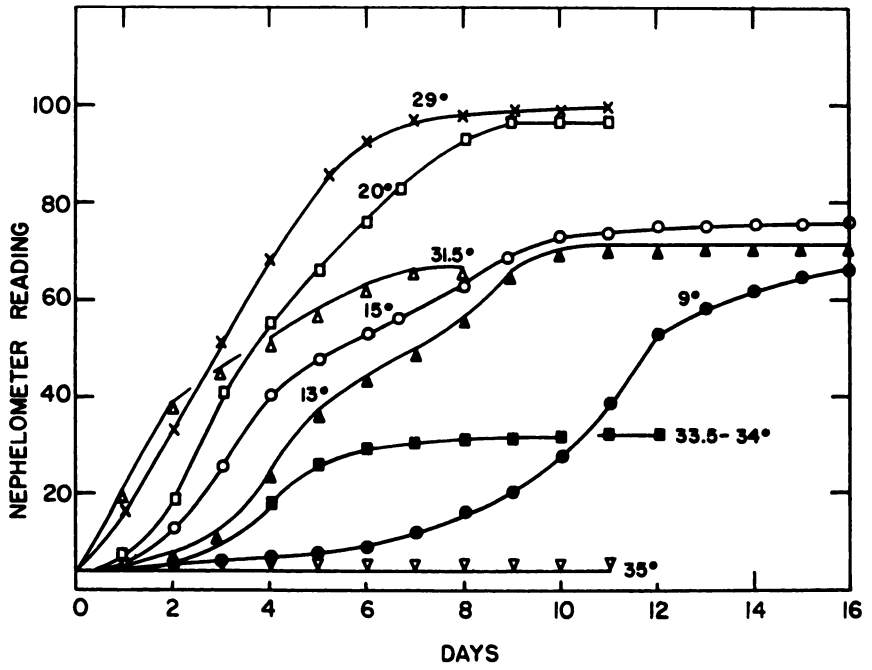

FIGURE 6. *Effect of incubation temperature on growth of leptospire ICFK.*

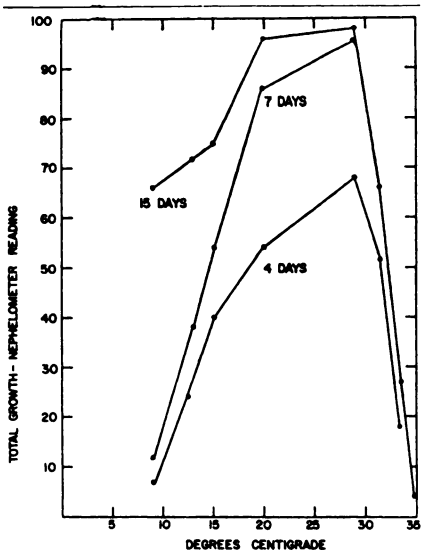

FIGURE 7. *Growth of leptospire ICFK at the end of 4, 7, and 15 days of incubation.*

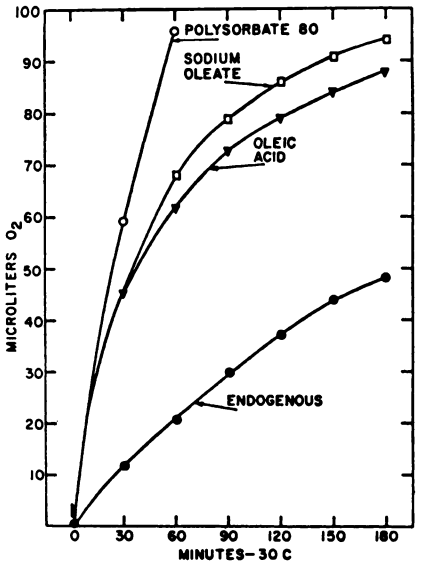

FIGURE 8. *Respiration of leptospire ICFK on complexes of albumin with polysorbate 80, sodium oleate, and oleic acid. Flask volume of 2.2 ml was composed of 0.2 ml 3N KOH in the center well, 1 ml of cell suspension, and the remaining 1 ml made of albumin detoxified lipid and buffer.*

Tributyrinase concentration. It has been found that some serotypes of lepto spires have both a cellular bound and soluble tributyrinase.[7] Therefore whole washed cells, sonicated cells, and cell-free supernatant culture medium were tested for tributyrinase concentration.[7] From each sample or dilution of sample to be examined, a 0.025 ml aliquot was delivered to a corresponding well in the tributyrin agar plate. The highest dilution in a two-fold series of dilutions which caused a demonstrable clearing reaction in the opaque agar was the "tributyrinase titer." The quantity of the tributyrinase present in 0.025 ml of this dilution was defined as one "diffusion unit" of enzyme. With whole cells there were 2.5 to 2.9 μg dry weight/diffusion unit. Sonication of whole cell suspensions did not result in a liberation of more tributyrinase. When culture supernatants derived from cultures started with as few as 500,000 cells/ml were tested periodically during the growth cycle and at maximum growth it was not possible to demonstrate an extracellular tributyrinase.

Respiratory activity. The oxygen uptake of washed cells of the frog kidney isolate on complexes of bovine albumin with polysorbate 80, sodium oleate, and oleic acid is shown in Fig. 8. The Q_{O_2} values for the previously mentioned substrates were 17.9, 11.3, and 9.9 respectively. During short term respiration studies with 50 micromoles of glucose/Warburg flask, 9.1% and 9.8% of the glucose disappeared when measured by the anthrone and enzymatic glucostat methods respectively. When the organism was propagated at 32.5 C the Q_{O_2} on bovine albumin-polysorbate 80 was 4.8 representing a 63% reduction in respiratory rate.

