Benzodiazepine Inhibition of the Calcium-Calmodulin Protein Kinase System in Brain Membrane

Abstract. *Benzodiazepines inhibit Ca^{2+}-calmodulin–stimulated membrane protein phosphorylation. The effects of the benzodiazepines on protein phosphorylation are stereospecific and produced by membrane-bound benzodiazepine. The potency of benzodiazepine kinase inhibition is correlated with the ability of the benzodiazepines to inhibit electric shock–induced convulsions. These findings provide evidence that some of the anticonvulsant and neuronal stabilizing effects of benzodiazepines may be modulated by the Ca^{2+}-calmodulin protein kinase system and indicate that this calmodulin-kinase system represents an identifiable benzodiazepine receptor in brain that is distinguishable by several criteria from the previously described high-affinity benzodiazepine receptor.*

Benzodiazepines are widely prescribed therapeutic agents (*1*). The demonstration of benzodiazepine receptors in brain membrane and their possible role in neuronal function have been reviewed (*2, 3*). However, the molecular mechanisms underlying the effects of the benzodiazepines on neuronal function have not been elucidated.

It is important to determine if the benzodiazepines regulate important biochemical systems involved in neuronal activity. The Ca^{2+}-stimulated protein kinase system initially described by this laboratory in rat (*4*) and human (*5*) brain may play a role in mediating the effects of Ca^{2+} on synaptic function, neurotransmitter release, and membrane stability (*6–8*). Furthermore, activation of this Ca^{2+}-kinase in brain membrane is modulated by the Ca^{2+} receptor protein calmodulin (*7, 8*). In this report we demonstrate that the benzodiazepines modulate the activity of the membrane-bound Ca^{2+}-calmodulin kinase system.

Membrane fractions depleted of calmodulin were prepared from rat brain by established procedures (*7*) and incubated with γ-^{32}P-labeled adenosine triphosphate (ATP) under standard conditions for studying endogenous Ca^{2+}-calmodulin–stimulated protein phosphorylation (*8*). After incubation, each reaction was terminated by the addition of sodium dodecyl sulfate (SDS) and subjected to SDS–polyacrylamide gel electrophoresis. We then measured the incorporation of radioactivity into specific membrane peptides by autoradiography or liquid scintillation counting (*4, 5*). Figure 1A shows that Ca^{2+} plus calmodulin stimulated the endogenous phosphorylation of several membrane proteins, producing the characteristic Ca^{2+}-stimulated phosphorylation pattern in brain with major phosphoproteins of molecular weight ranges of 100,000 to 200,000; 80,000 to 90,000; 60,000 to 63,000 (protein DPH-L); 50,000 to 53,000 (protein DPH-M); and 10,000 to 15,000 (*9*). In the absence of calmodulin, Ca^{2+} had no significant effects on the endogenous phosphorylation of most membrane proteins, except for those in two bands with molecular weights of approximately 46,000 and 12,000, respectively, whose phosphorylation was slightly stimulated by Ca^{2+} (Fig. 1A).

We determined the effects of benzodiazepines on the Ca^{2+}-calmodulin kinase system by incubating diazepam (Valium) under standard conditions with membrane protein in the presence of Ca^{2+} and calmodulin. Diazepam caused a significant inhibition of the endogenous Ca^{2+}-calmodulin–stimulated phosphorylation of several major membrane protein bands, but had no effect on the incorporation of ^{32}P-labeled phosphate into four other specific membrane proteins. Since diazepam did not inhibit the phosphorylation of all membrane proteins (Fig. 1A), it is possible that different protein kinases are affected selectively by the drug. The concentration curve for diazepam reveals that this benzodiazepine began to significantly inhibit protein phosphorylation at approximately 0.2 μM and produced maximum inhibition of the kinase at approximately 200 μM (Fig. 1B). The plasma concentrations of diazepam that produce clinical effects in man and rats are in the 0.1 to 50 μM range (*10*), and brain concentrations in animals are significantly greater than corresponding plasma concentrations (*10*). Thus, concentrations of diazepam in brain that produce clinical effects are adequate to significantly affect the Ca^{2+}-calmodulin kinase system.

