Table 1. Effect of chlorothiazide venoclysis (2.5 mg/kg prime plus 3.0 mg/kg hr) on the renal elimination of electrolytes in dogs pretreated for 3 days with 3 g of $NaHCO_3$ or NH_4Cl. Triplicate 10-minute clearances were measured in "control" and "drug" phases. Data are tabulated as: average "control" value/average "drug" value.

Na		K	Cl⁻	Urinary
Amt (μeq/min)	Reabsorption (%)	(μeq/min)	(μeq/min)	*p*H
Pretreatment with $NaHCO_3$				
143/400	98.9/95.8	26/41	17/131	7.4/7.9
Pretreatment with NH_4Cl				
8/273	99.8/95.1	9/27	15/280	5.7/5.8

causes an increased excretion of retained fluid. In this respect, it resembles the organomercurial diuretic agents from which it differs in structure and apparent mode of action. The chloruresis distinguishes this agent from carbonic anhydrase inhibitors that cause a predominant increase in bicarbonate rather than chloride excretion when administered to the dog and to man.

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4. Chlorothiazide is the nonproprietary name for 6-chloro-7-sulfamyl-1,2,4-benzothiadiazine-1,1-dioxide. Diuril is the trademark of Merck & Co., Inc. for this compound.

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Marine Fungus Requiring Vitamin B_{12}

Thraustochytrium globosum, an obligately marine fungus (Phycomycete) isolated from littoral sea water, has been found to require vitamin B_{12} for growth in a defined medium (*1*). This is the first instance of a fungus known to require an exogenous supply of vitamin B_{12}. The finding is therefore of interest to comparative biochemists because it places the fungi among the groups of organisms (bacteria, algae, animals) in which this vitamin is an essential metabolite, rather than with the higher plants which neither require nor produce vitamin B_{12} (*2*). It is further of considerable ecological interest: the B_{12} requirement of many members of the phytoplankton (*2, 3*) and the production of vitamin B_{12} by various marine bacteria (*4*) have provided evidence for the occurrence of this metabolite in ocean water.

Investigation of the determinative importance of vitamin B_{12} in marine ecology has been hampered by difficulties in assaying sea water. Extraction procedures necessary for assays with the usual microorganisms are time consuming and result in the loss of 5 to 25 percent of the vitamin (*5*). The best direct assay so far described (using the marine Chrysophyte, *Monochrysis lutheri*) requires 3 to 4 weeks and includes possible responses to pseudovitamin B_{12} and factor A (*6*). There are obvious advantages in assay organisms requiring shorter incubation periods. It would also be desirable to have available assay organisms providing a range of specificities suitable for distinguishing the biologically active forms of the vitamin. Since *Thraustochytrium globosum* requires an incubation period of only 9 to 14 days, it is presented as a possible assay organism. This fungus also responds to vitamin B_{12III}, but not to pseudovitamin B_{12}, factors A, B, G, or H, or thymidine (in the presence or absence of 1 mg/100 ml each of adenine, guanine, and uracil). Determination of the effect of the presence of methionine on the B_{12} requirement is complicated by the toxicity of this amino acid.

The basal medium used in the determination of the B_{12} requirement of this fungus is given in Table 1. Fresh glass-distilled water was used in the preparation of all solutions and media. The *p*H of the medium (at twice the final concentration) was adjusted to 7.5 with NaOH before the addition of agar. Glucose and $NaHCO_3$ were added aseptically after autoclaving. Experimental media were dispensed in 10-ml lots in 25-ml glass-capped erlenmeyer flasks. After sterilization and inoculation, the flasks were sealed between Pyrex kitchen trays with transparent cellulose tape and incubated for 10 days at 15°C. Optical density, as determined with a Klett-Summerson photoelectric colorimeter with No. 42 filter, was used as a measure of growth.

Inocula depleted of vitamin B_{12} were prepared by transferring 0.2 ml from cultures grown with 5 μg of vitamin B_{12} per milliliter to tubes containing 5 ml of basal medium. These inocula were incubated for 1 to 2 weeks at 15°C, to a population density of 1.0 to 1.7×10^5 cells per milliliter. One drop from such a culture was used to inoculate each experimental flask.

