hour; 1-hour cultures contained mostly free cells and small, loose clusters and there was no further aggregation during the rest of the cultivation period. Concentrations of puromycin as small as 0.5 μg/ml (the lowest tested) were inhibitory. The effect was concentration dependent. The puromycin block was reversible in that cells exposed for 6 hours to the inhibitor resumed aggregation, after a short lag period, when transferred to normal medium (Fig. 1*c*). To test whether the block to aggregation might not be due primarily to interference by puromycin with the repair of possible dissociation damage to cells, cells were cultured in normal medium before exposure to the inhibitor. Puromycin added to 6-hour cultures, when aggregation was already well advanced, stopped reversibly further aggregation in about 90 minutes (Fig. 1*d*). Addition of adenosine triphosphate (ATP) ($4 \times 10^{-3}M$) did not prevent the inhibitory effect of puromycin.

It appears that unimpaired protein synthesis is required for progressive reattachment and aggregation of these cells. The finding that cell aggregation is also reversibly inhibited by ribonuclease (*3*) seems relevant here. The evidence suggests therefore that, under our experimental conditions, RNA-dependent synthesis is essential for regeneration of mutual adhesiveness of the cells and that the products involved turn over, change, or become inactivated rather rapidly, at least as long as the cells remain free. Formation of the initial small clusters by some of the cells, prior to the onset of the inhibition plateau, could be due to their residual reserves of the essential constituents, to the time required for an effective suppression of protein synthesis by puromycin, or to factors not susceptible to the inhibitor.

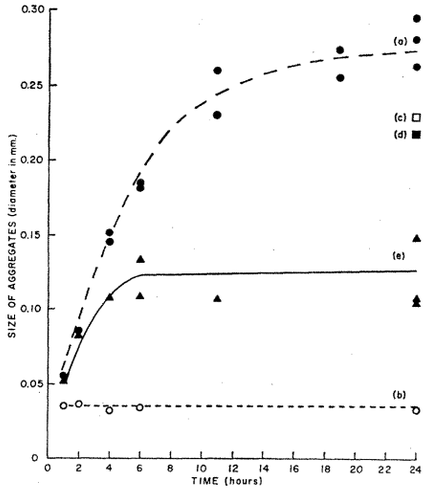

Fig. 1. Size of aggregates in suspension cultures of trypsin-dissociated neural retina cells from 10-day chick embryos: (*a*) controls; (*b*) in medium with puromycin (5 μg/ml); (*c*) cultures maintained for 6 hours in puromycin and then transferred to normal medium for the duration of the incubation period; (*d*) cultures maintained for 6 hours in normal medium and then transferred to medium containing puromycin; (*e*) in medium with actinomycin (0.5 μg/ml). Each point represents an average of measurements on several cultures in one experiment.

In considering further the prompt suppression of cell aggregation by blocking RNA-dependent protein synthesis it was of interest to examine whether inhibition of synthesis of new RNA by actinomycin would affect aggregation of these cells. It was found that actinomycin inhibited aggregation, albeit at a rate significantly slower than puromycin (Fig. 1*e*). In the presence of actinomycin D (0.5 μg/ml or higher concentrations) the cells continued to aggregate for about 4 hours, though at a declining rate as compared with controls; thereafter no increase in size of aggregates was detectable. The same effect was obtained after 1-hour exposure of cells to the inhibitor. Actinomycin added to 6-hour cultures in normal medium blocked further aggregation after about 5 hours. The time required for the full effect of actinomycin could reflect the life time of the existing RNA involved in the processes tested; however, it could also be due to the rate of binding of the drug with DNA. Under the conditions studied the effect of actinomycin was irreversible.

The usefulness of our exploratory results is that they suggest a hitherto unexploited and potentially promising approach to various aspects of cell-contact and cell-association phenomena. An adequate interpretation of these data and their comparison with other work bearing on this topic (*7*) require further information, particularly with reference to protein and RNA turnover and to other types of cells. The present findings agree with the postulation that biosynthetic processes are involved in the attachment mechanisms and contact properties of embryonic cells; hence they suggest that cell contact phenomena may be subject to controls which regulate these processes. It is conceivable that production of extracellularly functioning materials with specific cell-binding roles may be a major aspect of this matter; the recent isolation of aggregation-promoting materials from live sponge cells (*8*) and other evidence (*3*) point in this direction. Accordingly, the possibility exists that the block to aggregation by inhibitors of protein synthesis might be due to interference with formation of such materials. Their susceptibility to inhibition would be likely to vary with different kinds of tissues, cells, and functional or developmental states of cells (*9*).

