artifact of normal culturing procedures is dubious. If a cell population stabilized in the immortal state, the proportion of uncommitted cells would s...l be extremely small. Thus, if function depends on the presence of healthy cells (*20*), then senescence could be a result of the increasing fraction of old and terminally committed cells (*21*). The idea that the replicative limit is related to terminal differentiation (*1, 2, 22*) is in accord with the more general hypothesis of programmed aging (*23*), and the culture of human fibroblasts provides the best model for experimentation.

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References and Notes

1. T. B. L. Kirkwood and R. Holliday, *J. Theor. Biol.* **53**, 481 (1975).
2. R. Holliday, L. I. Huschtscha, G. M. Tarrant, T. B. L. Kirkwood, *Science* **198**, 366 (1977).
3. For $M = 60$, there are 62 classes of cells (U, C_1, C_2, C_3, . . . C_{60}, D). The following expressions apply to the fraction of cells in each class for $g > 60$ when uncommitted cells are lost at $g = 43$.

$$U = 0$$

$$C_i = 2^{g-60}(0.725)^{g-i}(0.275)/N$$

$$D = \frac{0.275}{0.725N}\sum_{j=1}^{g-60}(1.45)^j$$

where

$$g = 61, 62, \ldots 103$$
$$i = g - 43, g - 42, \ldots 60$$

$$N = 2^{g-60}\sum_{i=g-43}^{60}(0.725)^{g-i}(0.275) + \frac{0.275}{0.725}\sum_{j=1}^{g-60}(1.45)^j$$

For all other i, $C_i = 0$.
4. P. Good, *Cell Tissue Kinet.* **5**, 319 (1972).
5. H. Kay, *Lancet* **1965-II**, 418 (1965); P. Good, *J. Theor. Biol.* **64**, 261 (1977); P. Absher and R. Absher, *ibid.* **72**, 627 (1978); C. B. Harley and S. Goldstein, *J. Cell. Physiol.* **97**, 509 (1978).
6. V. J. Cristofalo, *Gerontologia* **22**, 9 (1976).
7. R. A. Vincent, Jr., and P. C. Huang, *Exp. Cell Res.* **102**, 31 (1976); W. W. Nichols, D. G. Murphy, V. J. Cristofalo, L. H. Toji, A. E. Greene, S. A. Dwight, *Science* **196**, 60 (1977).
8. Since some dividing cells may not utilize exogenous thymidine for DNA synthesis (*6*) or may cycle more slowly than the labeling period of 30 to 40 hours, the fraction of unlabeled cells may actually overestimate the fraction of nondividing cells.
9. The fraction of uncommitted cells = $U/N = (1 - P)^g$ or $g = \log (U/N)/\log (1 - P)$. When $U/N < 10^{-6}$ and $P < .05$, $g > 269$. When $P = .275$ and $U = 1$, $g = 7 \log N$. When $N > 2 \times 10^8$, $g > 60$. See also figure 3 in (*1*).
10. L. Hayflick and P. S. Moorhead, *Exp. Cell Res.* **25**, 585 (1961); L. Hayflick, *ibid.* **37**, 614 (1965).
11. Data from these bottleneck experiments (*2*) were not analyzed statistically for goodness of fit to the predictions of the commitment theory.
12. The probability of finding at least one uncommitted cell in a culture of 2×10^6 cells is $1 - (1 - P^{60})^{2 \times 10^6} = .00831$. Thus in N' cultures of 2×10^6 cells, the probability that at least one culture contains an uncommitted cell is $1 - (1 - .00831)^{N'}$.
13. L. Hayflick, personal communication.
14. For references, see S. Goldstein, E. J. Moerman, J. S. Soeldner, R. E. Gleason, D. M. Barnett [*Science* **199**, 781 (1978)].
15. S. Goldstein, *N. Engl. J. Med.* **285**, 1120 (1971); J. W. Littlefield, *Variation Senescence and Neoplasia in Cultured Somatic Cells* (Harvard Univ. Press, Cambridge, Mass., 1976).
16. C. Zavala, G. Herner, P. J. Fialkow, *Exp. Cell Res.* **117**, 137 (1978).
17. J. R. Smith, O. M. Pereira-Smith, E. Schneider, *Proc. Natl. Acad. Sci. U.S.A.* **75**, 1353 (1978).
18. E. C. Hadley, D. Kress, V. J. Cristofalo, *J. Gerontol.* **34**, 170 (1979).
19. J. R. Smith and K. I. Braunschweiger, *J. Cell. Physiol.* **98**, 597 (1979).
20. T. B. L. Kirkwood, *Nature (London)* **270**, 301 (1977).
21. S. Goldstein and C. B. Harley, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 1862 (1979).
22. V. J. Cristofalo, *Adv. Gerontol. Res.* **4**, 45 (1972); G. M. Martin, C. A. Sprague, T. H. Norwood, W. R. Pendergrass, *Am. J. Pathol.* **74**, 137 (1974); E. Bell, L. F. Marek, D. S. Levinstone, C. Merrill, S. Sher, I. T. Young, M. Eden, *Science* **202**, 1158 (1978).
23. G. M. Martin, *Am. J. Pathol.* **77**, 484 (1977).
24. We thank L. Hayflick for allowing us to cite his unpublished observations. Supported by grant MT 3515 from the Medical Research Council of Canada during the tenure of a Centennial Scholarship from the National Research Council of Canada to C.B.H.
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Doridosine: A New Hypotensive *N*-Methylpurine Riboside from the Nudibranch *Anisodoris nobilis*