Under standard conditions, diazepam inhibited both the initial rate and net levels of endogenous membrane protein phosphorylation (Fig. 1C). These inhibitory effects were not overcome by increasing ATP concentrations from 5 to 300 μM and Ca^{2+} concentrations from 5 to 1000 μM. Thus diazepam did not indirectly affect phosphorylation by competing with ATP and Ca^{2+} or by altering the concentration or breakdown of these agents under standard conditions, but directly affected the enzymes controlling the net level of incorporation of ^{32}P-labeled phosphate into protein. The net level of phosphorylation of brain protein is regulated by the balance between protein kinase and protein phosphatase activity. By selectively inhibiting protein phosphatase activity (with Zn^{2+}) without inhibiting kinase activity (*11*), we found that diazepam still significantly inhibited membrane protein phosphorylation under conditions where no protein phosphatase activity was observed. Diazepam therefore affects the net level of membrane protein phosphorylation by inhibiting protein kinase activity. To demonstrate that the effects of diazepam on protein phosphorylation are a general property of the benzodiazepines, we calculated for several representative benzodiazepines the concentrations producing a half-maximum inhibition of protein phosphorylation (K_i); these concentrations (micromolar) reflect their relative inhibitory protencies: Ro5-5345, 5.3; Ro5-5807, 7.0; Ro5-2180, 7.5; diazepam, 8.1; B10(+), 18; clonazepam, 20; oxazepam, 23; nitrazepam, 45; chlordiazepoxide, 79; Ro5-4864, 82; flurazepam, 122; B10(−), > 1000.

Since trifluoperazine inhibits protein phosphorylation by inactivating calmodulin, it was important to determine if the benzodiazepines also inactivate calmodulin or instead interact with the enzyme system on the membrane (*12, 13*). We therefore incubated diazepam (5 μM) under standard conditions in the presence of Ca^{2+} over a wide range of calmodulin concentrations (0.1 to 75 μg per reaction mixture). In contrast to trifluoperazine, the diazepam inhibition of membrane protein phosphorylation was not significantly affected by increasing calmodulin concentrations in excess of diazepam, suggesting that the benzodiazepines do not bind to calmodulin but actually interact with the membrane-bound Ca^{2+}-kinase system.

To further demonstrate that the benzodiazepines interact with the membrane-bound kinase system, we incubated calmodulin-depleted membrane with or without various concentrations of benzodiazepines and then isolated the membrane by centrifugation (*14*). Control and benzodiazepine-treated membrane fractions were resuspended in plain buffer and incubated under standard conditions in the presence or absence of calcium or calmodulin (Table 1). The incorporation of ^{32}P-labeled phosphate into membrane protein was determined by standard pro-

0036-8075/81/0731-0546$00.50/0

cedures. Membrane-bound diazepam, chlordiazepoxide, and Ro5-4864 caused statistically significant inhibition of the endogenous Ca^{2+}- and calmodulin-stimulated membrane protein phosphorylation (Table 1). These results indicate that membrane-bound benzodiazepines can modulate the activity of the Ca^{2+}-calmodulin protein kinase system.

The pharmacological effects of the benzodiazepines are stereospecific (*2, 3*). We therefore determined whether the effect of these drugs on the Ca^{2+}-calmodulin kinase system is also stereospecific. Using the pharmacologically active and inactive benzodiazepine enantiomers B10(+) and B10(−) (*15*), respectively, we found that B10(+) produced statistically significant inhibition of Ca^{2+}-calmodulin–stimulated protein phosphorylation when added directly to the phosphorylation reaction mixture or when the drug was incubated with the membrane preparation. The B10(−) enantiomer, however, had no significant effect on membrane protein phosphorylation in either type of phosphorylation experiment (Fig. 1C and Table 1).

Our findings show that the benzodiazepines stereospecifically modulate the activity of the Ca^{2+}-calmodulin kinase system in brain membrane preparations, and that the membrane site mediating the effects of benzodiazepines on protein phosphorylation may represent an identifiable biochemical system regulated by a stereospecific benzodiazepine receptor. Thus, there may be a relation between kinase inhibition and some of the pharmacologic effects of the benzodiazepines and it would be of interest to compare the membrane site modulating kinase activity with the benzodiazepine receptor (*2, 3*).