M. H. Moscona
A. A. Moscona
Department of Zoology, University of Chicago, Chicago, Illinois

References and Notes

1. A. Moscona, *Exptl. Cell Res.* **22**, 455 (1961).
2. ———, *Nature* **190**, 408 (1961); B. Garber, *Develop. Biol.* **7**, 630; Y. Kuroda, *Exptl. Cell Res.* **30**, 446 (1963).
3. A. Moscona, *J. Cell. Comp. Physiol. Suppl. 1*, **60**, 65 (1962); in *Biological Interactions in Normal and Neoplastic Growth* (Little, Brown, 1962), p. 113.
4. Y. Takeda, S. Hayashi, H. Nakagawa, F. Suzuki, *J. Biochem. Tokyo* **48**, 169 (1960); T. Hultin, *Experientia* **17**, 410 (1961); A. M. Nemeth and G. L. de la Haba, *J. Biol. Chem.* **237**, 1190 (1962).
5. J. Hurwitz, J. J. Furth, M. Malany, M. Alexander, *Proc. Natl. Acad. Sci. U.S.* **48**, 1222 (1962); E. Reich, R. M. Franklin, A. J. Shatkin, E. L. Tatum, *ibid.*, p. 1238; E. H. Davidson, V. G. Allfrey, A. E. Mirsky, *ibid.* **49**, 53 (1963).
6. R. E. Madden and D. Burk, *J. Natl. Cancer Inst.* **27**, 841 (1961).
7. E. H. Davidson, V. G. Allfrey, A. E. Mirsky, *Proc. Natl. Acad. Sci. U.S.* **49**, 53 (1963).
8. A. A. Moscona, *ibid.* **49**, 742 (1963); T. Humphreys, *Develop. Biol.* **8**, 27 (1963).
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Melatonin Synthesis in the Pineal Gland: Control by Light

Abstract. *In rats placed in continuous darkness for 6 days, there is a striking increase in the activity of melatonin-synthesizing-enzyme (hydroxyindole-O-methyl transferase) in the pineal gland, but no change in the activity of monoamine oxidase. Since melatonin appears to have a hormonal role in mammals, and its synthesis is confined to the pineal gland, the inhibition of hydroxyindole-O-methyl transferase by light may constitute a mechanism of neuroendocrine regulation.*

Melatonin (5-methoxy-*N*-acetyltryptamine) is produced in the mammalian pineal gland by the *O*-methylation of *N*-acetylserotonin (*1*, *2*). Although the *O*-methylating enzyme, hydroxyindole-*O*-methyl transferase (HIOMT), has

Table 1. The effect of illumination on the activity of hydroxyindole-O-methyl transferase (HIOMT) and monoamine oxidase (MAO) in the pineal glands of female rats. The activity of HIOMT is expressed as $\mu\mu$moles of C^{14}-labeled melatonin synthesized per hour; MAO activity is expressed as $\mu\mu$moles of C^{14}-labeled indoleacetic acid formed per hour.

Treatment	Duration of treatment	Weight of pineal gland (mg)	Activity			Estrus (%)
			HIOMT		MAO (per mg)	
			Per gland	Per mg		
Groups of 10 rats, each rat aged 21 days at onset of treatment						
Dark	50 days	1.51 ± 0.08	30.1 ± 5.14	20.1 ± 3.03	1410 ± 218	23
Diurnal lighting	50 days	1.13 ± 0.06*†	5.5 ± 1.39*	4.9 ± 1.21*	1140 ± 175	50
Light	50 days	0.95 ± 0.05*	3.0 ± 0.74*	3.1 ± 1.02*	1460 ± 161	78
Groups of 10 rats, each rat aged 78 days at onset of treatment						
Dark	6 days	1.28 ± 0.03	22.8 ± 6.11	17.6 ± 4.57	1370 ± 20	50
Light	6 days	0.93 ± 0.02*	4.1 ± 2.62*	3.9 ± 1.55*	1640 ± 300	60
Groups of 6 rats, treated from birth						
Dark	55 days	1.26 ± 0.02	13.1 ± 0.64	10.3 ± 0.60	1160 ± 80	29
Light	55 days	0.86 ± 0.09*	4.1 ± 1.66*	4.8 ± 2.09*	1010 ± 120	67