The growth response to increasing concentrations of vitamin B_{12} is illustrated in Fig. 1. The points on this graph represent the average of three experiments, each performed in duplicate. In individual experiments, variation between duplicate flasks seldom exceeded the error of the colorimeter; the greatest differences between experiments ranged from 9 Klett units (5 μμg of vitamin B_{12} per milliliter) to 21 Klett units (100 μμg/ml). The greater variation at higher vitamin concentration may reflect the fact that these cultures are still in the exponential phase of growth at 10 days. Growth in the presence of 100 μμg of vitamin B_{12} per milliliter reaches the stationary phase at 14 days. Since the Klett reading given by cultures grown with lower (for example, 10 μμg/ml) concentrations of vitamin B_{12} remain constant from the 8th to at least the 14th day, it is feasible to use a longer incubation period when greater precision at higher vitamin levels is desired. The sensitivity of the response is the same as that of the more prominent fresh-water assay organisms, *Lactobacillus leichmannii, Euglena gracilis,* and *Ochromonas malhamensis.*

Table 1. Basal medium for demonstrating the B_{12} requirement of *Thraustochytrium globosum.*

Nutrient	Amount
NaCl	2.5 %
KCl	0.1 %
$MgSO_4 \cdot 7H_2O$	0.5 %
KH_2PO_4	0.01 %
$CaCO_3$ (in acid)	0.02 %
Na_2EDTA	0.05 %
$(NH_4)_2SO_4$	0.02 %
Zn (as sulfate)	1.0 mg/100 ml
Mn (as sulfate)	1.0 mg/100 ml
Fe (as sulfate)	0.2 mg/100 ml
Cu (as sulfate)	0.002 mg/100 ml
Co (as sulfate)	0.02 mg/100 ml
B (as boric acid)	0.02 mg/100 ml
Mo (as sodium molybdate)	0.02 mg/100 ml
NaH · glutamate	0.05 %
L-Lysine	1.0 mg/100 ml
Thiamine · HCl	20.0 μg/100 ml
Glucose	0.1 %
$NaHCO_3$	0.02 %
Agar	0.1 %

The practicability of assaying vitamin

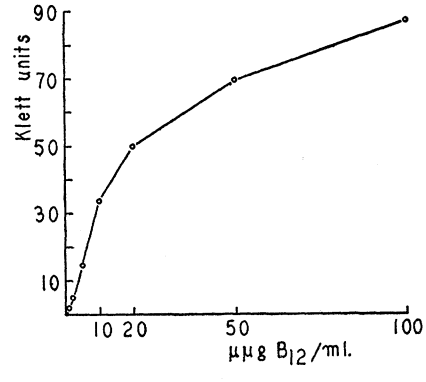

Fig. 1. Growth of *Thraustochytrium globosum* in the presence of various concentrations of vitamin B_{12}.

B_{12} in sea water was tested with water collected in July 1957 from two stations in Long Island Sound and frozen within 5 hours after collection. For the assay, the sea-water samples were enriched with glucose, glutamate, lysine, thiamine, and agar at the level of the basal medium. Five- and 2.5-ml portions of each sample were diluted with full-strength basal medium (without bicarbonate) to a final volume of 10 ml. Undiluted samples were inhibitory to *Thraustochytrium globosum* in the presence or absence of added vitamin B_{12}. Consistent results and satisfactory recovery of added vitamin B_{12} (5 μμg/ml) were obtained with one sample at both dilutions; with the other, only at the greater dilution. Both samples assayed 16 to 20 mμg of vitamin B_{12} per liter—a level somewhat higher than that found for unfiltered ocean water at other locations and at various seasons by Cowey (*5*) and by Droop (*6*) (0.2 to 4.0 mμg/lit and 5 to 10 mμg/lit, respectively).

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References and Notes

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4 November 1957

Propagation of Infectious Canine Hepatitis Virus in Porcine Kidney Tissue Culture

Infectious canine hepatitis virus has generally been considered to be a host-specific agent (*1*). Numerous attempts have been made, in this laboratory and elsewhere, to adapt this virus to embryonated eggs, mice, rabbits, and other animals, with essentially negative results. The successful propagation of infectious canine hepatitis virus in dog-kidney tissue cultures with the production of a typical cytopathogenic effect has been reported (*2, 3*). By continuous passage in dog-kidney tissue culture, the virus was modified so that it did not produce disease when it was inoculated into susceptible dogs (*3*). Previous attempts in this laboratory to adapt virulent infectious canine hepatitis virus to pig-kidney explants were unsuccessful. This report shows that the virus can be successfully propagated in pig kidney epithelial cells after a series of passages in dog-kidney tissue cultures.

