mates (*2*). However, in women taking oral contraceptives, the increase was abolished and amounts of acids were significantly lower. The precise role of olfactory mechanisms in human sexual behavior needs to be clarified. We have scant information on the physiological importance of these volatile constituents in the human, although it is of interest that the same substances (copulins) (*1*) possess sex-attractant properties in other primates. Furthermore, human vaginal secretions have been demonstrated to possess this activity in cross-taxa experiments with rhesus monkeys (*4*). Constant vaginal douching results in the destruction of the normal bacterial flora. The current vogue this practice enjoys is based on widely felt anxieties about genital odors which may, in fact, be wholly unnecessary and quite misplaced.

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References and Notes

1. R. P. Michael, E. B. Keverne, R. W. Bonsall, *Science* **172**, 964 (1971).
2. R. P. Michael, D. Zumpe, E. B. Keverne, R. W. Bonsall, *Recent Progr. Horm. Res.* **28**, 665 (1972).
3. R. P. Michael and E. B. Keverne, *Nature (Lond.)* **218**, 746 (1968); R. F. Curtis, J. A. Ballantine, E. B. Keverne, R. W. Bonsall, R. P. Michael, *ibid.* **232**, 396 (1971).
4. R. P. Michael, *Acta Endocrinol. Suppl.* **166**, 322 (1972).
5. We thank Dr. David Paintin, St. Mary's Hospital and the Samaritan Hospital for Women, London, for cooperation in providing facilities at his clinic.
6. Extracts were concentrated to 50 μl and analyzed on 10 percent FFAP columns in a Perkin-Elmer gas chromatograph programmed from 50° to 220°C. The area of the pentanol peak and calibration constants (determined with authentic compounds) were used to calculate amounts of acids present. Recoveries varied from 45 percent (acetic acid) to 67 percent (methylpentanoic acid). Overall coefficients of variation ranged from 6 percent (butanoic acid) to 18 percent (acetic acid). Control tampons contained 12.8 ± 1.7 μg ($N = 31$) of acetic acid only. This blank was not subtracted.
7. Biomedical Computer Program Series—10 V. Data for the first 7 days were excluded because samples were not collected reliably during the menstrual flow. The remaining days of each cycle were divided into five equal periods. Data from six subjects could not be used for statistical analysis because of empty cells (missed samples). Preliminary tests showed a lack of subject-time interaction. Pairwise comparisons by the Scheffé test showed period 2 (late follicular) to be significantly higher than period 4 (midluteal) ($P < .05$).
8. We thank Dr. Michael Kutner, Department of Biometry, Emory University, for the analyses in (*7*). This work was supported by NIMH grant MH19506. We thank the Grant Foundation for providing essential equipment, and we thank also the women who participated in this study.

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Microtubule-Macrotubule Transitions: Intermediates after Exposure to the Mitotic Inhibitor Vinblastine

Abstract. *Vinblastine treatment of microtubule protein or intact microtubules assembled in vitro produced bifilar rings and bifilar helices. Suspensions of rings and helices were demonstrated to bind [^3H]colchicine, a diagnostic property of microtubule protein. Macrotubules are suggested to consist of tightly coiled helices formed by longitudinal compacting of loosely coiled protofilament pair intermediates.*

After chemical and physical treatments which in general disrupt microtubules, larger diameter macrotubules may appear in the cytoplasm and nucleus of a variety of cell types (*1*). Prominent among the chemical agents which induce macrotubules is the *Vinca* alkaloid, vinblastine sulfate (VLB). Paracrystals induced by VLB (*2*) are reported to be the result of the lateral aggregation of macrotubules (*1*). Since VLB-induced paracrystals bind colchicine (*3*), and, since paracrystals and microtubules exhibit a high binding affinity for the same specific antibodies (*4, 5*), it seems probable that macrotubules consist of microtubule protein. VLB can also induce the formation of

