*AN ADRENERGIC CONTROL SYSTEM IN TETRAHYMENA**

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The discovery by Janakidevi *et al.*[1] that the ciliated protozoan *Tetrahymena* contains epinephrine and norepinephrine led Blum *et al.*[2] to examine the effect of reserpine on these ciliated protozoa. It was found that reserpine inhibited the growth of these cells and depleted the catecholamine content. The growth inhibition was partially reversed by addition of glucose to the medium.[2] These observations suggested that *Tetrahymena* might contain other components of the catecholamine system found in metazoa and prompted us to examine the effects of a variety of pharmacological agents on the growth of *Tetrahymena*. We have found that the growth of *Tetrahymena* is sensitively inhibited by certain α and β adrenergic blocking agents and other drugs which are known to interact with the catecholamine system in mammals. Because one of the major effects of the catecholamines in metazoa is to control the metabolism of glycogen, we also examined the effects of some of these agents on the glycogen content of *Tetrahymena* and have found effects on glycogen content consistent with the view that an adrenergic metabolic control system is operative in *Tetrahymena*. Abstracts of part of this work have been published.[3, 4]

Materials and Methods.—Tetrahymena pyriformis, strain HSM, were grown axenically in Erlenmeyer flasks with Morton closure tops at 25° without shaking. The flasks were filled to less than one fifth their nominal capacity for all growth experiments, and to less than one tenth nominal capacity for all experiments in which glycogen concentration was to be measured. Cells were counted with a Coulter counter (Coulter Co., Hialeah, Fla.).

Two media were used. Medium A consisted of 1% proteose peptone and 0.05% liver extract in 0.02 *M* potassium phosphate at pH 6.5. Medium DK was essentially the synthetic medium described by Dewey and Kidder,[5] supplemented with 0.04–0.07% proteose peptone, as specified. Generally, 25 ml of medium was used, and five ml of water, or water containing the reagents to be studied, was added at zero time. The media were sterilized by autoclaving. All other chemicals except triiodothyronine were dissolved in water, the pH adjusted to near neutrality, and sterilized by passage through ultramicro fritted glass filters. Triiodothyronine was suspended in water with vigorous stirring and then boiled for 2 min in a screw-cap test tube.

Glycogen was assayed by the phenol-sulfuric acid method as described by Dubois *et al.*[6] Cells were chilled in ice and washed twice by centrifugation for 1 min in a clinical centrifuge, using a buffer consisting of 0.08 *M* Tris and 0.036 *M* NaCl, pH 7.5. In this buffer, there was little or no cell lysis.[7] After the second wash, the cells were resuspended in ice-cold 0.086 *M* NaCl, counted, and suitable aliquots were taken in quadruplicate for glycogen assay. The absorbance was measured in a Spectronic 20 colorimeter. Glucose standards were run with each assay, and all results were computed directly from the glucose standard line. In the case of cells grown in the absence of added glucose, virtually all of the color developed in the assay was due to the glucose units of glycogen, but with cells grown in the presence of glucose there may have been large amounts of intracellular glucose.[8] For the present purposes, we are concerned only with the total glucose residues of the cell, and have referred to this as glycogen for convenience.

Chemicals were obtained from the following sources: reserpine phosphate, Ciba Pharmaceutical Company; dibenzyline, tranylcypromine sulfate, and the sodium salt of l-triiodothyronine, Smith, Kline, and French; dichloroisoproterenol (DCI), Aldrich Chemical Company; guanethidine, Ciba Pharmaceutical Company; corticosterone, Mann Research Laboratories; Segontin, Hoechst Pharmaceuticals, Inc.; Catron, Lakeside Laboratories, Inc.; desipramine, Geigy Pharmaceuticals; Inderal, Ayerst Laboratories; 3′5′-cyclic adenosine 5′-phosphate, Pabst Labora-

tories; L-epinephrine and 3-iodo L-tyrosine, Calbiochem; metaraminol bitartrate, Merck, Sharp and Dohme; α-methyl-meta-tyrosine, octopamine, L-β-3,4 dihydroxyphenylalanine, caffeine, aminophylline, and ethylene diamine, Sigma Chemical Company. All other chemicals were reagent grade.