DISCUSSION

Glucose was stimulatory to growth of the frog kidney isolate and even high concentrations (3,600 μg/ml) did not exert an inhibitory effect upon growth. Although glucose was stimulatory to growth, the long chain fatty acid supplied in the form of polysorbate 80 had to be present in the medium in order to demonstrate the effect. A semi-solid medium without glucose supplementation was adequate for isolation, and continuous subcultures could be made in agarose medium (free of normal agar impurities) which emphasized the need of this leptospire for fatty acid. The inhibition of leptospiral growth by 2-deoxy-D-glucose in an adequate lipid medium has been reported.[9] It is interesting that a mere alteration of the number 2 carbon atom of glucose can have a specific growth-inhibiting action which in some way is related to the leptospire's mechanism of utilizing lipids in the growth process. Conventionally one would think that such a sugar analogue would be acting as a competitive inhibitor of a hexokinase. Further work on the enzymatic makeup of leptospires is very much needed since there are no data in the literature to indicate whether such key enzymes as hexokinase, aldolase, and glucose-6-phosphate dehydrogenase are present.

The most frequently mentioned vitamins regarding leptospiral nutrition are vitamins B_1 and B_{12}. Both vitamins were required for maximum growth of the frog-kidney isolate. The phosphorylated forms of thiamine were not as effective in supporting growth as thiamine hydrochloride. Wessman[18,19] has reported concentrations of 0.01 to 0.1 μg/ml of the thiamine phosphates capable of supporting maximum growth of *Pasteurella haemolytica* while comparable levels of thiamine hydrochloride were inadequate.

The question arises as to whether leptospires require thiamine as an intact molecule or if the pyrimidine moiety or the thiazole moiety is the active portion of the molecule or if both moieties must be supplied simultaneously to support growth. There is also a possibility that some leptospires do not require thiamine for growth. Other possibilities include the use of modified thiamine molecules which reflect an alteration of either the pyrimidine or thiazole halves of the vitamin. Oxythiamine in which the NH_2 group is replaced by a hydroxyl group in the pyrimidine half of the molecule was almost equivalent to thiamine in growth supporting activity. In contrast, pyrithiamine where there is a replacement of the S-group on the thiazole ring, was inactive in supporting growth.. Our further study of the activity of thiazole and pyrimidine moieties confirmed the importance of the thiazole fragment as the active portion of the molecule. These findings suggest that leptospires are capable of synthesizing pyrimidine. Johnson and Rogers[13] have reported that ^{14}C labeled pyrimidines do not enter the cell and that pyrimidine analogues do not inhibit leptospiral growth. Studies in our laboratory with two *Leptospira pomona* strains, one causing renal infection in hamsters when inoculated intraperitoneally with as few as 10 cells versus the other long time laboratory cultivated strain where not even 600 x 10^6 cells could cause renal infection, did not reveal marked differences in their thiamine requirement. In contrast to this it was not possible to show a thiamine requirement for a strain of leptospira #3055 isolated by L. E. Hanson, Univ. Illinois. Whether such findings could be extended to other members of this genus is not known but might be considered from a taxonomic point of view in lieu of the recent interest to separate the leptospires into two main groups of pathogens and nonpathogens. Certain cellular activities may be related to the need for thiamine or its moieties, as when Peters and Nelson[15] reported the pyrimidine fragment of thiamine responsible

for lipase production of *Candida lipolytica*. Depletion of thiamine in the cells of staphylococci[12] and propionic-acid producing bacteria[16] have been reported and there is an indicated need for such studies with leptospires. A better understanding of the localization of leptospires in animal hosts could be investigated from a pharmacological approach similar to the work with the coccidiostat, amprolium,[17] a thiamine antagonist.

Since the first isolations of leptospires, little information related to the temperature requirements for growth and various cellular activities has been reported. The optimum temperature for growth of the frog-kidney isolate was 29 C. From the results obtained at 31.5 C and 33.5-34 C one would suspect there are temperature-dependent systems in certain leptospires which we have yet to understand. There might be some correlation between antibody formation in poikilothermic vertebrates with the observed temperature of growth sensitivities.[11] Our previous studies[6] on the effect of temperature upon the growth of *L. pomona* were done with different media, using static culture, and severely laboratory adapted cultures, thus comparisons are difficult to make. A further insight into the temperature sensitivities of leptospires might come from a study of the effects[3] on biologic systems of high order phase transitions in water.

At this time the absolute identity of the frog-kidney isolate has not been determined but the organism shows antigenic affinities with some members of the *L. javanica, L. canicola, L. pyrogenes,* and *L. autumnalis* group (personal communication—Dr. W. F. McCulloch, Univ. of Iowa). Johnson and Harris[14] have suggested that the lower limit for growth of pathogenic leptospires is 13 C yet the frog kidney isolate was capable of growth at 9 C in well aerated medium. Such deviations in biology are to be expected and further studies with field isolates and laboratory adapted cultures of leptospires are clearly warranted if we are to explain the preference of leptospires to grow *in vitro* at temperatures well below the body temperatures of the hosts they parasitize.

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ABSTRACT

Reed, Austin and J. Guy Cousineau. 1967. Epidemics involving the common eider (*Somateria mollissima*) at Ile Blanche, Quebec. Naturaliste Can. 94: 327-334.

An estimated 1000 adult female eiders died in 1964 and 700 died in 1966, of total nesting populations of about 4000. Necropsy and bacteriologic studies revealed *Pasteurella multocida* as the cause of death. There was no mortality among other avian residents, including gulls and cormorants. The source of infection remains unknown.