* Differs from dark-treated, $p < 0.001$. † Differs from light-treated, $p < 0.05$.

been sought in a wide variety of mammalian (*3*) and bird (*4*) tissues, it has been found only in the pineal gland. *N*-Acetylserotonin is formed by the *N*-acetylation of serotonin (5-hydroxytryptamine); this process is catalyzed by an enzyme found in many tissues, including the pineal gland (*2*). Since the mammalian pineal gland contains very large stores of serotonin (*5*), it seems likely that, under certain conditions, the activity of HIOMT may be the rate-limiting factor controlling the synthesis of melatonin in vivo. Pineal serotonin is also, presumably, metabolized by monoamine oxidase (*5*).

The mass (*6*), morphology (*7*), and biochemical composition (*8*) of the mammalian pineal gland can be altered by varying the amount of light or darkness to which the animal is exposed: constant exposure to light decreases the weight of the pineal gland in the rat; this effect does not require the presence or normal functioning of the pituitary gland, gonads, adrenals, or thyroid (*9*). Constant illumination also decreases the size of pinealocyte nucleoli and the level of cytoplasmic basophilia. This has been interpreted as indicating a decrease in pineal RNA synthesis (*7*). The reverse histologic picture is found when rats are placed in continuous darkness.

It has recently been demonstrated that the administration of minute amounts of melatonin subcutaneously over long periods inhibits ovarian growth and the incidence of estrus in young rats (*10*). Larger doses produce a decrease in the size of seminal vesicles in male rats (*11*), and inhibit the response of the thyroid to methylthiouracil (*12*). The natural occurrence of melatonin in a certain tissue, peripheral nerve (*13*), which cannot synthesize it (*3*), but can concentrate it from the circulation (*10*), has been taken as evidence that melatonin is a hormone normally present in the mammalian circulation (*10*). The physiological disposition of intravenously-administered tritiated melatonin is altered in the rat exposed to continuous light, while minute doses of this compound interfere with the persistent estrus induced by light (*10*). Since the actions and disposition of melatonin seem to be related to light exposure in the mammal, studies were undertaken to determine whether the capacity of the rat pineal gland to synthesize melatonin from its immediate precursor was also subject to photic regulation. Our experiments show that exposure of rats to constant darkness for as little as 6 days induces a striking increase in the activity of HIOMT in the pineal gland.

Sprague-Dawley female rats were placed in constant light (*7*), or constant darkness, except for a 25-watt red light bulb which was used while the cages were cleaned, in special rooms equipped with double-door light baffles and air conditioning. Light or darkness did not affect body weight, nor did they influence the length of gestation or litter size among newly-conceived pregnant rats. Vaginal smears were checked daily for about a week prior to enzyme assay in all groups, to confirm the presence of light-induced constant estrus. Animals were killed while still in their dark or light environment. Pineals were quickly dissected, weighed, and homogenized in 1.0 ml of 0.5*M* phosphate buffer, *p*H 7.9. Two or three pineal glands were pooled for each assay. A 0.6 ml sample was assayed for HIOMT activity, and a 0.1 ml sample was used for assay of monoamine oxidase activity, by methods previously described (*3*, *14*).

When immature, 21-day-old rats were kept in constant darkness for 50 days, they showed a highly significant increase in HIOMT activity in the pineal gland (Table 1); pineal glands of rats maintained in constant light had one-half the HIOMT activity of animals exposed to diurnal lighting, and only one-tenth that of littermates exposed to constant darkness. Constant darkness was associated with a significant increase in the weight of the pineal gland, when compared with glands of rats kept in constant light; pineal glands of animals exposed to diurnal lighting were intermediate in weight. When HIOMT activity was expressed per unit weight of gland, rats exposed to constant darkness had more than six times the activity of light-treated littermates. Neither treatment produced any change in the activity of monoamine oxidase in the pineal gland, whether the activity was expressed per gland or per unit weight. Animals kept in continuous light for 50 days showed an increased incidence of estrous phases (proestrus, estrus, or metaestrus), while those in darkness were largely in diestrus.