Results.—Effect of reserpine on growth and on glycogen content: In the experiment shown in Figure 1, 20 ml from a culture in early stationary phase was transferred into each of two flasks containing 45 ml fresh proteose peptone medium, and 3.5 ml of either water or reserpine in water was added. The control cells began exponential growth and their glycogen content decreased to about 0.4 mg/10^6 cells. As the culture entered stationary phase, the glycogen content increased to about 1.2 mg/10^6 cells and, finally, after about 50 hours in stationary phase, the cells rapidly utilized their glycogen reserves. The cells exposed to reserpine also grew exponentially, but at a slower rate. The initial decrease in glycogen content occurred at about the same rate and to the same extent as the control cells, but the reserpine-treated cells then failed to show any net synthesis of glycogen.

In the experiment shown in Figure 1, glycogen was synthesized largely by gluconeogenesis from amino acids. It was therefore of interest to inquire whether reserpine would inhibit net glycogen synthesis when the cells were also supplied with glucose. In the experiment shown in Figure 2 it can be seen that in the presence of glucose, 2.13×10^{-5} *M* reserpine caused only a small inhibition of growth, as expected from our earlier observations.[2] After about six hours, however, the net rate of synthesis of glycogen was greatly inhibited. With increasing reserpine, growth inhibition increased and there was an increasing inhibition of the initial rate of glycogen synthesis. These experiments demonstrate that reserpine interferes with glycogen synthesis in the presence or absence of exogenous glucose, and also suggest that the growth-inhibitory effect of reserpine is independent of its effect on glycogen metabolism. It should be mentioned that preliminary experiments indicated no large effect of reserpine on the RNA or protein levels of these cells.

Effect of α- and β-adrenergic blocking agents: Dibenzyline, a drug which presum-

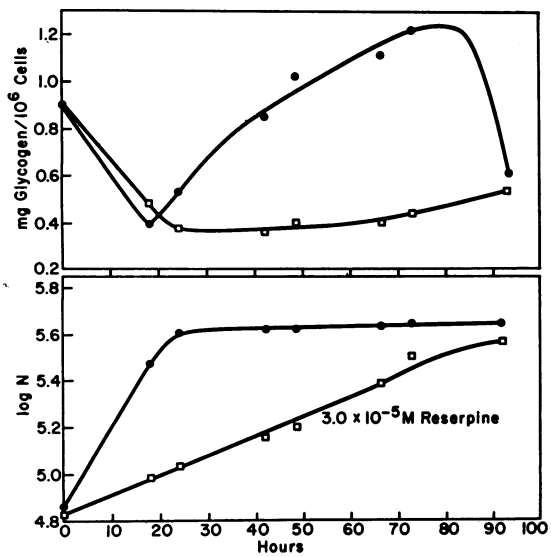

FIG. 1.—Effect of reserpine on growth and glycogen synthesis in the absence of exogenous glucose. Flasks initially contained 45 ml of medium A and 3.5 ml water or reserpine in water.

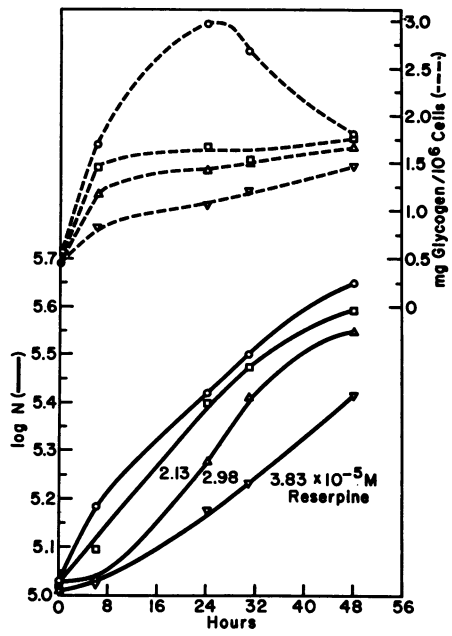

FIG. 2.—Effect of reserpine on growth and glycogen synthesis in the presence of exogenous glucose. The flasks initially contained 25 ml of cells in medium DK supplemented with 0.05% proteose peptone. At zero time glucose, reserpine, and water were added so that the concentration of glucose in each flask was 18.3 mM, the volume was 27.3 ml, and the reserpine concentrations were: ○, none; □, $2.13 \times 10^{-5} M$; △, $2.98 \times 10^{-5} M$; ▽, $3.83 \times 10^{-5} M$.

ably has an irreversible effect on α-adrenergic receptor sites,[9] slightly inhibits the growth of *Tetrahymena* at 0.1 mM, but considerably potentiates the growth-inhibitory effect of reserpine. With dibenzyline alone at higher concentrations, increasing inhibition of growth is observed.

