reported by Whittlestone(14) on milk-ejecting activity of high potency preparations of both oxytocin and vasopressin, prepared in the Cornell Laboratories, are of interest in connection with the question of the inherent oxytocic activity of vasopressin. He found that an oxytocic preparation of approximately 500 units per mg(6.13) showed a milk-ejecting activity equivalent to its oxytocic activity and that a vasopressin preparation of 400 pressor units per mg(7) showed a milk-ejecting activity equivalent to that which would be expected of approximately 80 units of oxytocin. Cross and van Dyke(15) have made similar comparisons in lactating rabbits by the method of Cross and Harris(16), using an oxytocin preparation with a potency of about 500 units per mg, and the 600 unit per mg vasopressin preparation of the present study, both of which had been prepared in the Cornell Laboratory. The results of Cross and van Dyke were in good agreement with those reported by Whittlestone.

Summary. Evidence has been obtained with a highly purified preparation that vasopressin, in addition to its pressor and antidiuretic activities, possesses intrinsic oxytocic action as one of its pharmacological properties, in contrast with purified oxytocin which appears not to possess pressor or antidiuretic activity. For each 100 units of vasopressor-antidiuretic activity there is intrinsic "oxytocic" activity represented by approximately 5 units as determined by the isolated rat uterus assay or by approximately 13-15 units by the chicken blood pressure depressor assay. The evidence that vasopressin possesses milk-ejection activity about one-fifth that of oxytocin has been discussed.

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Assay of Botulinum a Toxin with Goldfish.* (19925)

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The purpose of this report is to describe the successful use of the common goldfish as an assay animal for botulinum toxin. Goldfish have the obvious advantage that they are inexpensive and can be maintained healthy in a very small space.

The goldfish has been used extensively as a laboratory animal in toxicity tests. Pittenger and Vanderkleed(1), Pittenger(2), and Lapenta(3) observed specific reactions in

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goldfish when treated with digitalis and with toxins. They obtained highly reproducible results and advocated the use of goldfish in the standardization of doses of these agents. Stevenson, Helson, and Reed(4) showed that goldfish can be poisoned with botulinum toxin. On the basis of these findings and in view of the need for an animal less expensive and easier to handle than the mouse, it was decided to try to develop an assay technic for botulinum toxin using the common goldfish.

Materials and methods. A preparation of botulinum A toxin containing 6 x 10^6 mouse 50% lethal doses per ml was used in these experiments. The preparation was obtained through the courtesy of Dr. Eugene M. Sporn at Camp Detrick, Frederick, Md. It was stored at 4°C for about a year prior to use, but the mouse assay was kindly carried out by Dr. Sporn at the time the experiments on goldfish were under way. In most tests, 10-fold dilutions of the toxin sample were made with specially prepared .1 M phosphate buffer at pH 7 containing 0.2% gelatin. Common goldfish, *Carassius auratus,* from 2-3 in. in length and from 2-4 g in weight were used in these experiments. The diluted toxin solutions were introduced intraperitoneally. After an examination of the internal anatomy of the goldfish, it was decided that the least amount of damage to vital organs would be caused if the needle were inserted between the pelvic fins and directed toward the backbone. During the injection, the fish were held in a small net between the thumb and the forefinger of the left hand. Twenty-seven gauge, ¼ in. needles were used, and 0.05 ml of solution was injected into each fish. Usually 10 fish per dilution were used. After the inoculation, the fish were stored in 3 gallon glass aquaria, not aerated. The criterion chosen for deciding whether or not a fish was dead was the complete cessation of movements of the mouth and opercula. Deaths which occurred within 12 hours after injection were classified as non-specific. End points were calculated by an algebraic modification(5) of the Reed and Muench method(6). They were calculated on the basis of mortality ratios observed 36 hours after inoculation. Experiments were carried out to determine the effect of temperature on the 50% mortality end point in fish. To this end the fish were held in the aquaria at various temperatures after inoculation. Those fish held at 35° were first acclimatized to that temperature prior to the inoculation. This was done by raising the temperature of a thermostatic bath containing the fish approximately 1° per day.