Abstract. *A new* N-*methylpurine riboside (doridosine), probably* N^1-*methylisoguanosine, was isolated from the digestive glands of a nudibranch. Doridosine produces prolonged hypotension and bradycardia in anesthetized rats, decreases the rate and the amplitude of contraction of guinea pig atria in vitro, and causes the heart rate in anesthetized mice to be reduced by 50 percent for many hours after which the animals recover completely.*

We recently reported that some common California dorid nudibranchs contain several pharmacologically active substances (*1*). We now report the isolation, chemical characterization, and pharmacological properties of one of these, a new *N*-methylpurine riboside named doridosine, isolated from aqueous extracts of the digestive glands of *Anisodoris nobilis*.

Specimens of *Anisodoris nobilis* were collected subtidally and intertidally in Monterey Bay, California. The progress of purification was assayed on spontaneously beating guinea pig atria (*1*). The activity was determined from the amplitude of contraction after 1 minute as a percentage of the control. The logarithmic dose-response relationship was linear over a 15-fold range.

Pooled digestive glands (128 g, wet weight) were homogenized and dialyzed (Spectrapor 1 tubing; molecular weight cutoff, 6000 to 8000) at 5°C against one volume of distilled water changed five to eight times. The combined dialyzates were concentrated and lyophilized, and the residue was extracted with methanol. The syrup resulting from vacuum evaporation of the methanol was chromatographed on a column (Bio-Gel P-2) by elution with distilled water. The lyophilized combined active fractions were extracted with methanol, and the concentrate was chromatographed on silica gel (Woelm, 0.03 to 0.06 mm, 2.2 by 22 cm) with a mixture of tertiary butyl alcohol, ethyl acetate, water, and acetic acid (40:10:2:1). The active fractions (400 through 700 ml) were concentrated, and the residue was crystallized from a mixture of tertiary butyl alcohol, methanol, and water (2:1:1) to give 31 mg (about 1 mg of doridosine per gram of dry digestive gland). These crystals were further crystallized from a mixture of methanol and tertiary butyl alcohol and twice washed with 0.25 and 0.10 ml of acetic acid