Inderal (propanolol), one of the most potent β-adrenergic blocking agents known,[10] has a complex effect on the growth of *Tetrahymena*. At 0.1 mM it initially causes some growth inhibition which increases with increasing exposure to the drug. In the presence of reserpine there is at first no additive inhibition and even a slight protection against the inhibitory effects of reserpine. At later times an inhibition is observed which appears to be independent of the reserpine effect (data not shown).

Dichloroisoproterenol (DCI) is a potent β-adrenergic blocking agent which prevents the stimulatory effects of catecholamines on heart adenyl cyclase[11] and prevents the epinephrine-induced conversion of phosphorylase B to phosphorylase A in skeletal muscle.[12] It also inhibits the epinephrine-induced rise in 3′,5′-cyclic AMP levels in adipose tissue and the release of free fatty acids by adipose tissue.[13] At 0.1 mM, DCI has no effect on the growth of *Tetrahymena*, but markedly interferes with glycogen synthesis after about five hours of exposure (Fig. 3). At this concentration of DCI, there is a small but definite potentiation of the growth-inhibitory effect of reserpine which also does not become noticeable for at leat five hours.

Thus, *Tetrahymena* is sensitive to both α- and β-adrenergic blocking agents and in the case of DCI, at least, there is an inhibition of net glycogen synthesis at a concentration which has no effect on growth.

Effect of triiodothyronine: In mammalian tissues there is considerable evidence of an interrelation between the effects of thyroid hormones and the catecholamines.[14] It is well known that hyperthyroid animals are hypersensitive to epinephrine. Haugaard and Hess[15] have shown that thyroxine increases the per cent phos-

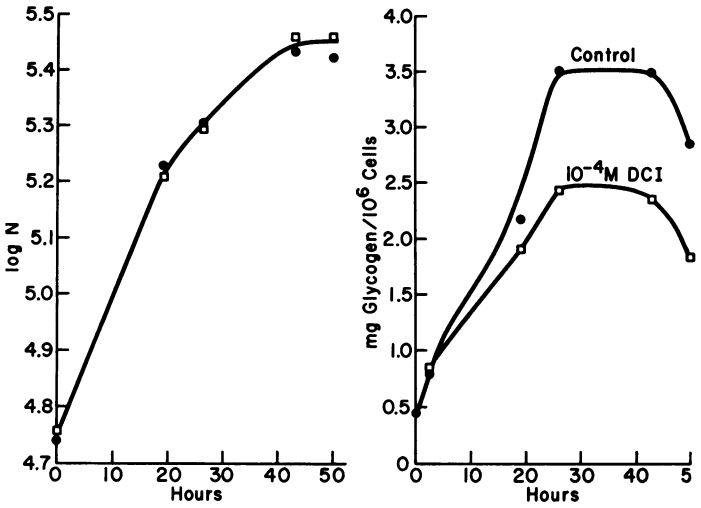

FIG. 3.—Effect of dichloroisoproterenol on glycogen synthesis. Flasks contained 25 ml of cells in medium DK supplemented with 0.06% proteose peptone. At zero time, glucose (16.7 mM final concentration) and DCI were added as indicated in a total of 5 ml of water.

phorylase A in the rat heart and Hornbook *et al.*[16] showed that reserpine prevents this increase. At 1.2×10^{-5} *M*, triiodothyronine causes little or no inhibition of the growth of *Tetrahymena* (Fig. 4). At higher concentrations, increasing inhibition of growth is obtained. A concentration of triiodothyronine which causes no inhibition of growth by itself potentiates the growth-inhibitory effect of reserpine. Thyroxine was less effective than triiodothyronine.

