causal relationship between ribonuclease susceptibility and antiviral activity. Both effects could be independent and result from important, and as yet undetermined, changes in three-dimensional structure. Colby and Chamberlin (*4*) followed the rate of ribonuclease degradation by measuring the increase of optical density at 260 nm of various ribonucleotide polymers and did not find any correlation between their efficiency of viral inhibition and sensitivity to pancreatic ribonuclease for the polynucleotides tested. However, the rate of breakdown of the polymers by intracellular nucleases may differ from their sensitivity to pancreatic ribonuclease. Nevertheless, it is tempting to causally relate the parallel increase of antiviral activity and resistance to ribonuclease degradation in poly r(AS-US).

As the substitution of a sulfur atom for an oxygen atom in the phosphate groups of poly r(A-U) results in a significant potentiation of the capacity to induce interferon production, it is likely that other, more active polynucleotides will also demonstrate greater activity upon sulfur substitution.

Note added in proof: Because of the excellent susceptibility of the rabbit to interferon inducers, poly r(A-U) and poly r(AS-US) were compared for their interferon inducing capacity in rabbits. Five to 15 and 600 to 1000 unit/ml of circulating interferon were detected 2 to 4 hours after intravenous injection of 20 μg of poly r(A-U), (preparation 2) and poly r(AS-US) (preparation 2), respectively. Thus, substitution of thiophosphate for phosphate in poly r(A-U) resulted in a more than 40-fold increase of interferon production in the rabbit.

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References and Notes

1. A. K. Field, A. A. Tytell, G. P. Lampson, M. R. Hilleman, *Proc. Nat. Acad. Sci. U.S.* **58**, 1004 (1967).
2. ———, *ibid.* **61**, 34 (1968); J. Vilcek, M. H. Ng, A. E. Friedman-Kien, T. Krawciw, *J. Virol.* **2**, 648 (1968); M. S. Finkelstein, G. H. Bausek, T. C. Merigan, *Science* **161**, 465 (1968); J. S. Youngner and J. V. Hallum, *Virology* **35**, 177 (1968); F. Dianzani, P. Cantagalli, S. Gagnoni, G. Rita, *Proc. Soc. Exp. Biol. Med.* **128**, 707 (1968).
3. E. De Clercq and T. C. Merigan, *Nature* **222**, 1148 (1969).
4. C. Colby and M. J. Chamberlin, *Proc. Nat. Acad. Sci. U.S.* **63**, 160 (1969).
5. F. Eckstein and H. Gindl, *Fed. Eur. Biochem. Soc. Letters* **2**, 262 (1969).
6. The homopolyribonucleotides poly rA, rU, rI, and rC were purchased from Miles Laboratories. Two different preparations of (poly rI)·(poly rC) were used, made up from different commercial samples of poly rI and rC.
7. H. Matzura and F. Eckstein, *Eur. J. Biochem.* **3**, 448 (1968). The polymerization mixture did not contain phosphoenolpyruvate or pyruvate kinase. Protein was removed by extraction with a mixture of chloroform and isoamylalcohol. The polymers were isolated by chromatography on Sephadex G25 and, finally, Sephadex G100. Before the polymerization of poly r(AS-US) was stopped approximately 0.5 mg of deoxyribonuclease I (Worthington) was added. Ultraviolet absorption maxima (H_2O, *p*H 6.5) (maximum wavelength, 259 nm) and minima (minimum wavelength, 232 nm) were identical for the two polymers. Sedimentation values ($S_{20,w}$): poly r(A-U) (preparation 1), 7.7; poly r(A-U) (preparation 2), 7.62; poly r(AS-US) (preparation 1), 3.2 (less accurate, limited number of determinations); poly r(AS-US) (preparation 2), 5.0.
8. T. C. Merigan and M. S. Finkelstein, *Virology* **35**, 363 (1968).
9. M. Chamberlin, R. L. Baldwin, P. Berg, *J. Mol. Biol.* **7**, 334 (1963).
10. M. Riley, B. Maling, M. J. Chamberlin, *ibid.* **20**, 359 (1966).
11. M. J. Chamberlin and D. L. Patterson, *ibid.* **12**, 410 (1965).
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Cytogenetic Studies in Rats of Cyclohexylamine, a Metabolite of Cyclamate

Abstract. *Cyclohexylamine, the major known metabolite of cyclamate, was tested in vivo for possible cytogenetic effects. In rats injected with this metabolite, there was a direct relation between dose concentration and percentage of spermatogonial and bone marrow cells showing chromosomal breaks. Single chromatid breaks predominated with infrequent exchange figures.*

Consumption of cyclamate has now reached such a level that 13 million pounds were produced in 1967 and a production of 20 million pounds is projected for 1970 (*1*). At the time of its introduction, it was believed to be almost totally excreted and to have no metabolic products. Several investigators have since found that cyclamate is metabolized to cyclohexylamine (CHA) in dogs and man (*2*). The increase in the consumption of the cyclamates and the realization that metabolic products exist necessitates a reexamination of the entire subject of cyclamate safety.