The Ca^{2+}-calmodulin kinase has been implicated in the modulation of synaptic function, neuronal excitability, and seizure discharge (*4–8*). Phenytoin, a major anticonvulsant that antagonizes many effects of Ca^{2+} in brain, is also a potent inhibitor of the Ca^{2+}-calmodulin kinase system (*4, 5*). Diazepam and other benzodiazepines are also good anticonvulsants, and diazepam has striking structural similarities to phenytoin (*16*). Using established procedures for correlating membrane binding affinity with pharmacological potency (*17*), we compared the inhibition potencies of benzodiazepine kinases (represented by their K_i values) to their pharmacological potencies in several test systems to determine whether some of the anticonvulsant or other properties of these drugs are correlated with their effects on kinase activity. We found a highly significant correlation between the pharmacological potencies of benzodiazepines in inhibiting electric shock–induced convulsions (*17*) and their kinase inhibition potencies (r = .931, $P < .001$). Phenytoin also inhibits electric shock–induced convulsions, but is not very effective in inhibiting pentylenetetrazol-induced seizures, muscle tension, or anxiety (*18*). We found no significant correlation between benzodiazepine potency of kinase inhibition and benzodiazepine inhibition of muscle relaxant action, rotorod performance, electric shock–induced fighting, conditioned avoidance tests, or pentylenetetrazol-induced convulsions (*17*).

The concentrations of benzodiazepines required to occupy 50 percent of the benzodiazepine receptor are in the nanomolar range compared to the micromolar range for calmodulin-kinase inhibition, suggesting that the stereospecific effects on the kinase may be mediated by a distinct receptor site. This difference is also apparent if one compares the relative kinase inhibitory and membrane binding potencies of the benzodiazepines with their pharmacological effects. The affinities of the benzodiazepine receptor show no correlation with inhibition of electric shock–induced seizures (*17*). However, inhibition of electric shock–induced seizures show a high correlation with potency for kinase inhibition. Conversely, kinase inhibitory potency is not correlated with the pharmacological potencies of benzodiazepines in test systems where the results are significantly correlated with the relative affinities of these drugs for the membrane receptor (*17*) [for example, muscle relaxant action, inhibition of shock-induced fighting in mice, and antagonism of pentylenetetrazol convulsions (*17*)]. The various conditioned avoidance tests in rats and cats