In the experiment shown in Figure 4 there was no significant inhibition of growth, but triiodothyronine markedly inhibited net glycogen synthesis after about six hours. When both triiodothyronine and DCI were added to the culture, net glycogen synthesis was completely inhibited after six hours of incubation, but again there was no significant inhibition of growth.

Effect of caffeine: The methyl xanthines are known to inhibit the specific nucleotide phosphodiesterase which converts cyclic 3′,5′-AMP to 5′AMP.[17] Caffeine, for

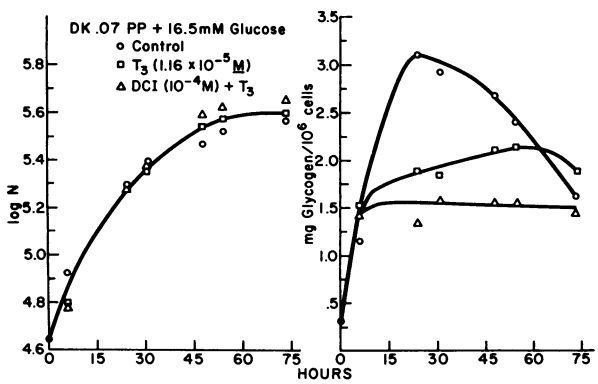

FIG. 4.—Effect of triiodothyronine on glycogen synthesis. To flasks containing 25 ml of cells in medium DK supplemented with 0.07% proteose peptone were added glucose (16.5 mM final concentration) and triiodothyronine (T_3) or both DCI and T_3, as indicated, in a total volume of 5 ml of water.

example, inhibited the activity of the purified enzyme by about 20 per cent at a concentration of 1 mM, and aminophylline was more potent than caffeine.[17] When 1.34 mM caffeine was added to *Tetrahymena*, there was little inhibition of growth, but some potentiation of the growth-inhibitory effect of reserpine. At 2.68 mM significant growth inhibition developed after about 15 hours' exposure and, in the presence of reserpine, growth virtually ceased after this time. At 4 mM caffeine (growth curves not shown), growth was inhibited completely for over 140 hours, demonstrating a remarkable dose-response effect of caffeine. Aminophylline (the ethylene diamine salt of theophylline) was more potent than caffeine, and ethylene diamine itself did not inhibit growth. Although the growth rate of cells treated with 1 mM aminophylline was slightly inhibited, their glycogen content was about 50 per cent higher than that of the control cells. That aminophylline and DCI affect the glycogen content of *Tetrahymena* in opposite directions is in accord with the probable modes of action of these drugs in mammalian cells.

Since the methyl xanthines inhibited growth, it was of interest to inquire whether 3′,5′-cyclic AMP would alter the growth of *Tetrahymena*. There was no effect of 1 mM 3′,5′-cyclic AMP on the growth of *Tetrahymena* in the absence of reserpine. In the presence of high concentrations of reserpine, both 5′AMP and 3′,5′-cyclic AMP (at 1 mM) partially protected against the growth inhibitory effects of the reserpine, with the cyclic AMP being more effective. There was, however, no significant effect of the 3′,5′-cyclic AMP on the net rate of glycogen synthesis in the reserpine-treated cells.

Effect of monoamine oxidase inhibitors: The work described above strongly suggested that *Tetrahymena* possessed a catecholamine system with a number of remarkable similarities to the system described in mammalian liver, nerve, and

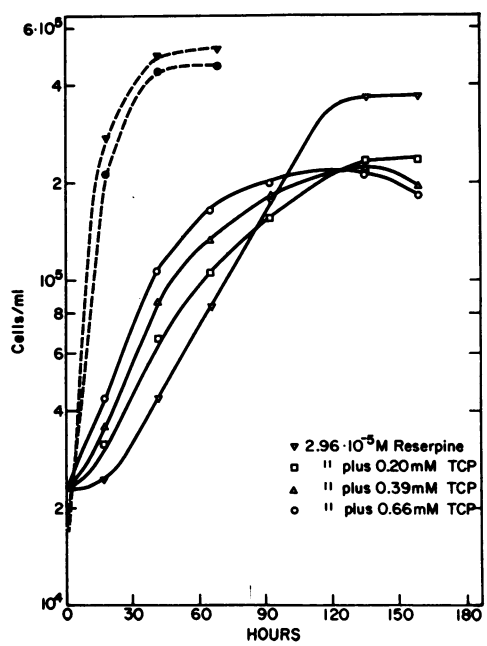

FIG. 5.—Reversal of reserpine effect by tranylcypromine (TCP). Each flask contained 25 ml of cells in medium A. At zero time, TCP and reserpine were added in a total of 2 ml water. Growth curves for cells without reserpine are shown by dotted lines and solid symbols (▼, no TCP; ●, 0.66 mM TCP). Growth curves for cells with 2.96×10^{-5} *M* reserpine are shown by solid lines.