TABLE I. Effect of Toxin Concentration on Mortality Ratios.

Dilution	Mortality ratio
Uninj. control	0/10
Buffer control	0/10
10^{-2}	10/10
10^{-3}	9/10
10^{-4}	2/10
10^{-5}	0/10

TABLE II. End Points Calculated from Independent Assays on Same Preparation of Toxin.

3.8
4
3.8
3.5
4.1
3.5
3.6

Experimental results. A representative experiment showing the effect of toxin concentration on mortality ratios is shown in Table I. The end point of this particular experiment calculated by the method of Reed and Muench is 3.6. Reproducibilities of the end points were determined by carrying out seven independent titrations on the same toxin preparation. The results are shown in Table II. The mean end point is 3.8 and the standard deviation of the values is 0.24. Since these end points are expressed as logs of concentrations, this result indicates that end points are reproducible within about a 2-fold concentration of toxin.

Experiments were carried out using 4-fold and 2-fold dilutions to determine whether there was any effect of dilution interval on the end point calculation. Values of 4.1 and 3.5 were obtained respectively. It was observed that mortality ratios varied from zero to 1 over a concentration range of between 10 to 100 fold. On the basis of this result, it was decided that no substantial advantage would ensue from the use of dilution intervals less than 10-fold.

Burgen *et al.*,(7) observed that tempera-

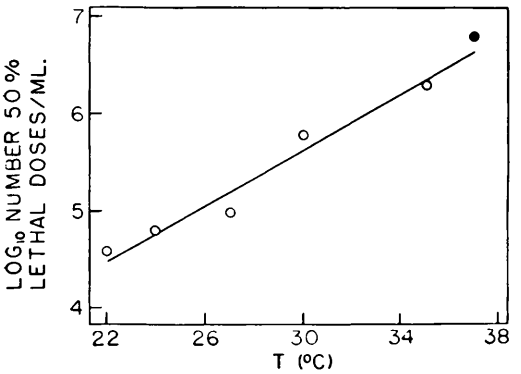

FIG. 1. Variation of the 50% lethal dose of botulinum A toxin in goldfish with temperature. Open circles–goldfish assays; closed circle–mouse assay.

ture variation affected the response of rat phrenic nerve-diaphragm preparations to botulinum A toxin. In view of this result, the effect of temperature on the 50% mortality end point of botulinum A toxin in goldfish was investigated. It was found that the end point values were increased and that the fish at higher temperatures died earlier than those receiving the same dose but kept at lower temperatures. The data obtained are plotted in Fig. 1. They show that the logarithm of the titer expressed as 50% lethal doses per ml increases with the temperature in essentially a linear manner. The straight line was fitted to the data by the method of least squares. When this line is extrapolated to 37°, a value is obtained which is in excellent agreement with the assay value for the toxin preparation in mice.

It should be pointed out that the apparently excellent agreement between the extrapolated value for goldfish at 37° and the value for mice may be in part fortuitous. These end points are determined as 50% lethal doses per unit volume of toxin solution. There is about a five-fold difference in body weight between the mouse and the goldfish in this experiment. On this basis, the susceptibility per unit body weight would be higher for the mouse than for the fish. Furthermore, the mouse end point was calculated from mortality ratios at the end of 48 hours and the fish end points were calculated from mortality ratios observed after 36 hours. Thus, the experiments in fish and in mice are not absolutely comparable. Nevertheless, it is apparent that a large fraction of the difference between the end point observed with fish at room temperature and the end point observed with mice can be attributed to the effect of temperature.