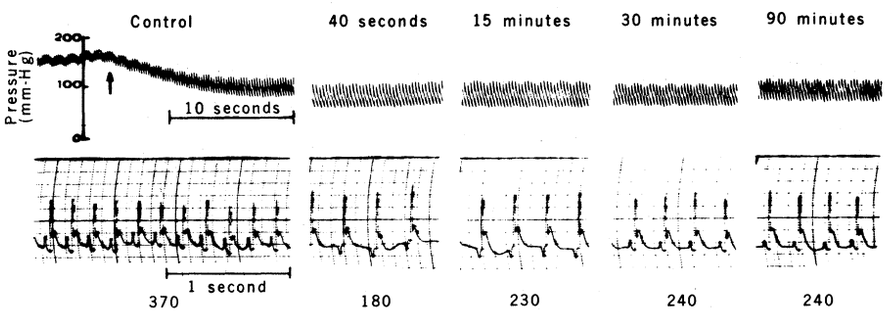

Fig. 1. Arterial pressure (upper tracing) and electrocardiogram (lower tracing) of a rat anesthetized with sodium pentobarbital. Arterial pressure was recorded from one carotid artery on a Grass polygraph by means of a Statham model P23 AC transducer. Electrocardiograms from lead 2 were recorded simultaneously on a separate polygraph at higher chart speed. Doridosine (2 mg/kg) was injected into the saphenous vein beginning at the arrow; the injection (0.52 ml) lasted 140 seconds. Figures below the electrocardiograms are heart rates per minute.

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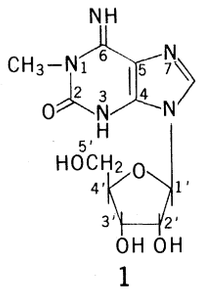
to give 28 mg [melting point, 250° to 255°C, the specific rotation relative to the sodium D line at 20°C, $[\alpha]^{20}_D = 66.2°$ (concentration = 0.42 g per 100 ml, methanol); values of the wavelength at the maximum molar absorptivity, $\lambda_{max}(\epsilon)$, at *p*H 6.5 are 292 nm (8500), 248 nm (6500); at *p*H 1.5, 282 nm (9000); and at *p*H 12, 286 nm (7500), 254 nm (6000)].

High-resolution mass spectrometry (*2*) establishes the molecular formula as $C_{11}H_{15}N_5O_5$ (mass-to-charge ratio *m/e* = 297.1075). The base peak, $C_6H_7N_5O$ (*m/e* 165.0651) corresponds to a methylguanine (−H) fragment or isomer thereof. The proton nuclear magnetic resonance (NMR) spectrum of doridosine is as follows: 100 MHz, δ, parts per million downfield from external tetramethylsilane (signal multiplicity, signal area), 3.50 (singlet, 3 H), 3.88 (doublet, 1 H), 4.29 (multiplet, 1 H), 4.35 (multiplet, 1 H), 4.51 (triplet, 1 H), 5.87 (doublet, 1 H), 8.00 (singlet, 1 H). Comparison of the low-resolution mass spectra, NMR, and ultraviolet spectra of doridosine with published data (*3*) establishes that doridosine is not N^1-, N^2-, N^3-, or N^7-methylguanosine. Direct comparison of the NMR spectra of doridosine with those of isoguanosine (*4*) and N^6-methylisoguanosine (*5*) reveals a remarkable correspondence except for the methyl signal. In these known compounds and doridosine the signals for the β-D-ribose moiety are essentially superposable. Two-dimensional, thin-layer chromatography demonstrated that doridosine is different from the known common methylpurines so far reported from the hydrolysis of transfer RNA (*6*). These properties, the stability at *p*H 12 (*7*) (eliminating the N^3-methyl possibility), and the unperturbed β-D-ribose NMR signals (making the N^3-methyl possibility unlikely) lead us to the tentative conclusion that doridosine is the previously unreported N^1-methylisoguanosine (**1**):



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Doridosine reduces the rate and amplitude of contraction of spontaneously beating guinea pig atria in vitro. A new stable level was reached 1 to 3 minutes after administration, and no recovery occurred during 10 minutes of recording. The amplitude of contraction was reduced 50 percent at a concentration of 3.75 mg/liter. This effect was completely reversed when the atria were washed with fresh medium.