An experiment was performed to determine whether lighting conditions could produce a difference in HIOMT activity in the pineal gland of mature rats, and within a relatively short period. When 78-day-old animals were exposed to constant darkness for 6 days, there was a fivefold increase in the melatonin-forming enzyme activity (Table 1) compared with animals kept in continuous light. Pineal glands of rats kept in constant darkness for 6 days were appreciably heavier than those of littermates maintained in light. Exposure to light for 6 days induced no significant change in the estrous cycle, or in the activity of monoamine oxidase in the pineal gland.

Another experiment was designed to determine whether exposure to constant light or darkness from birth produced correspondingly greater changes in the activity of HIOMT in the pineal gland, compared with the changes observed in the short-term experiments. The effect of darkness upon HIOMT activity was no greater in animals exposed for 55 days, from birth, than in adult rats treated for 6 days (Table 1).

Again, exposure to constant light was associated with decreased weight of the pineal gland, unchanged monoamine oxidase activity, and an increase in the incidence of estrus.

About one third of the animals kept in constant light had no detectable HIOMT activity in the pineal gland. Male rats and female guinea pigs, which were not in constant estrus, also responded to constant light exposure with decreased HIOMT activity and unchanged monoamine oxidase activity.

That exposure to light can inhibit selectively the activity of the enzyme required for the synthesis of the pineal hormone, melatonin, is of especial interest in view of the possibility that this enzyme limits the rate of melatonin synthesis in vivo. Since melatonin appears to be released from the pineal gland into the circulation, and to act on distant organs as a hormone (*10*), the relationship presented here between illumination and HIOMT activity in the pineal gland may describe a new kind of neuroendocrine regulatory mechanism: control by light of the availability of a hormone by regulating the activity of an enzyme required for its synthesis. It is suggested that one means by which light exposure may induce an increase in ovary growth and in the incidence of estrus (*15*) is by inhibiting the synthesis of melatonin, a hormone which inhibits both these functions (*10*).

Information relating to environmental lighting could be transmitted to the pineal gland by several routes: (i) retinal stimulation could be transmitted to the pineal gland through nerve pathways; (ii) light could act indirectly by influencing other circulating hormones, which in turn would affect enzyme activity; or (iii) light could impinge directly upon the mammalian pineal gland.

Previous work, in which light exposure was found to be related to the RNA content of the pineal gland (*7*), by histochemical estimations, suggests that the differences in enzyme activity described here may actually represent differences in the net rate of synthesis of enzyme protein. The fact that HIOMT activity in the pineal gland can be increased in the absence of light in adult animals, treated for a short period, as effectively as in immature animals treated from birth, suggests that cyclic variations in the activity of this enzyme may occur physiologically, perhaps even diurnally. Since light exposure and the incidence of estrus both show periodicity, it is possible that diurnal variations in light may influence the estrous cycle by influencing the synthesis of melatonin.

RICHARD J. WURTMAN
JULIUS AXELROD
LAWRENCE S. PHILLIPS*
Laboratory of Clinical Science, National Institute of Mental Health, Bethesda 14, Maryland