heart cells. To test this analogy further, we next inquired whether compounds known to be monoamine oxidase inhibitors in mammalian cells would affect the growth of *Tetrahymena*. Tranylcypromine (TCP) is a monoamine oxidase inhibitor which reverses the sedative effect of reserpine in rats.[18] At concentrations of TCP of 1 mM or more, the growth of *Tetrahymena* can be completely inhibited. Up to 0.66 mM, however, there is practically no inhibition of growth (Fig. 5). At concentrations up to 0.66 mM, TCP initially reverses the growth inhibition due to reserpine. As the concentration of TCP increases, the reversal effect is more rapidly succeeded by a complete inhibition of growth and, after about 120 hours, by a tendency toward the disappearance of some cells, presumably as a result of cell lysis.

Catron (J.B. 516; α-methyl phenethylhydrazine) is a potent and long-acting monoamine oxidase inhibitor which can alter the inotropic response of the heart[19] and increases the norepinephrine content of the soluble fraction of rabbit brain.[20] At concentrations much above 10^{-5} M, it completely inhibits the growth of *Tetrahymena*. Even at 7.2×10^{-6} M it causes noticeable inhibition of growth, but, unlike tranylcypromine, it markedly potentiates the inhibitory effect of reserpine (Fig. 6).

Effects of other drugs: Desipramine (desmethylimipramine) is a dibenzazepine compound used clinically for the relief of mental depression. Its mode of action is unknown, but according to Goodman and Gilman[21] it seems to require an intact store of catecholamines in the central nervous system to be effective. As can be seen in Figure 6, 4.1×10^{-5} M desipramine significantly inhibits the growth of *Tetrahymena* and strongly potentiates the growth-inhibitory effects of reserpine.

Segontin (N-(3-phenyl-2-propyl)-3,3 diphenylpropylamine lactate) decreases the concentration of norepinephrine in the heart, brain, and adrenal gland of the rat and has several other similarities to reserpine despite its entirely different structure.[22]

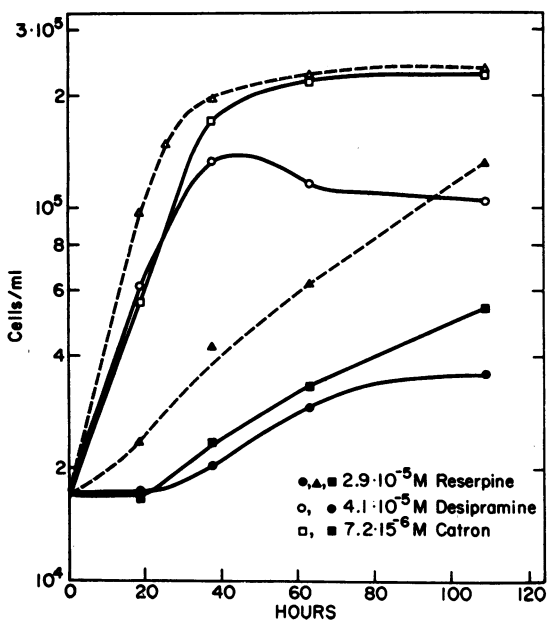

FIG. 6.—Potentiation of the growth-inhibitory effect of reserpine by desipramine and by Catron. Each flask contained 25 ml of cells in medium DK supplemented with 0.07% proteose peptone. At zero time, drugs at the indicated concentrations were added in a total of 5 ml of water. Open symbols indicate cells without reserpine. Solid symbols indicate cells with 2.9×10^{-5} M reserpine.