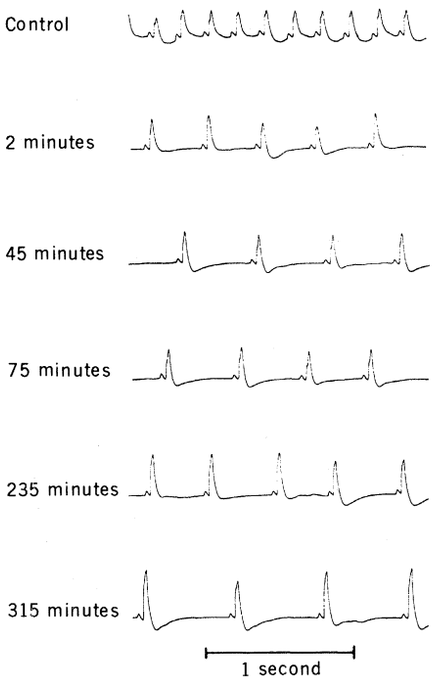

Fig. 2. Electrocardiogram of a mouse anesthetized with sodium pentobarbital recorded from lead 1 on a Grass polygraph. Doridosine (10 mg/kg) was injected intraperitoneally immediately after the control record was taken.

The negative inotropic effect of doridosine is dependent upon the Ca^{2+} concentration of the medium. At a doridosine concentration of 1.25 mg/liter, the amplitude (as a percentage of the control) in ten experiments was 58.4 ± 4.2 percent at 1.5 m*M* Ca^{2+}, 65 ± 0.7 percent at 3 m*M* Ca^{2+} (standard), and 78.9 ± 2.7 percent at 6 m*M* Ca^{2+}. At both high and low calcium concentrations the differences from the 3 m*M* concentration were significant ($P < .001$ and $< .01$, respectively).

Adenosine deaminase (calf intestinal mucosa enzyme, Sigma) did not inactivate doridosine in experiments that did inactivate adenosine after a few minutes. Doridosine still was not inactivated at a tenfold increase in enzyme concentration and after 12 hours of incubation.

Purified doridosine produced hypotension and bradycardia in rats (Fig. 1). The effects became apparent almost immediately. After only 1 minute, the heart rate fell from 370 to 150 beats per minute and the arterial pressure fell by about 50 percent. The arterial pressure and heart rate remained at very low levels for the full 2 hours and 45 minutes of the experiment. This prolonged action is in sharp contrast to the brief hypotensive action of adenosine and its analogs (*8*), histamine, and hypotensive peptides (*9*). The changes in the electrocardiograms are minor and indicate little or no interference with conduction of the impulse within the heart.

Administration of doridosine to mice established the duration of bradycardia as at least 5 hours, with complete recovery after 24 hours (Fig. 2).

The occurrence of unusual purine bases and ribosides in marine invertebrates is uncommon but not unprecedented. Most have been reported in sponges (*10*) and none in nudibranchs. The hypotension, bradycardia, coronary dilation, and relaxation of smooth muscle in mammals produced by adenosine and its analogs are well known (*11*), but the action lasts only a few minutes because the compounds are either taken up by tissues or are deaminated by adenosine deaminase (*8*). In contrast, doridosine acts for many hours. It is not deaminated by adenosine deaminase and is even effective after intraperitoneal injection in mice. The structure of doridosine is consistent with the conclusions of others (*8*, *12*) that maximum activity requires the presence of a sugar moiety at C-9 and an imino group at C-6. Evidently the N^1-methyl group of doridosine is responsible for its prolonged action, perhaps through a blocking of deamination by adenosine deaminase. Experiments with this long-acting analog of adenosine may help to explain the mechanism of hypotensive action of this group of substances.

Note added in proof: After this report was accepted for publication, an abstract of a German patent appeared (*13*) describing the isolation of a 1-methylisoguanosine from an Australian sponge (*Tedania*) and its synthesis.