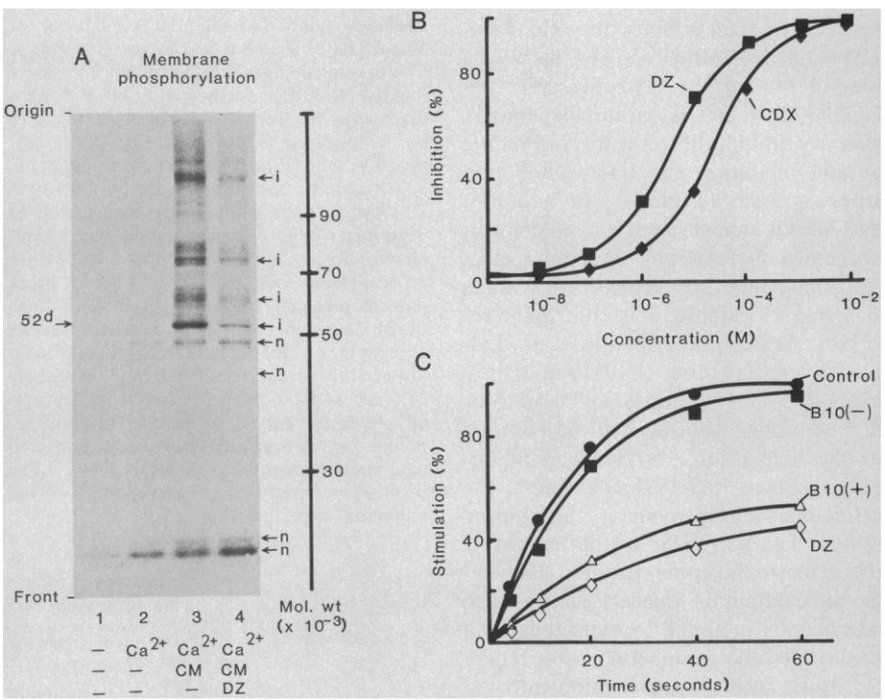

Fig. 1. (A) Autoradiograph showing the effects of diazepam on Ca^{2+}-calmodulin (*CM*) stimulated protein phosphorylation in brain membrane. The standard reaction mixture for studying endogenous protein phosphorylation (final volume, 100 μl) contained 100 μg of membrane protein, 50 m*M* Pipes buffer (*p*H 7.4), 5 m*M* $MgCl_2$, 0.2 m*M* EGTA (minus Ca^{2+}) or 0.2 m*M* EGTA plus 0.5 m*M* $CaCl_2$ (plus Ca^{2+}), 12 μ*M* [γ-^{32}P]ATP (5 to 10 Ci/mmole, New England Nuclear), with or without 5 μg of calmodulin, and 2 μl of dimethyl sulfoxide (DMSO) or 2 μl of diazepam dissolved in DMSO giving a final concentration in the reaction tube of 15 μ*M* diazepam. Reactions were incubated for 1 minute under standard conditions and prepared for autoradiography as described (*4*). The autoradiograph shown was representative of 20 independent experiments (*i*, major inhibited proteins; *n*, noninhibited membrane proteins). (B) Drug concentration curves of diazepam (*DZ*) and chlordiazepoxide (*CDX*) for inhibition of Ca^{2+}-calmodulin membrane protein phosphorylation. The inhibition of incorporation of ^{32}P-labeled phosphate into the 52,000-dalton (*52d*) protein is expressed as the percentage of the maximally inhibited condition which was designated 100 percent. The data give the means of ten determinations: the largest standard error was ± 5.5 percent. (C) Time course of the Ca^{2+}-calmodulin–stimulated phosphorylation of the 52,000-dalton protein in the absence (*Control*) or presence of diazepam (10 μ*M*), and the pharmacologically active and inactive benzodiazepine enantiomers (40 μ*M*), B10(−) and B10(+). The data present the means of ten determinations; the largest standard error was ± 4.2 percent.

(*17*) are not significantly correlated with either receptor affinity or kinase inhibition, raising the possibility that other benzodiazepine receptors may exist to mediate these actions.

The high-affinity benzodiazepine receptor may play a role in the modulation of the γ-aminobutyric acid (GABA) receptor in brain (*19*); the benzodiazepine binding of this receptor is affected by GABA agonists and antagonists (*20*). Two potent GABA antagonists, picrotoxin and bicuculline, that we tested on the Ca^{2+}-calmodulin kinase system had no significant effects on endogenous Ca^{2+}-stimulated membrane protein phosphorylation over a wide range of concentrations. The GABA agonist, muscimol, also had no significant effect on the Ca^{2+}-calmodulin kinase activity. Thus, the Ca^{2+}-kinase benzodiazepine receptor, in contrast to the high-affinity membrane receptor, is not affected by GABA agonists or antagonists. Another distinction between these receptors can be seen in their affinity for Ro5-4864. The benzodiazepine Ro5-4864 has a very low affinity for the benzodiazepine receptor (*2, 3*), but is essentially equally effective to chlordiazepoxide in binding to and inhibiting the Ca^{2+}-calmodulin kinase system (Table 1). In addition, Ro5-4864 is similar in potency to chlordiazepoxide in inhibiting maximum electric shock–induced convulsions in mice (*17*). A lower affinity benzodiazepine receptor has been described in brain, kidney, liver, and lung (*2, 3*) that tightly binds Ro5-4864, but does not significantly bind clonazepam, in marked contrast to the high-affinity benzodiazepine receptor. Since Ro5-4864 does affect the calmodulin-kinase system, this kinase system may be related to the peripheral tissue benzodiazepine receptor. However, the calmodulin-kinase system is also significantly inhibited by clonazepam, in contrast to the peripheral tissue receptor; thus, further investigation into the similarities between these receptors is necessary.