At 2×10^{-5} M, it strongly inhibits the growth of *Tetrahymena*. Unlike reserpine, Segontin causes an almost complete loss of cell glycogen.

The following compounds did not appreciably inhibit the growth of *Tetrahymena* at the concentrations indicated, nor did they alter the growth-inhibitory effect of reserpine: corticosterone at 0.93 μg/ml; epinephrine at 9.2 μg/ml; isoproterenol at 60 μg/ml; guanethidine at 24 μg/ml; octopamine, 1 mM; metaraminol, 0.75 mM; α-methyl-meta-tyrosine, 1 mM; L-β-3,4 dihydroxyphenylalanine, 1 mM; 3-iodotyrosine, 0.1 mM; tyramine, 0.58 mM.

Discussion.—Tetrahymena pyriformis contains two catecholamines, epinephrine and norepinephrine, and is sensitive to a third substance which acts as a hormone, triiodothyronine. It is sensitive to the classical α or β adrenergic site blocking agents, dibenzyline, propanolol, and dichloroisoproterenol. Growth is inhibited by the methyl xanthines, by monoamine oxidase inhibitors, and by two compounds known to affect the catecholamine system in the brain. Growth is sensitively inhibited by reserpine, and reserpine also depletes the store of catecholamines of these calls. We may approach these observations in two ways.

First, we may inquire whether the extensive analogy between the sensitivity of *Tetrahymena* and the sensitivity of mammalian brain, liver, and heart cells to this series of drugs is no more than a coincidence. Most of the drugs tried may have more than one mode of action, and perhaps any drug which inhibits growth of *Tetrahymena* also causes, indirectly, a change in the metabolic control patterns which could be interpreted as a result of changes in the catecholamine system. In fact, there can be no satisfactory answer to this argument until more data become available on the mode of action of these drugs in *Tetrahymena* and, in most cases, in the mammalian cell as well. For example, the present data suggest the presence of monoamine oxidase in these cells, but we have not yet assayed cell homogenates for the presence of this enzyme. The reversal of the reserpine inhibition of growth by tranylcypromine superficially resembles the reversal of reserpine sedation in rats, but we have no explanation for either of these phenomena. In *Tetrahymena*, it is possible to inhibit net glycogen synthesis without appreciably inhibiting growth, and it seems clear that reserpine, at least, must affect growth at a separate locus from the one at which it affects glycogen metabolism.

Alternatively, we may accept the analogy at face value. It is well known that one major locus of action of the catecholamines in the mammalian cell is on the control of phosphorylase and glycogen synthetase. In *Tetrahymena*, glycogen synthesis is highly sensitive to drugs (reserpine, DCI) and to triiodothyronine, which are known to influence the catecholamine system. In both nerve cells and *Tetrahymena*, reserpine depletes the catecholamines. Tranylcypromine reverses the sedative effects of reserpine in the rat and reverses the growth-inhibitory effects of reserpine in *Tetrahymena*. These observations suggest that *Tetrahymena* possesses a fully developed catecholamine system. It is worth mentioning that *Tetrahymena* is rather insensitive to hexamethonium and related compounds.[23] It seems probable, therefore, that *Tetrahymena* are simpler than such tissues as brain or liver, not only on structural grounds, but perhaps also in not possessing the acetycholine system which further complicates analyses of mammalian tissues. Thus, *Tetrahymena* may provide a very useful single-cell system for the study of the fundamental metabolic actions of drugs which affect the central nervous system, such as Segontin and desipramine.

If the action of these drugs on *Tetrahymena* is indeed analogous to their action on mammalian cells, then it appears that most of the major components of the catecholamine system originated before the metazoa developed and presumably acted as a metabolic control system at the intracellular level before they were adapted for endocrine and neurotransmitter functions in the metazoa. (*See* Note added in proof.)

Summary.—The protozoan *Tetrahymena* appears to possess a fully developed catecholamine metabolic control system as evidenced by the effects of adrenergically reactive drug on the growth rate and glycogen content of these cells.

Note added in proof: It has recently been found (Janakidevi, K., V. C. Dewey, and G. W. Kidder, *Arch. Biochem. Biophys.*, **113**, 758 (1966)) that *Tetrahymena* contains serotonin. Some of the drugs effects reported here may therefore also involve changes in serotonin concentration.

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