The most difficult areas in which to determine a cause-and-effect relationship in the human population are in the assessment of carcinogenicity, mutagenicity, or teratogenicity after exposure of the subject to a specific compound. Because of the long latent period between exposure and expression of effects as well as the high background rate of damage, it is difficult to detect effects of a given agent in the population even after years of exposure. Induction of chromosome damage is the method we used to evaluate potential carcinogenic, mutagenic, or teratogenic effects of this cyclamate metabolite.

Holtzman strain albino male rats (170 to 200 g) received daily intraperitoneal injections for 5 days of 50, 40, 20, 10, or 1 mg/kg of CHA (*3*) (adjusted to *p*H 7.3 with HCl); controls received distilled water. For each dose at least 20 (usually 30) animals were used. The total amount of CHA given to the rats was 250, 200, 100, 50, or 5 mg/kg. The rats were killed 24 hours after the last injection of CHA. Cytogenetic studies were performed on both somatic and germinal cells.

Colcemid (*4*) (4 mg/kg) was administered intraperitoneally 5 to 5.5 hours before the tissue was taken. The bone marrow cells were collected from the femur by aspiration into Hanks' balanced salt solution (HBSS). The cells were then centrifuged at 1000 rev/min for 10 minutes, washed once again with HBSS, and exposed to hypotonic KCl solution (0.055*M*) for 30 minutes; they were then centrifuged and fixed in a mixture of methanol and acetic acid (3:1) for at least 30 minutes and usually for 24 hours. The fixative was changed once during this time; an air-dry preparation was then made on the slides. The slide preparations were stained by Giemsa (10 percent) with NH_4OH (5 ml of 0.15*N* NH_4OH per 100 ml of stain) for 5 minutes, rinsed in acetone, cleared with xylene, and mounted.

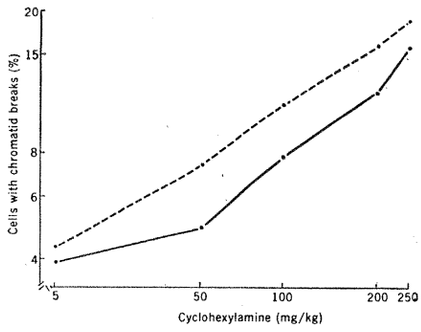

Fig. 1. The percentage of cells with breaks in relation to the concentration of cyclohexylamine. Hatched line, spermatogonial cells; solid line, bone marrow cells.

After orchidectomy, the spermatogonial cells were prepared by removing the tunica albuginea and releasing the seminiferous tubules in a petri dish. Hypotonic solution (0.25 percent trypsin in 0.055*M* KCl) was added to the free tubules, and the tubules were teased apart with forceps. After the tissue was exposed to the hypotonic solution for 40 minutes, it was fixed by adding an equal volume of a mixture of ethanol and acetic acid (3:1).

The seminiferous tubules were softened with acetic acid (45 percent) on the slide, the cover slip was added, and the tubules were squashed. The spermatogonial cells were immediately analyzed with the phase-contrast microscope.

The slides of bone marrow and spermatogonial tissue from each animal were read, and the percentage of breaks (percentage of cells with one or more breaks) was recorded. For each dose, at least 625 metaphase spreads from the bone marrow and 500 metaphase spreads from the spermatogonial cells were analyzed.

The mean percent breakage for the spermatogonial cells was 4.4, 7.6, 11.2, 16.2, and 19.2, whereas the mean percent breakage for the bone marrow cells was 4.0, 5.12, 8.0, 12.16, and 16.28 for 5, 50, 100, 200, and 250 mg/kg of cyclohexylamine, respectively (Fig. 1). The control values for the spermatogonial and bone marrow cells were 1.8 and 2.72 percent, respectively. A dose-response relationship between the concentration of cyclohexylamine and the percentage of breaks is evident. Analysis of means on the transformed data with the *t*-test indicates that the number of spermatogonial cells with one or more breaks was significant at the 95 percent confidence limit, when compared to the control, at a concentration of 1 mg/kg administered in five equal doses over a 5-day period. The analysis of bone marrow cells found significance (99 percent confidence limit) at 10 mg/kg but not at 1 mg/kg.