Our results indicate that the effects of the benzodiazepines on the Ca^{2+}-calmodulin kinase system may be mediated by a distinct benzodiazepine receptor in brain with different binding affinities, pharmacological effects, sensitivity to GABA agonists and antagonists, and potencies for Ro5-4864 from the previously described benzodiazepine receptor (*2*). The ability of benzodiazepines to inhibit both electric shock– and pentylenetetrazol-induced seizures may reflect their interaction with both the membrane Ca^{2+}-kinase system and the high-affinity membrane receptor, making the benzodiazepines potentially more diversified anticonvulsant drugs. Thus, regulation of the Ca^{2+}-calmodulin kinase system by membrane-bound benzodiazepines represents a specific biochemical system involved in modulating calcium's actions through calmodulin on nerve function that may mediate some of the anticonvulsant and neuronal stabilizing effects of the benzodiazepines. The Ca^{2+}-calmodulin kinase represents an identifiable biochemical membrane system that can be modulated by the benzodiazepines.

Table 1. Effects of benzodiazepines on endogenous Ca^{2+}-calmodulin–stimulated membrane protein phosphorylation. For these studies we used two different brain membrane preparations: unequilibrated brain membrane (*7*) or equilibrated brain membrane (*14*). The equilibrated membranes had been incubated in the presence or absence of 20 μ*M* diazepam, 90 μ*M* chlordiazepoxide, 90 μ*M* Ro5-4864, 50 μ*M* B10(+), and 50 μ*M* B10(−), before being isolated by centrifugation and resuspended in buffer for use in the membrane phosphorylation studies (*14*). To measure phosphorylation we incubated the membrane preparations for 1 minute under standard conditions (Fig. 1A) in the absence (control) or presence of Ca^{2+} and calmodulin. Unequilibrated membranes were incubated with or without 10 μ*M* diazepam, 70 μ*M* chlordiazepoxide, 70 μ*M* Ro5-4864, 50 μ*M* B10(+), or 50 μ*M* B10(−) added to the reaction tubes. The previously equilibrated membranes were incubated without further addition of benzodiazepines since these were already bound to the membranes. After incubation, the incorporation of ^{32}P-labeled phosphate into the 52,000-dalton membrane protein (protein DPH-M) in each sample was determined as described in Fig. 1. Results qualitatively similar to those shown with the 52,000-dalton protein were obtained with several other phosphoproteins. Data give the mean values of 20 determinations and are expressed as percentages of the maximally stimulated condition (100 percent): 100 percent represents 21.6 and 14.8 pmole of phosphate incorporated per milligram of membrane protein per minute for the unequilibrated membranes and equilibrated membranes, respectively. The results presented were representative of ten individual experiments.

Conditions	Endogenous membrane protein phosphorylation	
	Control (%)	Calcium plus calmodulin (%)
Unequilibrated brain membrane		
No drug	5.1	100
Diazepam	4.6	47.1*
Chlordiazepoxide	5.2	56.2*
Ro5-4864	5.1	58.6*
B10(+)	4.9	53.3*
B10(−)	5.0	99.2
Equilibrated brain membrane		
No drug	8.3	100
Diazepam	7.6	67.2*
Chlordiazepoxide	8.0	72.3*
Ro5-4864	8.1	74.2*
B10(+)	7.9	73.4*
B10(−)	8.2	99.7

*$P < .001$ compared to the no-drug condition for unequilibrated and equilibrated membranes, respectively.

