lacks the concentric mesophyll layer, but yet has photorespiratory rates below those of C_3 plants. Kranz anatomy may not be a requirement for the low photorespiration observed in C_4 plants.

Photosynthetic rates of the two *Mollugo* species are also given in Table 1. Under the conditions used, the rates are approximately equal. The relatively low photorespiration ratio of *M. verticillata* may be responsible for the nearly identical photosynthetic rates in spite of the different leaf anatomies and primary products of the two species.

Mollugo verticillata is, as we have shown, intermediate in at least four features which are diagnostic for C_4 photosynthesis: leaf anatomy, cell ultrastructure, photorespiration, and primary photosynthetic products. This "continuum" of C_4 expression within closely related species is not surprising, since C_4 photosynthesis probably arose independently in many areas as a result of similar environmental pressures (*18*). Since selective pressures for the development of C_4 photosynthesis exist as gradients, transitional (ecotonal) species are also likely to exist. *Mollugo verticillata* appears to be such a species.

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19. We thank Dr. Norman Terry, Department of Soils and Plant Nutrition, University of California, Berkeley, for determining the photosynthetic rates by infrared gas analysis.
20. This work was supported in part by grant GB-12964 from the National Science Foundation and by a grant-in-aid of research from Society of the Sigma Xi.

4 March 1974

Cyclic Guanosine and Adenosine 3′,5′-Monophosphates in Canine Thyroid: Localization by Immunofluorescence

Abstract. *When guanosine 3′,5′-monophosphate (cyclic GMP) and adenosine 3′,5′-monophosphate (cyclic AMP) are localized in canine thyroid by a fluorescence immunocytochemical procedure, distinct staining patterns for each nucleotide are seen: cyclic AMP is distributed throughout the follicular cell cytoplasm before and after administration of thyroid-stimulating hormone, while cyclic GMP is localized to the follicular cell membrane in the control state, and increased cytoplasmic fluorescence is visualized after acetylcholine. These data provide histological evidence that correlates with cyclic nucleotide tissue measurements, suggesting diverse roles of the two nucleotides in thyroid function.*

Guanosine 3′,5′-cyclic monophosphate (cyclic GMP) is present in almost all mammalian tissues, but its role in cell function is poorly understood. In some tissues such as kidney and heart, the addition of cyclic GMP or its dibutyryl derivative causes effects antagonistic to those of adenosine 3′,5′-cyclic monophosphate (cyclic AMP), although exogenous cyclic GMP acts as a weaker cyclic AMP in most tissues. An agonist-antagonist hypothesis for interreaction of these nucleotides has been suggested and the subject has been reviewed recently (*1*).

Cyclic GMP could also be involved in the action of acetylcholine (ACh) at muscarinic receptor sites. Acetylcholine and other cholinergic compounds cause an increase in the concentration of cyclic GMP in a variety of tissues, and this rise is blocked by prior treatment of the tissues with atropine (*2*).

To gain insight into possible roles of cyclic GMP in cell function and to examine further the relation of cyclic GMP to the action of ACh we have applied the technique of fluorescence immunocytochemistry (*3*) in the localization of cyclic GMP to thyroid tissue and have contrasted the localization of this cyclic nucleotide with that of cyclic AMP. We have used thyroid tissue for these studies because thyroid-stimulating hormone (TSH) has been shown to increase the concentration of cyclic AMP but not that of cyclic GMP, whereas ACh increases concentrations of cyclic GMP but not of cyclic AMP (*4*). We report distinct differences in localization of cyclic AMP and cyclic GMP in canine thyroid under basal conditions and after hormonal stimulation and suggest that the diverse roles of these two nucleotides and their intracellular localization may be related.

