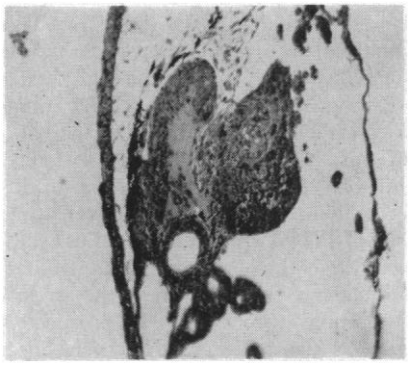

Fig. 1. Neural and nephric differentiation in a ventral explant that received a CO_2 shock.

The use of CO_2 is particularly advantageous because this gas can readily penetrate into the cells and because it does not disaggregate the cells or cause the marked cytolysis that followed the alkaline shock treatments. Modification of the "A" part of the culture medium with a 0.005*M* acetate buffer to a *p*H of 3.7 was not effective in inducing dorsal tissues.

No dorsal structures were observed in the explants that received alkaline shocks, but neuroid tissue and parts of the brain were frequently noted in explants that had received CO_2 shocks. An example of such differentiation is illustrated in Fig. 1. Less frequently, muscle, nephric tubules, and notochord were present in the explants. Of the ventral tissues that received CO_2 (*p*H 3.7) shocks, 50 percent showed neuroid tissue, 40 percent had well-defined brain vesicles, while in 10 percent of the cases no dorsal structures were present. The latter percentage is far above that of such tissues appearing in the other types of culture. One possible interpretation of the mechanism of CO_2 induction is that the acid shock released soluble protein and ribonucleoprotein from the yolk platelets (*6, 8*) and that this initiated the synthesis of cytoplasmic protein and subsequent differentiation of the dorsal tissues.

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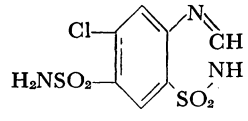
4 November 1957

Electrolyte Excretion as Influenced by Chlorothiazide

In 1953 we reported that *p*-carboxybenzenesulfonamide produced (i) both natriuresis and chloruresis in dogs and (ii) that there was a causal relationship between the (plasma) concentration of drug presented to the kidney and the saluretic effect (*1*). These two attributes of that compound appeared to be inconsistent with the more classical concepts of the nephrotropic action of a carbonic anhydrase inhibitor (*2*). That agent was not sufficiently potent to warrant extended clinical study.

This premonitory indication that saluresis might be a clinically important attribute of a potent carbonic anhydrase inhibitor that fulfilled certain specific pharmacodynamic criteria has influenced our studies of renal electrolytes. In addition, the secondary attributes for an essentially specific nephrotropic agent of this category have been considered by us to include good absorption from the gastrointestinal tract, a volume of distribution essentially limited to extracellular fluid, a high concentration ratio of the compound in nephron/plasma, and high carbonic anhydrase inhibitor activity (*1*).

In the course of this study, it was found that an unusual heterocyclic acid increased substantially the excretion of sodium and chloride by the dog. This agent was synthesized by Novello and Sprague (*3*), who identified it as 6-chloro-7-sulfamyl-1,2,4-benzothiadiazine-1,1-dioxide and assigned to it the following structure:



The compound is referred to herein as chlorothiazide or Diuril (*4*).

Qualitatively, at equal dosages and under similar experimental conditions, chlorothiazide induces changes in electrolyte excretion that more nearly resemble those known to be caused by the organomercurial diuretic agents than the response generally considered to attend the administration of a carbonic anhydrase inhibitor. Under reasonably normal conditions of acid-base balance, the effect of chlorothiazide is to increase the excretion of sodium and chloride preponderantly. The excretion of potassium and bicarbonate is increased slightly, if at all. However, the effect of the drug on the ratio of sodium to chloride excreted per unit time varies according to the state of electrolyte balance of the dog.

In the two experiments summarized in Table 1, the dogs were pretreated on three successive days with moderate amounts (3.0 g/day) of $NaHCO_3$ or NH_4Cl, the last dose being administered just prior to the control phase of the test. When the animal was pretreated with $NaHCO_3$, chlorothiazide caused a substantial increase in chloride excretion that was quite inadequate to cover the profound increase in sodium elimination. The increase in urinary *p*H reflects a substantial increase in bicarbonate excretion in the drug phase which more elaborate renal clearance experiments have demonstrated. In the NH_4Cl experiment, chlorothiazide caused a marked increase in chloride excretion which was essentially equivalent to the amount of sodium eliminated. Under the conditions of this experiment, the excretion of bicarbonate is negligible and the effect on potassium elimination or urinary *p*H is not impressive.

The oral or parenteral administration of chlorothiazide to dogs made edematous by an excessive daily intake of a mineralocorticoid, NaCl, KCl, and water resulted in a prompt loss of fluid with reduction of weight to normal only as long as the drug was administered (Fig. 1). During the 100-day test, the apparently normal dog received 6 mg of 9α-fluorohydrocortisone, 2 g of NaCl per kilogram, and 150 mg of KCl plus 1000 ml water orally per day in addition to the Na and K contained in its diet. During the coadministration of 5 mg/kg of chlorothiazide orally twice daily from the 23rd through the 34th day, body weight returned to control value. When the drug was withdrawn, the dog promptly gained weight. After the single injection of the saluretic agent on the 78th day, the dog lost 0.7 kg of body weight within 24 hours. The attendant decrease in weight was maintained when the drug was administered orally until chlorothiazide therapy (5 mg/kg twice daily) was withdrawn on the 88th day. Thereafter, the dog began to gain weight under influence of the steroid. The ability of this agent to counteract the sodium and fluid retention induced by steroids has been confirmed by the repetition of this type of experiment in other dogs and by conventional renal clearance experiments in this species.

Chlorothiazide is an orally active saluretic (or diuretic) agent. Its predominant effect is to increase the excretion of sodium and chloride. Secondarily, it

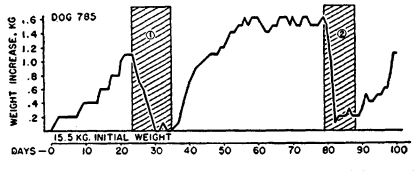


Fig. 1. Reversal by chlorothiazide of weight gain induced by the injection of 9α-fluorohydrocortisone and a high salt intake.

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.

18 October 1957

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

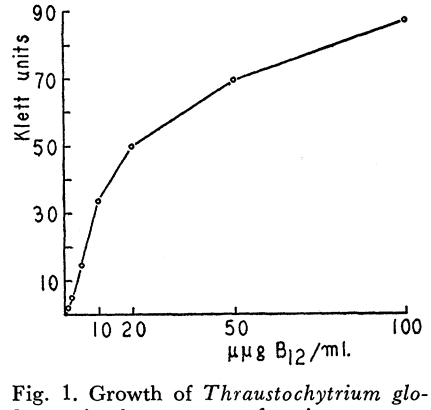

Fig. 1. Growth of *Thraustochytrium globosum* in the presence of various concentrations of vitamin B_{12}.