ROBERT J. DELORENZO*
SUSAN BURDETTE
JOY HOLDERNESS
Department of Neurology,
Yale Medical School,
New Haven, Connecticut 06510

References and Notes

1. D. J. Greenblatt and R. I. Shader, *Benzodiazepines in Clinical Practice* (Raven, New York, 1974).
2. J. F. Tallman, S. M. Paul, P. Skolnick, D. W. Gallager, *Science* **207**, 274 (1980); R. Squires and C. Braestrup, *Nature (London)* **255**, 732 (1977).
3. H. Möhler and T. Okada, *Science* **198**, 849 (1977); D. W. Gallager, P. Mallorga, W. Oertel, R. Henneberry, J. Tallman, *J. Neurosci.* **1**, 218 (1981).
4. R. J. DeLorenzo, *Biochem. Biophys. Res. Commun.* **71**, 590 (1976); ——— and G. H. Glaser, *Brain Res.* **105**, 381 (1976); R. J. DeLorenzo, G. Emple, G. H. Glaser, *J. Neurochem.* **28**, 21 (1977); R. J. DeLorenzo and S. D. Freedman, *Epilepsia* **18**, 357 (1977).
5. R. J. DeLorenzo, *Brain Res.* **134**, 125 (1977).
6. ——— and S. D. Freedman, *Biochem. Biophys. Res. Commun.* **77**, 1036 (1977); *ibid.* **80**, 183 (1978).
7. H. Shulman and P. Greengard, *Nature (London)* **271**, 478 (1978); *Proc. Natl. Acad. Sci. U.S.A.* **75**, 5436 (1978).
8. R. J. DeLorenzo, S. D. Freedman, W. B. Yohe, S. C. Maurer, *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1838 (1979); R. J. DeLorenzo, in *Antiepileptic Drugs: Mechanisms of Action*, G. H. Glaser, J. K. Penry, D. M. Woodbury, Eds. (Raven, New York, 1980), pp. 399–414; R. J. DeLorenzo, *Ann. N.Y. Acad. Sci.* **356**, 92 (1980); B. Burke and R. J. DeLorenzo, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 991 (1981); R. J. DeLorenzo, *Fed. Proc. Fed. Am. Soc. Exp. Biol.*, in press.
9. This pattern of endogenous Ca^{2+}-stimulated protein phosphorylation was originally described in rat and human brain (*4–6*) and manifest prominent bands in the 50,000- to 60,000-dalton range (designated proteins DPH-M and DPH-L). Most of the endogenous substrates for this Ca^{2+}-kinase system are distinct (*8*) from the well-described endogenous phosphorylation patterns of brain proteins produced by the cyclic nucleotide kinase systems reviewed by P. Greengard [*Nature (London)* **260**, 101 (1976)].
10. Plasma concentrations of diazepam producing clinical effects in the rat and man were determined from the studies of A. J. Bond, D. M. Hailey, M. H. Lader, *Br. J. Clin. Pharmacol.* **4**, 51 (1977); Z. P. Horovitz, J. J. Piala, J. P. High, J. C. Burke, R. C. Leaf, *Int. J. Neuropharmacol.* **5**, 405 (1966); N. E. Booker and G. G. Celesia, *Arch. Neurol.* **29**, 191 (1973); A. J. Christmas and D. R. Maxwell, *Neuropharmacology* **9**, 17 (1970); D. J. Greenblatt, E. Woo, M. D. Allen, P. J. Orsulak, R. I. Shader, *J. Am. Med. Assoc.* **240**, 1872 (1978). Brain concentrations of diazepam and other benzodiazepines were significantly higher than corresponding blood concentrations in mouse, rat, and guinea pig [S. Garattini, E. Mussini, F. Marcucci, A. Guaitani, in *Benzodiazepines*, S. Garattini *et al.*, Eds. (Raven, New York, 1973), pp. 75–98].
11. R. J. DeLorenzo and P. Greengard, *Proc. Natl. Acad. Sci. U.S.A.* **70**, 1831 (1973); H. Maeno, T. Ueda, P. Greengard, *J. Cyclic Nucleotide Res.* **1**, 37 (1975).
12. W. Y. Cheung, *Science* **207**, 19 (1980).
13. B. Weiss and R. M. Levin, *Adv. Cyclic Nucleotide Res.* **9**, 285 (1978).
14. Membrane protein (Fig. 1) was suspended (0.5 μg/ml) in 50 m*M* Pipes buffer (*p*H 7.4) in the presence or absence of various concentrations of benzodiazepines (Table 1) in a total volume of 5 ml and kept at 4°C for 30 minutes. The tubes were then centrifuged at 4°C for 30 minutes at 150,000*g*. The resultant membrane pellets were immediately suspended in 5 ml of 50 m*M* Pipes buffer (*p*H 7.4) at 4°C and used as a source of protein for phosphorylation studies. Equal por-