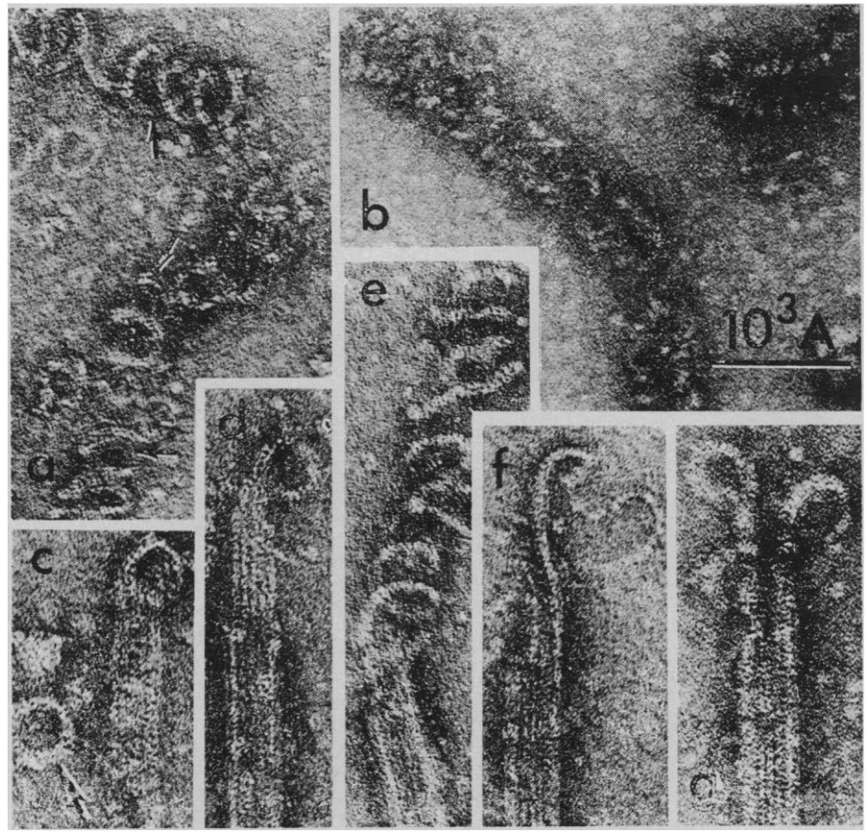

Fig. 1. MTP and microtubules after treatment with VLB. MTP extracted from porcine brain was incubated with $10^{-4}M$ VLB at 4°, 23°, and 37°C. Helices and rings were stained with 1 percent uranyl acetate and could be identified within 1 minute of VLB incubation. Microtubules were assembled by incubating MTP at 37°C for 20 to 30 minutes and cooling the suspension to 23°C. Assembled microtubules were subsequently treated with $10^{-4}M$ VLB. (a) Loosely coiled helices from VLB-treated MTP. Arrows point to regions where the bifilar nature of helices is evident. (b) Helices from VLB-treated MTP exhibiting compacting of coils. (c to g) Unraveling ends of assembled microtubules after VLB treatment. The apparent continuity between a microtubule protofilament pair and loosely coiled helix in (e) suggests the rationale of what is graphically illustrated in Fig. 2b. Samples of VLB-treated intact microtubules revealed: (i) no unraveling at 1 minute, (ii) unraveling ends and a few short free helices at 4 minutes, and (iii) unraveling ends and many free or apparently attached long helices at 10 minutes; the scale represents 1000 Å.

helical and ringlike structures (*5–7*) that are apparently related to the more highly organized paracrystals (*6*). We now report direct in vitro evidence which suggests that VLB-induced helices may be early stages of macrotubule formation and that these helices bind colchicine, which is a diagnostic property of microtubule protein (*8*). In addition, by examining the specific effects of VLB on intact microtubules, evidence suggesting a conversion to macrotubules by reorientation or stabilization of microtubule protofilament pairs has been obtained.

Microtubule protein (MTP) was extracted from pig brain essentially by the procedure of Borisy and Olmsted (*9*). Aqueous VLB (Velban, Lilly) was added to cold brain extract or to assembled microtubules to a final concentration of $10^{-4}M$. Microtubules were assembled by incubating cold brain extract at 37°C for 20 to 30 minutes; microtubule suspensions were then cooled to 21° to 23°C, and VLB or water (controls) were added. [^{3}H]Colchicine (New England Nuclear) with a specific activity of 5 c/mmole was dissolved in assembly medium (*9*) and adjusted to a final concentration of $2 \times 10^{-7}M$ by addition to appropriate protein suspensions.