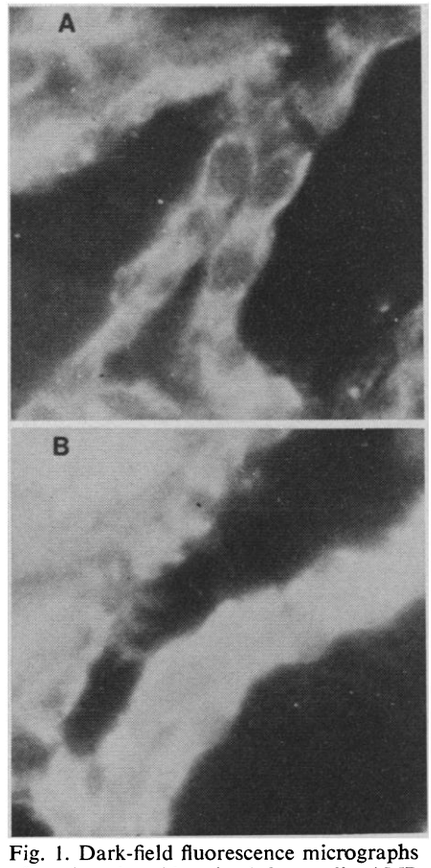

Fig. 1. Dark-field fluorescence micrographs of canine thyroid stained for cyclic AMP (×400). (A) Before TSH stimulation. (B) Twenty minutes of incubation with TSH (50 milliunits per milliliter), illustrating an apparent increase in cytoplasmic staining.

In each flask four thyroid slices (approximately 10 to 15 mg each) were first incubated in 2 ml of Krebs-Ringer bicarbonate buffer, *p*H 7.4, for 10 minutes at 37°C, with agitation and continuous gassing with 95 percent oxygen and 5 percent CO_2. The tissues were then incubated in the presence of $10^{-2}M$ theophylline with and without the addition of 100 milliunits of bovine TSH per milliliter or $10^{-3}M$ ACh or carbachol. An acetylcholinesterase inhibitor, physostigmine, was also added in a concentration of $3 \times 10^{-4}M$ with the cholinergic stimulants.

After a 20-minute incubation, a sam-

ple of tissue was frozen on a cryostat chuck for sectioning, and the remainder was placed in liquid nitrogen for subsequent assay of the concentrations of cyclic AMP and cyclic GMP. Radioimmunoassays of cyclic nucleotides revealed (i) no significant change in either cyclic AMP or cyclic GMP concentration in the presence of theophylline alone, (ii) a mean (± standard error) increase in cyclic AMP concentration after TSH from 0.3 ± 0.06 to 2.1 ± 0.12 pmole/mg, and (iii) a mean (± S.E.) rise in cyclic GMP with cholinergic stimulation from 0.03 ± 0.02 to 0.08 ± 0.03 pmole/mg. Histochemical localization of both nucleotides was determined by an indirect immunofluorescent technique on frozen cryostat-cut sections 4 to 6 um in thickness with the use of highly specific immunoglobulin (IgG) from rabbit antiserums to either cyclic AMP or cyclic GMP, as prepared by the method of Steiner *et al.* The frozen sections were dried in air on slides and treated in sequence for 10 minutes with each of the following: antibody to nucleotide (1:4 dilution of antibody to cyclic AMP and 1:8 dilution of antibody to cyclic GMP) or control serum; phosphate buffered saline (PBS); fluorescein-labeled goat antiserum to rabbit IgG in a 1:4 dilution; and PBS. The slides were mounted with 50 percent glycerine in PBS. To demonstrate the specificity of the procedure, we showed that similarly prepared IgG fractions from unimmunized rabbits failed to produce significant staining. Furthermore, passage of the specific antiserum over an affinity column of Sepharose coupled to the antigen eliminated the specific nucleotide staining pattern. In the latter procedure, modified as described by Cuatrecasas *et al.* (*6*), 10 ml of Sepharose (Sigma) was mixed with an equal volume of water and activated with 10 ml of freshly prepared solution of cyanogen bromide (100 mg/ml). The *p*H was rapidly adjusted to and maintained at *p*H 11 by titration with 4*N* NaOH. The activated Sepharose was washed with 20 volumes of cold 0.1*M* sodium bicarbonate (*p*H 9.0) and then suspended in the buffer to a volume of 10 ml. Then 1 to 1.5 ml of a solution of cyclic AMP or cyclic GMP, containing 30 mg of the nucleotide, was added to the activated Sepharose, and the mixture was gently agitated for 20 hours at 4°C. The complex of Sepharose and cyclic nucleotide was washed extensively with buffer and stored at 20°C. Antiserum to either nucleotide, 0.5 ml of a 1:4 or 1:8 dilution, was filtered through a 2-ml Sepharose column poured in a disposable pipette. The effluent antiserum together with 2.0 ml of buffer washing was dialyzed against water for 2 hours, lyophilized, and reconstituted to the original 0.5-ml volume. Varying dilutions of each of the antiserums were subsequently tested by incubation with radioactive cyclic AMP or cyclic GMP to demonstrate that the ability to bind to the radioactive marker had been removed. The antiserum used as control in the immunofluorescent staining bound less than 5 percent of radioactive marker as compared to 50 percent binding by antiserums which had been passed through control Sepharose columns or columns containing Sepharose coupled to the other nucleotide. In two experiments, antibody was eluted from the column with 6*M* guanidine hydrochloride (Eastman). The effluent was collected in normal rabbit serum, dialyzed, and lyophilized. When incubated in appropriate dilutions, the normal rabbit serum was now able to bind labeled antigen specifically, suggesting recovery of the specific antibody from the column after treatment with guanidine hydrochloride.