tions of each membrane fraction were incubated under standard conditions for studying membrane protein phosphorylation, and the incorporation of ^{32}P-labeled phosphate into membrane protein was measured as described previously (*4–6*). Benzodiazepines were routinely dissolved in 100 percent dimethyl sulfoxide (DMSO) and added to the membrane suspension at a dilution of 1:500. Control mixtures received an equal amount of DMSO.

15. B10(+) and B10(−) represent the pharmacologically active (+) and inactive (−) benzodiazepine enantiomers corresponding to Ro11-6896 and Ro11-6893, respectively.
16. A. Camerman and N. Camerman, *Science* **168**, 1457 (1970).
17. Potencies of benzodiazepine kinase inhibition (represented by K_i values) were compared to the relative pharmacological potencies of these drugs in several test systems: inhibition of maximum electric shock–induced seizures in mice, cat muscle relaxant action, inhibition of electric shock–induced fighting of mice, antagonism of pentylenetetrazol-induced convulsions in mice, impairment of mouse rotorod performance, inhibition of rat and monkey conditioned avoidance [G. Zbinden and L. O. Randall, *Adv. Pharmacol.* **5**, 213 (1967); L. O. Randall, W. Schallek, L. H. Sternbach, R. Y. Ning, in *Psychopharmacological Agents*, M. Gordon, Ed. (Academic Press, New York, 1974), vol. 3, p. 175]. Correlation coefficients and statistical significance for each test system in comparison to kinase inhibition was determined for Ro5-5345, Ro5-5807, Ro5-2180, diazepam, clonazepam, oxazepam, nitrazepam, chlordiazepoxide, Ro5-4864, and flurazepam by standard procedures used to compare the affinity of the benzodiazepine receptor to pharmacological potency [H. Mohler and T. Okada (*3*); C. Braestrup and R. Squires, *Eur. J. Pharmacol.* **48**, 263 (1978)]. These references were also used to obtain statistically significant correlation of potency of membrane receptor affinity and pharmacologic potency. The median effective dose for Ro5-4864 in inhibiting maximum electric shock–induced seizures in mice is 36.1 mg/kg. These data were obtained from Dr. W. E. Scott and represents experimental work done at Hoffmann–La Roche, Inc.
18. D. M. Woodbury, in *Experimental Models of Epilepsy*, D. P. Purpura, J. K. Penry, D. Tower, D. M. Woodbury, R. Walton, Eds. (Raven, New York, 1972), p. 557.
19. L. L. Iversen, *Nature (London)* **275**, 477 (1978); J. F. Tallman, J. W. Thomas, D. W. Gallager, *ibid.* **274**, 383 (1978); T. Costa, D. Rodbard, C. B. Pert, *ibid.* **277**, 315 (1979).
20. This research [preliminary results of which were published in S. Burdette and R. J. DeLorenzo, *Neurology* **30**, 449 (1980)] was supported by PHS grant NS 1352 and RCDA NSI-EA-1-KO4-NS245 to R.J.D. We thank W. E. Scott from Hoffman–La Roche, Inc., for supplying the benzodiazepines, and J. McNamara, Y. Ehrlich, and G. H. Glaser for helpful discussions.
* Reprint requests and correspondence to R.J.D.

4 March 1981; revised 20 May 1981

Nullisomic *Tetrahymena:* Eliminating Germinal Chromosomes

Abstract. *Germinal and somatic functions in* Tetrahymena *are performed separately by the micro- and macronuclei, respectively. Cells with haploid micronuclei were mated with diploids to yield monosomic progeny. These were induced to undergo a form of self-fertilization, generating cells lacking both copies of one or more of the five chromosomes in the micronucleus while still possessing a complete macronuclear genome.*

Fertile strains of the ciliate *Tetrahymena thermophila* have two nuclei. The germinal micronucleus determines inheritance in the next sexual generation and the somatic macronucleus directs the phenotype of the cell. We recently recognized that the two nuclei can be manipulated independently; we can create heterokaryons with different micro- and macronuclear genotypes (*1*). The phenotype of these heterokaryons always reflects the genotype of the macronucleus. The micronucleus remains silent.