Microtubule protein treated with VLB for 30 minutes at either 0°, 23°, or 37°C will assemble into three general conformations, in agreement with previous studies (*6*): (i) loosely organized bifilar helices (Fig. 1a) continuous with or separate from (ii) tightly coiled bifilar helices (Fig. 1b), and (iii) bifilar rings (Fig. 1c, arrow). Helices could be identified within less than 1 minute after addition of VLB, and temperature had no measurable effect on the kinetics or nature of assembly. Helices and rings were also observed when VLB was added to the supernatant from a homogenate centrifuged at 230,000*g*, for 1.5 hours at 0° to 4°C, a procedure that removes a presumptive nucleating factor (*9*). Helices were stable for at least several hours and were only partially inhibited by colchicine added to the incubation mixture (Fig. 2a, $C + V$). In assembly medium no helices or microtubules formed in the presence of colchicine alone, no microtubules formed in VLB alone, and controls with no colchicine or VLB contained microtubules but no helices.

Colchicine binding sites were present in preparations containing VLB-induced helices (Fig. 2a, V), suggesting helices do contain MTP. Such sites could be competitively occupied by high concentrations of unlabeled colchicine (Fig. 2a, $C + V$). Other controls, such as the incubation of MTP at low temperature (Fig. 2a, W) and incubation of MTP in the presence of colchicine alone (Fig. 2a, C), gave only relatively low levels of colchicine binding in a sedimentable product which contained no microtubules or helices when examined by electron microscopy.

To examine the possible relationship of helices to microtubules, we added VLB at room temperature to microtubules previously assembled at 37°C, and the suspension was incubated for 30 minutes at room temperature. The treated suspension revealed the usual

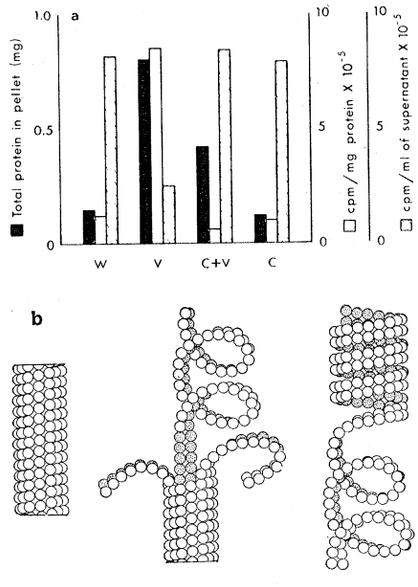

Fig. 2. (a) Binding of [^{3}H]colchicine to microtubule protein after treatment with VLB, colchicine, or both. MTP in assembly medium at 0° to 4°C was treated with water (W), $10^{-4}M$ VLB (V), $2 \times 10^{-3}M$ colchicine and $10^{-4}M$ VLB ($C + V$), and $2 \times 10^{-3}M$ colchicine (C). The suspensions were incubated for 30 minutes at the following temperatures: W, 0° to 4°C; V, $C + V$, and C, 37°C. Subsequently all were incubated at 37°C for 30 minutes with $2 \times 10^{-7}M$ [^{3}H]colchicine and centrifuged at 31,000*g* for 30 minutes at 37°C. The pellets were assayed for radioactivity and for total protein. V and $C + V$ contained bifilar helices. (b) Diagrammatic representation of macrotubule formation. (Left) Microtubule before incubation with VLB. (Center) Unraveling of microtubule end into protofilament pairs after treatment with $10^{-4}M$ VLB. (Right) Longitudinal compacting of protofilament pairs into macrotubule. Helices and macrotubules also form after VLB-treatment of MTP (presumably from subunits) under conditions where microtubules do not assemble.

free helices and rings, and, in addition, microtubules that appeared to be unraveling at their free ends (Fig. 1, c to g). Samples taken at intervals during the VLB incubation revealed a progressive increase in helices and rings. At 1 minute of incubation, microtubule breakdown was not evident. At 4 minutes of incubation, microtubule breakdown and short, loose helices were evident. At 10 minutes of incubation, longer helices, both loose and tight, were evident. Breakdown can occur at both ends of the microtubule. Interestingly, microtubules in VLB appeared to unravel by separation of pairs of longitudinal elements (protofilaments) accompanied by a recoiling of the separated pairs into helices, as shown in Figs. 1e and 2b.