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References and Notes

1. F. A. Fuhrman, G. J. Fuhrman, K. DeRiemer, *Biol. Bull.* **156**, 289 (1979).
2. These mass spectral studies were conducted by Dr. D. Thomas, SRI International, Menlo Park, Calif.
3. R. Hall, *The Modified Nucleosides in Nucleic Acids* (Columbia Univ. Press, New York, 1971).
4. The authentic sample was purchased from Vega Biochemicals, Tucson, Ariz.
5. This authentic sample was supplied by Prof. M. Ikehara, Department of Pharmaceutical Sciences, Osaka University [A. Yamazaki, I. Kumashino, T. Takenishi, M. Ikehara, *Chem. Pharm. Bull.* **16**, 2172 (1968)]. See also W.

Kampe, M. Thiel, K. Stock, W. Schauman, K. Dietman, South African Patent 6707.603 (25 April 1968); *Chem. Abstr.* **70**, 882122 (1973).
6. Dr. B. Vold, SRI International, Menlo Park, Calif., performed this study according to the procedure described in H. Rogg, R. Brambilla, G. Keith, M. Staehelin, *Nucleic Acid Res.* **3**, 285 (1976).
7. L. B. Townsend and R. K. Robbins, *J. Am. Chem. Soc.* **85**, 242 (1963).
8. L. B. Cobbin, R. Einstein, M. H. Maguire, *Br. J. Pharmacol.* **50**, 25 (1974).
9. E. G. Erdös, N. Back, F. Sicuteri, *Hypotensive Peptides* (Springer-Verlag, New York, 1966).
10. W. Bergmann and R. J. Feeney, *J. Org. Chem.* **16**, 981 (1951); W. Bergmann and D. C. Burke, *ibid.* **21**, 226 (1956); A. J. Weinheimer, C. W. J. Chang, J. A. Matson, P. N. Kaul, *Lloydia* **41**, 488 (1978); D. Ackermann and P. H. List, *Hoppe-Seyler's Z. Physiol. Chem.* **309**, 286 (1957); *ibid.* **323**, 192 (1961); H. Kanatani, H. Shirai, N. Nakanishi, T. Kurokawa, *Nature (London)* **221**, 273 (1969).
11. A. N. Drury and A. Szent-Györgyi, *J. Physiol.* **68**, 213 (1929); H. P. Baer and G. I. Drummond, Eds., *Physiological and Regulatory Functions of Adenosine and Adenine Nucleotides* (Raven, New York, 1979).
12. M. M. Wolf and R. M. Berne, *Circ. Res.* **4**, 343 (1956).
13. R. P. Gregson, R. J. Quinn, A. F. Cook, *Chem. Abstr.* **91**, 39792 (1979).
14. We thank Drs. David Thomas and Barbara Vold for analyses. Supported by grant NIH HL 22201. We acknowledge NSF instrument grants CHE-77-08810 and GP-28142 for the Varian XL-100 NMR with which these spectra were taken.

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Pentobarbital: Stereospecific Actions of (+) and (−) Isomers Revealed on Cultured Mammalian Neurons

Abstract. *Stereoisomers of the barbiturate anesthetic pentobarbital were applied to mouse spinal neurons growing in tissue culture. Intracellular recordings of neuronal membrane properties revealed that the (+) and (−) isomers caused direct changes in membrane potential and conductance on some but not all of the cells tested. The action of the (+) isomer was predominantly excitatory, whereas the (−) isomer produced predominantly inhibitory responses. The (−) isomer was considerably more effective in potentiating inhibitory responses to the transmitter gamma-aminobutyric acid. The results show that pentobarbital has multiple effects on neuronal excitability and demonstrate the presence of stereospecific sites of barbiturate action on central neurons.*