Tissue cultures were prepared from the kidney cortex of pigs of between 2 and 12 weeks of age by a trypsin digest method in which 0.33 percent trypsin was used. Two nutrient media were employed: (i) 0.5 percent solution of lactalbumin hydrolyzate in Earle's balanced salt solution, fortified with 10 percent inactivated horse or bovine serum (ELS medium); (ii) medium No. 199 (Parker) without serum. Each medium contained penicillin and streptomycin. Initial attempts to adapt infectious canine hepatitis virus to trypsinized-pig-kidney tissue cultures were made with virulent virus. From portions of liver from two dogs killed during the acute phase of infection, 20-percent suspensions in physiological saline were made, and the supernatants of these were inoculated into culture tubes containing ELS medium. Additional cultures were inoculated, in the same manner, with tissue culture fluid from the 14th dog-kidney tissue culture passage of infectious canine hepatitis virus. The cultures, incubated at 35°C, were observed for 7 days, after which time the fluids from each group of tubes were harvested and passed into additional tubes. Subpassages of each group of cultures were made three times, at 7-day intervals. At that time, the tissue culture fluids were inoculated into trypsinized-dog-kidney roller tubes, to test for the presence of virus. No virus could be detected.

Further efforts at adaptation were made with modified virus which had undergone 134 passages in dog-kidney cultures. Several pig-kidney tissue cultures in tubes containing ELS medium were inoculated in a manner similar to that described above, and subpassages were prepared. Commencing with the fifth tissue culture passage, a portion of the cells exhibited a cytopathogenic effect that resembled, to some degree, the changes previously observed in dog-kidney tissue cultures. This effect continued to occur, in an erratic manner, through the tenth passage. Beginning with the tenth passage, the nutrient medium was changed to No. 199. Thereafter, a complete cytologic change developed in the cells 3 to 6 days after inoculation and occurred regularly through 28 additional passages. The titer of the tenth pig-kidney passage was $10^{5.0}$, as was demonstrated by titrations in dog-kidney tissue culture. Titrations at intervals through the 38th passage ranged from $10^{4.5}$ to $10^{5.5}$. The affected cells became swollen, rounded-up, and highly refractile. Epithelial sheets broke up, and the cells formed small, grapelike clusters.

Serum neutralization tests to identify the cytopathogenic agent in pig-kidney tissue were performed at various passage levels. Infected tissue culture fluid prepared in tenfold dilutions was mixed with an equal amount of known infective-canine-hepatitis-positive canine antiserum. These mixtures were incubated for 2 hours in a 37°C water bath and inoculated, in 0.2-ml amounts, into each of several pig- and dog-kidney cultures. Up to 100,000 tissue culture infectious doses of virus were neutralized both in pig and dog cultures by the positive serum, while the usual cytopathogenic effect occurred in control cultures that contained the normal serum.

Tests were made in dogs to provide additional evidence that the cytopathogenic agent propagated in pig-kidney tissue culture was infectious canine hepatitis virus. Fourteen infectious-canine-hepatitis-susceptible dogs were used. Eight were inoculated subcutaneously with 19th-passage, and six with 34th-passage, material. Daily temperature recordings and observations for other signs of illness were made for 3 weeks, following which the animals were challenged with known virulent virus. No signs of illness were observed in the dogs inoculated with pig-adapted virus, and none of the dogs responded to challenge. Preinoculation serums were found to be negative (by a serum-neutralization test employing known infectious canine hepatitis virus that had been grown in dog-kidney culture); all dogs had developed neutralizing antibody titers of from 100 to 3200 three weeks later. The development of specific antibodies in the dogs further demonstrates that the cytopathogenic agent is infectious canine hepatitis virus. The lack of illness in the inoculated dogs indicates that the virus is of modified virulence and could be used for development of a vaccine.

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Action of Hydrocortisone on Respiration and Aerobic Glycolysis of Cultured Cells

In previous reports, inhibition of growth of fibroblasts by a single high dose of hydrocortisone was demonstrated (*1*). Cells derived from a single fibroblast, which had been isolated from a culture of normal subcutaneous tissue of the mouse, Earle's strain L, have been