We have now exploited this micronuclear silence to produce strains with micronuclei missing both copies of one of the five chromosomes. These nullisomics are viable because they have a normal macronucleus. They are extremely useful because, when crossed with a diploid, they yield progeny with monosomic micro- and macronuclei, facilitating genetic analyses of that chromosome. Since *T. thermophila* has a haploid set of five chromosomes (*2*), it should require the establishment of only five unique strains to have nullisomics for each of the chromosomes. Figure 1, A and B, demonstrates that the chromosomes in a normal diploid can readily be visualized during meiosis; identification of abnormal karyotypes is feasible.

Variations in micronuclear chromosome content can occur if meiotic missegregation is induced. One method for generating such meiotic misbehavior became possible when Nanney and Preparata (*3*) isolated cells with normal macronuclei but haploid micronuclei. Of importance here is that these cells still attempt to mate. During conjugation the haploid micronucleus undergoes meiosis, but lack of pairing partners for each of the chromosomes results in the generation of meiotic products that contain variable numbers of chromosomes. Thus crossing a haploid with a diploid should yield monosomics.

To isolate successful progeny of such a cross, a haploid strain was constructed which contained in its micronucleus the dominant cycloheximide (cy) resistance mutation *Chx* (*4*). It was crossed to a strain that had a micronucleus homozygous for the *Mpr* allele, a dominant mutation which, when expressed, confers resistance to 6-methylpurine (6-mp) (*5*). Progeny were selected by growing exconjugants in both cy and 6-mp; single cells were cloned following the drug selection.

Many of these progeny clones should be aneuploid. Cytological examination of the micronuclei during meiosis was employed to detect the monosomic clones. However, as can be seen in Fig. 1A, only the haploid number of chromosomes is evident during meiotic prophase in a diploid cell, since homologous chromosomes pair so completely. Monosomics can only be clearly identified at anaphase I by resolving all the chromosomes at both poles. To simplify the cytogenetic identification of monosomics, the progeny of the haploid-diploid cross were crossed to strain A*, and exconjugants were cloned to create nullisomics. Crosses to A* undergo an abnormal form of conjugation called genomic exclusion (*6*). In these crosses the non-A* mate undergoes the normal events preceding fertilization: meiosis, elimination of three of the four products, mitotic doubling of the retained haploid nucleus, and transfer of one of these to its partner. In contrast, A* has a defective micronucleus that is lost at meiosis. After transfer, the only germinal genome in each of the two cells is contained in the haploid nucleus from the non-A* parent. This zygote nucleus undergoes an endoreduplication (*7*); each conjugant now has an identical, fully homozygous genome. Thus the loss of the A* micronucleus leads to uniparental micronuclear inheritance; the new genome is homozygous and comes only from the non-A* parent. The next step in normal conjugation, development of a macronucleus from the zygote nucleus, fails to occur. The end result of this round of mating (round 1) is the generation, from each pair, of two cells with identical homozygous micronuclei derived solely from the non-A* parent, but with parental macronuclei (*8*). Thus a change in the germinal genome has been effected, but the cells' phenotypes (drug sensitivity, mating type, and so on) are unaltered.

When monosomics are crossed to A* and pairs of exconjugant clones established, the micronuclei of each pair of round 1 exconjugants should be fully homozygous, identical, and nullisomic or diploid for each chromosome pair. The nullisomics should still be viable, because the macronuclei are still parental. When the round 1 exconjugants are allowed to remate (round 2), they now not only undergo normal biparental fertilization, they also develop new macronuclei (*6*). Thus, if the original isolate from the double drug selection were monosomic, one would expect half the isolated pairs of round 1 exconjugant clones to be diploid and yield viable round 2 progeny and the other half to be

0036-8075/81/0731-0549$00.50/0