Since helices form from VLB-treated MTP in the cold and in the absence of a presumptive nucleating factor, intact microtubules are not requisite for their formation. Bifilar helices or rings produced from either the breakdown of microtubules or MTP directly are of similar dimensions and agree with the dimensions of VLB-induced helices (*5–7*). Since helices bind colchicine (Fig. 2a) and paracrystals bind colchicine and contain the two monomers of the 6*S* dimer of MTP (*3, 10*), it seems probable that helices and paracrystals represent assembly of MTP, but an assembly altered from microtubule assembly. The evidence from our experiments and from experiments of others indicates that VLB-induced helices are formed as a result of the *rearrangement or assembly and coiling of pairs of microtubule protofilaments.* Helices appear to undergo longitudinal compacting resulting in macrotubules; and, finally, in some cells, macrotubules aggregate into the characteristic VLB-induced paracrystals.

A unifying hypothesis for macrotubule induction by the wide variety of agents (*1*) is suggested: (i) Microtubule protofilaments form the stable unit that may assemble into microtubules (*11, 12*) or macrotubules. (ii) The association between adjacent protofilaments (*12, 13*) or protofilament pairs is labile. (iii) Protofilaments may spontaneously coil into larger diameter tubules (macrotubules). (iv) The various agents that induce macrotubules may differ in their specific effects at the level of the protofilament.

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References and Notes

1. G. E. Tyson and R. E. Bulger, *Z. Zellforsch. Mikrosk. Anat.* **141**, 443 (1973).
2. K. G. Bensch and S. E. Malawista, *J. Cell Biol.* **40**, 95 (1969).
3. A. Krishan and D. Hsu, *ibid.* **48**, 407 (1971).
4. A. Nagayama and S. Dales, *Proc. Natl. Acad. Sci. U.S.A.* **66**, 464 (1970).
5. S. Dales, D. C. Hsu, A. Nagayama, *J. Cell Biol.* **59**, 643 (1973).
6. R. Marantz and M. L. Shelanski, *ibid.* **44**, 234 (1970).
7. O. Behnke and A. Forer, *Exp. Cell Res.* **73**, 506 (1972); P. Filner and O. Behnke, *J. Cell Biol.* **59**, 99a (1973).
8. G. G. Borisy and E. W. Taylor, *ibid.* **34**, 525 (1967).
9. G. G. Borisy and J. B. Olmsted, *Science* **177**, 1196 (1972). At the suggestion of J. B. Olmsted a modification of the published procedure, omitting Mg^{2+}, increasing guanosine triphosphate concentration to 2.5 m*M*, and adjusting the *p*H of the assembly medium to 6.9, was utilized. Purification was achieved by microtubule assembly at 37°C followed by centrifugation and resuspension-disassembly of the pellet in fresh 4°C assembly medium.
10. J. Bryan, *J. Mol. Biol.* **66**, 157 (1972).
11. S. Granett, W. Dentler, G. B. Witman, J. L. Rosenbaum, *J. Cell Biol.* **59**, 119a (1973).
12. M. W. Kirschner, R. C. Williams, M. Weingarten, J. C. Gerhart, *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1159 (1974).
13. A. V. Grimstone and A. Klug, *J. Cell Sci.* **1**, 351 (1966); F. D. Warner and P. Satir, *ibid.* **12**, 313 (1973).

14. Supported in part by PHS grant GM 19537.

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Reversal of Catecholamine Refractoriness by Inhibitors of RNA and Protein Synthesis

Abstract. *The generation of adenosine 3′,5′-monophosphate (cyclic AMP) in response to catecholamines in the 2B subclone of RGC6 rat glioma cells previously exposed to norepinephrine and refractory to further norepinephrine addition is substantially increased by addition of inhibitors of RNA and protein synthesis. The time course of the effect of these inhibitors on cyclic AMP concentration suggests that rapid protein synthesis and turnover are involved in catecholamine refractoriness. Norepinephrine induction of cyclic nucleotide phosphodiesterase is demonstrable in RGC6 cells but not in the 2B subclone. Thus, catecholamine refractoriness cannot be attributed to induction of phosphodiesterase. This implies that induction of a protein or proteins, important in catecholamine refractoriness, affects the synthesis rather than the degradation of cyclic AMP.*