The effect of temperature on the mortality dose of botulinum A toxin can be interpreted in terms of conventional chemical kinetics. If the natural logarithm of the number of 50% mortality doses per ml is plotted against the reciprocal of the absolute temperature, a straight line can be fitted. From the slope of this straight line, a heat of activation for the over-all killing mechanism can be evaluated as 60,000 calories per mole. At least one positive statement can be made on the basis of this figure. It is much too high to be consistent with the assumption that the temperature effect is the result solely of a mechanism involving translational or rotational motion of the toxin particles. The reason for this is that such motion depends primarily upon viscosity which has a heat of activation of only about 4,000 calories per mole.

This heat of activation could arise from any one of a number of different kinds of mechanism. It could be the heat of dissociation of toxin aggregates or of toxin inhibitor complexes. It could be the heat of activation of a direct reaction between the toxin and some center for nerve impulse transmission. It could be the difference between the heat of association of some substance essential in nerve metabolism with its substrate and of the same substance with toxin behaving as an inhibitor. There is little evidence available to permit a selection among these and other possible alternatives. The idea that the heat of activation may be the heat of dissociation of essentially non-effective aggregates into effective smaller toxin units gains some support from the known dissociability of the toxin material at pH values above 7(8).

Summary. A method has been established for the assay of botulinum A toxin in goldfish. When 10 fish are inoculated per dilution and the dilution interval is 10-fold, the end point distribution was found to have a standard deviation of about 0.24 log unit. The end points are strongly temperature dependent; they decrease about a hundred-fold for a temperature decrease of 15°C. The end point

in fish at 37° has approximately the same value as that in mice.

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Blood Volume Studies in Pernicious Anemia and Non-Tropical Sprue. (19926)

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It is well recognized that patients with pernicious anemia in relapse tend to retain fluids. Clinically, this may be manifested by minimal edema of skin and subcutaneous tissues or by frank dependent edema. Clinical evidence of increased water retention may be evident during the early phase of effective therapy as shown by increase of peripheral edema and occasionally by the appearance of pulmonary edema. Meulengracht(1) demonstrated that persons in relapse showed decreased diuresis following ingestion of large quantities of water. Vaughan(2) reported retention of fluid in 12 patients with pernicious anemia in relapse from the onset of treatment to the peak of reticulocytosis after which there was a period of diuresis and return to normal fluid balance. Gibson(3) reviewing the work of many investigators, noted no general agreement in reports of plasma volume changes in patients with pernicious anemia. Using Evans blue dye (T-1824) he observed a fall in plasma volume and rise in red blood cell and total blood volume after treatment. He made no studies during the period of reticulocytosis. These variable results plus the frequently observed discrepancy of a sharply elevated reticulocyte peak after specific anti-pernicious anemia therapy, with no increase in hemoglobin or red blood cell values, prompted the following investigation. Hemodilution resulting from increased plasma volume could explain this seeming anomaly.

Methods. This study included one patient with non-tropical sprue and four persons with typical Addisonian pernicious anemia in relapse. Each case in the latter group showed hyperchromic macrocytic anemia, histamine-fast achlorhydria, and megaloblastic bone marrow. Roentgen examinations of gastrointestinal tract, urinalyses and blood chemistry determinations disclosed no abnormalities. The patients were treated with either intramuscular injections of liver extract, vit. B_{12} or citrovorum factor. The subject with non-tropical sprue showed hyperchromic macrocytic anemia, histamine-fast achlorhydria, megaloblastic bone marrow and flat glucose tolerance curve. There were frequent liquid stools containing abundant fatty acid crystals. Roentgen evidence of spasm, segmentation and "puddling" of the barium column in the small intestines was reported. He was treated with citrovorum factor intravenously. The results of this study will be presented elsewhere(4). In all patients hemoglobin and red blood cell determinations were made 3 times a week, leukocyte counts once a week, and reticulocyte estimations daily. Blood volume determinations were made twice weekly using P^{32} labeled erythrocytes(5). Red cell mass was calculated from the total blood volume and hemato-