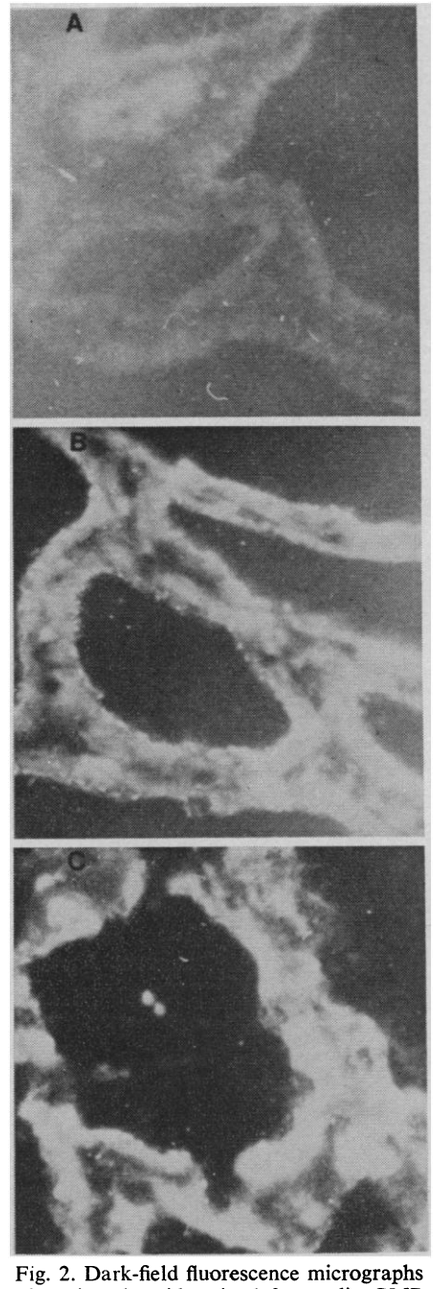

Fig. 2. Dark-field fluorescence micrographs of canine thyroid stained for cyclic GMP (×400). (A) Before stimulation with ACh employing antiserums in which the antibody to cyclic GMP has been removed by affinity chromatography, illustrating nonspecific fluorescence. (B) Before stimulation with ACh. Antiserum to cyclic GMP was used. Beaded fluorescence surrounding a follicle is illustrated. (C) After ACh stimulation. There is a more diffuse cytoplasmic distribution of cyclic GMP.

Using this method we found that in unstimulated thyroid tissue cyclic AMP is present diffusely throughout the thyroid follicular cell cytoplasm with little suggestion of nuclear or plasma membrane localization (Fig. 1A). Lesser amounts of staining were also found in the stroma between the follicular cells. Stimulation with TSH resulted in an observable increase in the intensity of follicular cytoplasmic fluorescence which appears in a coarse granular distribution (Fig. 1B). However, TSH did not appear to significantly alter the distribution of cyclic AMP from that of unstimulated tissue.

The pattern of localization of cyclic GMP was distinctly different from that of cyclic AMP. Cyclic GMP appeared predominantly in a beaded pattern along the thyroid follicular cell plasma membrane that bordered on the follicular colloid (Fig. 2B). After cholinergic stimulation the thin beaded appearance was reduced, the area of staining along the membrane was widened, and there was an increase in cytoplasmic staining (Fig. 2C). Theophylline itself did not affect the degree of staining. The TSH had no effect on the cyclic GMP staining pattern and ACh had no effect on the cyclic AMP staining pattern.

The diffuse cytoplasmic localization of cyclic AMP, and its cytoplasmic accumulation in the thyroid follicular cell after TSH stimulation, is consistent with the numerous thyroidal effects of TSH which reportedly are mediated by cyclic

AMP, such as iodide metabolism, hormone synthesis, phospholipid metabolism, and proteolysis (*7*). These functions do not appear to be dependent on alteration in the cell nucleus, and our histochemical findings are consistent with this conclusion. However, we cannot exclude an increase in nuclear cyclic AMP which may not have been detected by this staining method.