The rat glial tumor cell line RGC6 is highly responsive to β-adrenergic agonists, which elicit a transient rise in the intracellular concentration of adenosine 3′,5′-monophosphate (cyclic AMP) (*1*). After the peak cyclic AMP concentration is obtained, the readdition of hormones does not lead to greater accumulation of intracellular cyclic AMP (*2*). The accumulation of an inhibitory substance in the medium has been ruled out, since the medium when added to untreated cultures elicits the same response as fresh medium containing norepinephrine (NE). Similar observations have been reported for human astrocytoma cells (*3*) and human fibroblasts (*4*). We have shown (*5*) that the catecholamine-mediated induction of lactic dehydrogenase (E.C. 1.1.1.27) in RGC6 cells is mediated by cyclic AMP and is blocked by certain inhibitors of RNA and protein synthesis. During these studies we observed that in the presence of actinomycin D (AD) and acetoxycycloheximide (CH), the cells maintained nearly peak levels of cyclic AMP for up to 12 hours. We now report that RNA and protein synthesis appear to be involved in the refractoriness of the cells to catecholamines once the initial peak of cyclic AMP is obtained (*6*).

The 2B subclone of RGC6 cells was derived from RGC6 cells obtained from G. Sato's laboratory and was used between passages 4 and 26. No differences in cyclic AMP accumulation elicited by catecholamines were observed between high-passage cells and frozen stocks of cells at passages 4 to 6. The 2B clone is diploid and free of mycoplasma (*7*). The RGC6 cells were obtained from the American Type Culture Collection. Cells were grown at 37°C for 10 days to a monolayer of stationary cells in T-30 Falcon plastic flasks in Ham's F-10 medium (Gibco) supplemented with 10 percent virus-free fetal calf serum (Reheis, Armour) without antibiotics. At the time of the experiments, cultures were provided with serum-free fresh medium containing appropriate hormones or drugs and kept at 37°C. Cell number and total protein remained unchanged during the experiments. The incubation was terminated by decanting the medium, the cells were immediately rinsed with 5 ml of 0.9 percent saline at 4°C, and 5 percent trichloroacetic acid at 4°C was added. The cells were scraped off the flask with a rubber policeman, and denatured proteins were removed by centrifugation. Cyclic AMP was purified by column chromatography and assayed either by high pressure liquid chromatography (*8*) or by the binding assay (*9*) as modified (*10*). Identical results were obtained by either method. Protein was measured by the method of Lowry *et al.* (*11*).

Figure 1A shows the effect of 3 μ*M* NE, alone and in the presence of CH (5 μg/ml), on cell cyclic AMP concentrations in the 2B subclone at 1 and 6 hours. Cyclic AMP concentration was high 1 hour after NE addition, and was reduced almost to the control value by 6 hours. Cyclic AMP concentration reached a peak 20 to 30 minutes after addition of NE and was 70 percent of the peak value at 1 hour. Addition of CH, an inhibitor of protein synthesis, along with NE had no significant effect on cell cyclic AMP concentrations at 1 hour but had a dramatic effect at 6 hours. The presence of CH markedly reduced the decline in cyclic AMP normally observed in the presence of NE alone. Inclusion of AD (1 μg/ml) gave results identical to those for CH (data not shown). Under these conditions, CH inhibited 97 percent of pro-

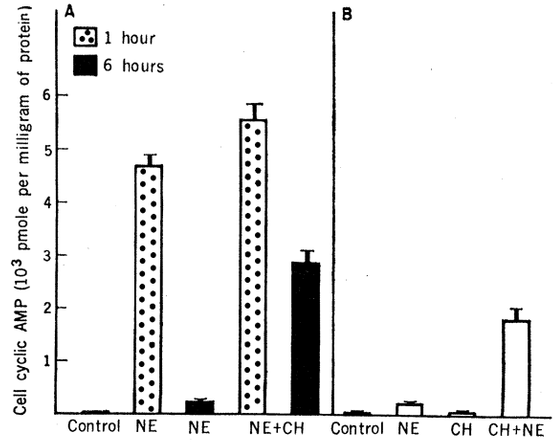

Fig. 1. (A) Effect of CH (5 μg/ml) on intracellular cyclic AMP in cells treated simultaneously with NE (3 μ*M*). Each bar represents the mean and standard error of the mean for four cultures. (B) Intracellular cyclic AMP in refractory cells treated with NE (3 μ*M*) or CH (5 μg/ml) for 1 hour. The combination of NE and CH relieved the refractory condition of the cells. Control cell cyclic AMP was 11 ± 1 pmole per milligram of protein. The NE was L-norepinephrine bitartrate.