Racemic mixtures of barbiturates are used clinically as hypnotics and general anesthetics. However, subhypnotic doses are sometimes associated with stimulation of the central nervous system (CNS) (*1*) and preanesthetic excitation (*2*) as well as a hyperalgesic state (*3*). The (+) isomer of the anesthetic barbiturate pentobarbital (PB) causes a transient period of extreme hyperexcitability before depressing excitability in the CNS, whereas the (−) isomer produces a relatively smooth and progressively deeper hypnotic state (*4*). Similar differences in effects on excitability have been observed with other asymmetrically substituted barbiturates that can be resolved into stereoisomers (*2, 5*). At the cellular level racemic mixtures of clinically important barbiturates have multiple depressant actions on the excitability of vertebrate central and peripheral neurons in vivo (*6*) and in vitro (*7-9*). We have studied the cellular mechanisms underlying the stimulant and depressant effects of the barbiturate isomers by applying them to cultured mouse spinal neurons and report here evidence of cellular actions that correlate well with the clinical effects of the isomers. The results provide electrophysiological evidence for stereoselective requirements for the pharmacological effects of PB.

Neurons were dissociated from embryonic spinal cords and grown in tissue culture according to methods described (*10, 11*). Using conventional recording techniques we obtained intracellular microelectrode recordings from spinal cord cells on the modified stage of an inverted phase microscope. Prior to the experiment, growth medium was replaced with recording medium consisting of Hanks solution buffered to *p*H 7.4 with 25 m*M* Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid]. We added 10 m*M* $MgCl_2$ in many experiments to suppress synaptic activity and allow clearer examination of membrane events. The (+) and (−) isomers of PB were dissolved in the recording medium and then applied to individual neurons by pressure from pipettes containing 1 to 200 μM barbiturate that were positioned within 50 μm of the cell surface. The final concentration of drug bathing the neuron is unknown but is likely to be close to that present in the pipettes. Gamma-ammobutyric acid (GABA) (1*M*; *p*H 3.5) (Sigma, St. Louis), was applied by iontophoresis from pipettes positioned within 5 μm of the cell surface.

In 62 percent of the cells studied (18 of 29 neurons) brief application of (+)-PB depolarized the membrane potential in a dose-dependent, reversible manner. The depolarizing response was usually prolonged and outlasted the drug application period (Fig. 1A). The amplitude of the response increased as the membrane was hyperpolarized with the "null potential" for the response extrapolating to a potential more depolarized than the threshold for action potential generation. In ten cells (+)-PB also evoked excitatory synaptic activity, as evidenced by the appearance of synaptic potentials. These are shown superimposed on the depolarizing excitatory responses in Fig. 1, A_2. The presence of increased Mg^{2+} was clearly not sufficient to block the evoked synaptic activity. The evoked synaptic activity could result from either direct stimulation of transmitter release from innervating terminals or from indirect stimulation of transmitter release due to excitation of neurons presynaptic to the recorded cell. Sustained application of 100 to 200 μM (+)-PB produced complex membrane effects in four cells. The responses consisted of a transient depolarization followed by a hyperpolarization and increase in membrane conductance that persisted for the duration of the application (Fig. 1, B_3). After termination of the (+)-PB application, the membrane potential first depolarized and then repolarized to the control level. The drug did not produce any effect on eight cells whose membrane properties resembled those of cells responsive to (+)-PB. Since the cultures contain a heterogenous mixture of cells, it is likely that different types of neurons with apparently similar electrical properties may display different drug sensitivities.

The (−) isomer of PB depressed excitability in 11 of 30 cells studied, causing an increase in membrane potential and conductance (Fig. 1A). The null potential of the inhibitory response shifted in a depolarizing direction when KCl recording pipettes were used and was similar to that of inhibitory GABA responses. These results suggest that (−)-PB, like GABA (*11*) and racemic PB (*9*) may activate a Cl^- conductance, although further research is required to prove this. When 100 μM (+)-PB and (−)-PB were applied simultaneously, (−)-PB markedly attenuated excitatory responses to (+)-PB and blocked (+)-PB–evoked transmitter release (Fig. 1, A_2). Since 100 μM approximates the concentration of racemic PB necessary to induce general anesthesia (*12*), the depressant effects of the (−) isomer correlate well with the depression of the CNS seen clinically. Whether (−)-PB antagonizes (+)-PB excitation directly at excitatory sites or indirectly through its effective shunting of membrane resistance remains to be established. In three of the cells responding to (−)-PB a transient weak excitatory response was observed followed by

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