The presence of cyclic GMP in intimate proximity to the plasma membrane was rather surprising for several reasons. First, unlike membrane bound adenylate cyclase, guanylate cyclase has been reported to be a soluble enzyme in most tissues examined (*8*). Finding the reaction product of this nucleotide in close association with the plasma membrane suggests that guanylate cyclase might be membrane bound and may become dissociated from plasma membrane during the process of tissue homogenization. Alternatively, cyclic GMP may have a high affinity for some membrane protein of the thyroid follicular cell.

These observations suggest that patterns of cyclic nucleotide localization as demonstrated by immunocytochemistry may be helpful in elucidating the roles of the cyclic nucleotides in cell function. The localization of cyclic GMP in close proximity to the thyroid follicular cell membrane adjacent to the follicular colloid raises the possibility that cyclic GMP may have a role in moderating thyroglobulin iodination since thyroglobulin iodination is thought to occur adjacent to the follicular cell membrane (*9*).

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19 November 1973; revised 20 February 1974

Pineal β-Adrenergic Receptor: Diurnal Variation in Sensitivity

Abstract. *The responsiveness of the pineal β-adrenergic receptor that regulates serotonin-*N*-acetyltransferase activity is nearly ten times greater at the end of the light period (0600 to 1800 hours) than at the end of the dark period (1800 to 0600 hours). These changes in sensitivity of the postsynaptic β-adrenergic receptor are related to diurnal changes in the release of noradrenaline from sympathetic nerves innervating the pineal. Supersensitivity of the receptor appears to result from decreased release of the neurotransmitter during daytime, and subsensitivity from increased release at night.*

Supersensitivity in cholinergic and in adrenergic nervous systems occurs after denervation, decentralization, or treatment with certain drugs. Subsensitivity is most frequently manifest as a result of a repetitive stimulus or repeated administration of drugs. A distinction is frequently made between presynaptic and postsynaptic changes in sensitivity. Presynaptic (or prejunctional) changes in sensitivity result from treatment with agents which change the action of nerve terminals on the release, uptake, or local metabolism of the neurotransmitter. Postsynaptic (or postjunctional) changes are changes in the responsivity of the receptor or of the end organ itself to a given stimulus (*1*).

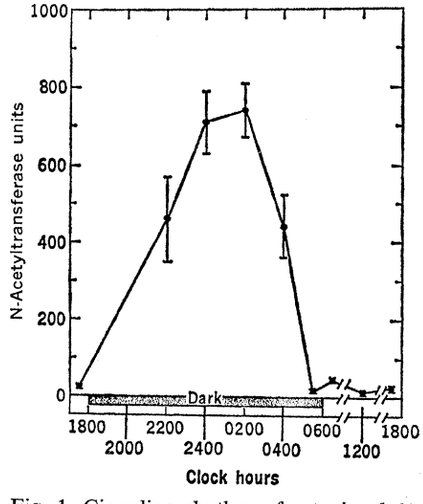

Fig. 1. Circadian rhythm of rat pineal *N*-acetyltransferase activity showing decline beginning 4 hours before the onset of light. Each point is the mean ± the standard error of six pineal glands; the results are expressed as picomoles of ^{3}H-labeled *N*-acetyltryptamine per pineal gland per 10 minutes.

Our laboratory has recently reported the phenomenon of supersensitivity and subsensitivity in the pineal gland (*2*). A β-adrenergic receptor in the pineal cells regulates the synthesis of serotonin-*N*-acetyltransferase (E.C. 2.3.1.5), an enzyme involved in the synthesis of the hormone melatonin (*3*). Removal of endogenous neurotransmitter noradrenaline by preliminary treatment of rats with reserpine, by denervation, or exposure to continuous light results in increased responsiveness of the enzyme to induction by the β-adrenergic agonist isoproterenol (*2*). Repeated administration of isoproterenol to normal animals without any surgical or other pharmacological intervention causes subsensitivity and abolishes the supersensitivity resulting from denervation or reserpine administration. Hence, the prior degree of interaction of neurotransmitter with the receptor determines the subsequent responsiveness of the organ to further stimulation (*1, 2*).

There is a marked circadian rhythm in the activity of serotonin-*N*-acetyltransferase, which increases 30- to 50-fold during the night as compared to daytime values (*4*). This rhythm persists in constant darkness and is abruptly abolished by light (*4, 5*) or by blockade of β-adrenergic receptors (*5*), which cause precipitous falls in enzyme activity.

The circadian rhythm of *N*-acetyltransferase appears to be driven by diurnal changes in the turnover (and presumably release) of noradrenaline from sympathetic fibers innervating the