References and Notes

1. A. B. Lerner, J. Case, Y. Takahashi, *J. Biol. Chem.* **235**, 1992 (1960); J. Axelrod and H. Weissbach, *Science* **131**, 1312 (1960).
2. H. Weissbach, B. G. Redfield, J. Axelrod, *Biochim. Biophys. Acta* **43**, 352 (1960).
3. J. Axelrod, P. D. MacLean, R. W. Albers, H. Weissbach, in *Regional Neurochemistry*, S. S. Kety and J. Elkes, Eds. (Pergamon, New York, 1961), pp. 307–311.
4. J. Axelrod, C. Winget, R. J. Wurtman, L. S. Phillips, in preparation.
5. N. J. Giarman, N. Day, *Biochem. Pharmacol.* **1**, 235 (195 ➡ N. J. Giarman, D. X. Freedman, L. Picard-Ami, *Nature* **186**, 480 (1960).
6. V. M. Fiske, K. Bryant, J. Putnam, *Endocrinology* **66**, 489 (1960); R. J. Wurtman, W. Roth, M. D. Altschule, J. J. Wurtman, *Acta Endocrinol.* **36**, 617 (1961).
7. W. Roth, R. J. Wurtman, M. D. Altschule, *Endocrinology* **71**, 888 (1962).
8. W. B. Quay and A. Halevy, *Physiol. Zool.* **35**, 1 (1962).
9. V. M. Fiske, J. Pound, J. Putnam, *Endocrinology* **71**, 130 (1962).
10. R. J. Wurtman, J. Axelrod, E. W. Chu, *Science* **141**, 277 (1963).
11. J. A. Kappers, *Intern. Congr. Zool., 16th, Washington, D.C., 1963*, abstracts, vol. 3, p. 238.
12. L. Baschieri, F. DeLuca, L. Cramarossa, C. DeMartino, A. Oliverio, M. Negri, *Experientia* **19**, 15 (1963).
13. A. B. Lerner, J. D. Case, R. V. Heinzelman, *J. Am. Chem. Soc.* **81**, 6084 (1959).
14. R. J. Wurtman, J. Axelrod, *Biochem. Pharmacol.*, in press.
15. V. M. Fiske, *Endocrinology* **29**, 187 (1941).

* Summer resident, National Institute of Mental Health. Present address: Harvard Medical School, Boston, Mass.

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Proteinpolysaccharide in Connective Tissue: Inhibition of Phase Separation

Abstract. *A macromolecule of protein and chondroitin sulfate (PP-L) inhibits sedimentation of barium-polystyrene sulfonate (BaPSS) and of calcium phosphate at low but not at high values of gravity. Sedimentation of BaPSS removes a large fraction of PP-L from solution, but sedimentation of calcium phosphate does not. The results suggest entanglement among linear polyanionic chains.*

The behavior of collagen fibrils or large particles, upon sedimentation in solutions containing hyaluronate, has led to the suggestion that the diffusely spread polyanionic hyaluronate chain may become entangled with the collagen fibrils (*1*) or mechanically inhibit the sedimentation of large particles (*2*). The anionic polysaccharides of connective tissue occur bound to protein as macromolecules called proteinpolysaccharides (PP). Their existence in solution as diffusely spread molecules may underlie some special properties of connective tissues, such as their capacity to hold water, their control of calcification, and the elasticity of cartilage (*3*).

The present report concerns phenomena which seem related to the diffuse nature in solution of a proteinpolysaccharide from cartilage called PP-L (*4*). This substance consists of about 15 percent protein, 70 percent chondroitin sulfate, and 5 percent keratan sulfate (*5*) and has a molecular weight above 10^6 (*6*). The phenomena investigated are the inhibition of precipitation of insoluble salts from water in the presence of PP-L, and the effect on PP-L of sedimenting these salts at high values of *g*.

Precipitates of two types were studied, calcium phosphate and barium-polystyrene sulfonate (BaPSS). Both precipitate readily from water and sediment completely at values below 100*g*. In the presence of 0.5 to 5.0 mg/ml of PP-L, only small amounts of the insoluble salts sediment when centrifuged at about 500*g*, leaving strongly opalescent supernatant solutions from which either calcium phosphate or BaPSS can be completely sedimented at high centrifugal speeds (30,000 to 100,000*g*). The calcium phosphate sediments contain 10 to 30 percent of the PP-L originally in solution while the BaPSS sediments contain 30 to 70 percent, depending on conditions.

A series of solutions was made up in 0.12*M* NaCl and 0.03*M* veronal buffer, each at *p*H 7.8 and containing in 11 ml, 40.5 μmole Ca^{++}, 41.4 μmole of phosphate, and the variable amount of PP-L indicated in Table 1, column 1. The phosphate was always added last. After standing 16 hours at either 25°C or 37°C, the solution containing no PP-L had a deposit of calcium phosphate with a clear supernatant solution; all others had progressively smaller deposits of calcium phosphate and more opalescent supernatant solutions. All were centrifuged at about 500*g* for 15 minutes and the residues left after drainage were called R1. The supernatant solutions were then centrifuged at 100,000*g* for 30 minutes and the residues after drainage were called R2.