Single chromatid breaks predominated, with infrequent exchange figures. The single chromatid breaks produced by CHA are the type of abnormality believed most likely to have mutagenic and carcinogenic significance. If multiple breaks occur, the result will, in the majority of cases, consist of loss of viability, which is less important in terms of mutagenicity or carcinogenicity (*5*).

Significance of cytogenetic effects varies with the biological system and the concentration of chemicals used. Stone *et al.* have reported on the effect of cyclamate in leukocyte cultures in vitro (*6*), and Sax and Sax have reported on the effect of cyclamates in plant cells (*7*). The present study demonstrates the effect of a metabolite of cyclamate, cyclohexylamine, in both somatic and spermatogonial cells of rats. The significant effect on the rat spermatogonial cell occurs at the comparatively low concentration of 5 mg/kg when CHA (1 mg/kg) is administered daily for 5 days. The present investigation places CHA in a rather limited group of agents, such as certain viruses (*8*), chemicals (*9*), and irradiation (*10*), which produce chromosome abnormalities in vivo. Our observations indicate potential mutagenic, carcinogenic, or teratogenic effects that have yet to be determined.

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References and Notes

1. R. A. Ballinger, *Agricultural Economic Report No. 133*, USDA (U.S. Government Printing Office, Washington, D.C., 1967).
2. S. Kojima and H. Ichibagase, *Chem. Pharm. Bull. Japan* **14**, 971 (1966); J. S. Leahy, M. Wakefield, T. Taylor, *Food Cosmet. Toxicol.* **5**, 447 (1967).
3. Cyclohexylamine was purchased from City Chemical Corp., New York. When it was tested for purity by gas chromatography, no peaks were observed other than the peak from CHA.
4. Colcemid, Ciba brand of synthetic colchicine.
5. P. Aula, W. W. Nichols, A. Levan, *Ann. N.Y. Acad. Sci.* **155**, 737 (1968).
6. D. Stone, E. Lamson, Y. S. Chang, K. W. Pickering, *Science* **164**, 568 (1969).
7. K. Sax and H. J. Sax, *Jap. J. Genet.* **43**, 89 (1968).
8. W. W. Nicholas, A. Levan, B. Hall, G. Ostergren, *Hereditas* **48**, 367 (1962).
9. E. Conen and G. S. Lansky, *Brit. Med. J.* **2**, 1055 (1961).
10. A. L. Brooks and F. W. Lengemann, *Radiat. Res.* **32**, 587 (1967).

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Studying Neural Organization in Aplysia with the Scanning Electron Microscope

Abstract. *Preliminary attempts have been made to employ the scanning electron microscope in the mapping of a simple nervous system, the abdominal ganglion of* Aplysia californica. *Early results are encouraging: neuronal fibers have been identified and traced over relatively long distances from their cell bodies to structures tentatively identified as synapses and to structures tentatively identified as electrotonic connections.*

Because the abdominal ganglion of the mollusk *Aplysia californica* currently is the object of intensive, concerted electrophysiological investigations, because the neuronal cell bodies of this ganglion are large and easily identified, and because its cells and its neuropile have been studied extensively with the light microscope and the transmission electron microscope (*1, 2*), this ganglion was chosen as the object of our first attempted application of scanning electron microscopy to mapping neural networks. Subsequent work has shown that relatively clean neuronal surfaces can be obtained in a vertebrate retina (*3, 4*). If the same success were achieved in the *Aplysia* neuropile, then mapping should be possible with the scanning electron microscope. The problems in *Aplysia* are similar to those in the retina. In both cases cleavage must be achieved along neuronal boundaries, and extraneous interstitial material such as remnants of glial cells (in the ganglion) or pigment epithelium (in the retina) must be extracted sufficiently to allow a clear view of the neuronal topography. Rather drastic preparation techniques were used in the case of the retina. It was demonstrated, however, that although these techniques caused considerable shrinkage, they conserved proportions, integrity, and topography of both gross and fine structures (*3*). The same fixation, extraction, dehydration, and drying techniques were applied to the *Aplysia* ganglion.

The abdominal ganglion of an *Aplysia californica* was removed from the animal and mounted dorsal side up in a small dissecting dish filled with filtered seawater. An H-shaped incision was made through the sheath in the following manner: one rostral-caudal cut from right connective to branchial nerve, one rostral-caudal cut from left