tation results in a sudden increase in the amount of transmitter released by the synapses of the sensory neurons as a result of activity in another neuronal pathway (Fig. 3). We suggest that these pathways contact the presynaptic terminals of the sensory neurons and regulate their transmitter release. The existence of presynaptic facilitation has been suggested in several synaptic systems on the basis of indirect evidence (*5, 10*); our data provide direct evidence for its occurrence.

We have so far examined only the synaptic connections made between sensory neurons and motor cells. We have not yet examined the connections between sensory neurons and interneurons or between interneurons and motor cells. Thus we cannot exclude other mechanisms as contributing to the behavioral sensitization.

Our results indicate that, although habituation and sensitization are different, they act on a common locus: the presynaptic terminals of the sensory neurons (Fig. 3). Both of these short-term behavioral modifications act by modulating the release of transmitter for periods ranging from several minutes to an hour or more.

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8. The connections between sensory and motor neurons were studied by inserting a single microelectrode into a sensory neuron; the electrode was connected to a Wheatstone bridge circuit for intracellular stimulation and recording. A low-resistance, double-barreled electrode was placed into a motor cell, usually gill motor neuron L_7 (Fig. 1A). The signals from the motor cell were led to a cathode follower and then to a d-c and a high gain a-c amplifier (Tektronix No. 2A61). To minimize baseline noise, the band width of the a-c amplifier was set between 0.6 and 60 hertz. Data were tape-recorded and later displayed (Brush recorder No. 440).
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13. Since the evoked signal is small, failures might have not been recognized accurately by visual inspection. We therefore averaged all the failures encountered in five experiments with a PDP-8 computer. We also averaged the background activity 2 seconds before the occurrence of the triggered presynaptic spikes. In all cases, the averages of the failures and of the background activity were indistinguishable. We observed no time-locked depolarizations, which might have suggested an undisclosed evoked EPSP hidden in the baseline noise (*9*). In addition we examined the possibility that the failures occurred because of a conduction block in the terminals of the sensory cells. If this were so, the failures should have occurred in groups. To determine whether the failures tended to occur in groups, we used the Poisson equation e^{-m}, e^{-2m}, . . . and found that the number of successive failures did not exceed that predicted by the Poisson equation.
14. By assuming that the first peak in a histogram was q, we also examined the fit between the observed data in a histogram of EPSP amplitudes and the values predicted by a Poisson distribution. Knowing $\bar{E}$, we could derive m. In 31 of 38 regions, the predicted values were not statistically different from the observed values ($P > .05$). For a given region, three to seven quantal classes were used with a minimum of five predicted responses in each class. The predicted and observed distributions were compared in a chi-square table with 2 degrees of freedom fewer than the number of quantal classes used.
15. The value of q was obtained from the median value of the first peak of evoked responses after the peak of failures (Fig. 2A). To qualify as a peak we demanded (i) a minimum of five responses and (ii) that the two adjacent bins be lower by one or more units.
16. In three other experiments, we could not produce failures after 300 stimuli. We estimated q from the coefficient of variation (based on the assumption of a Poisson distribution). The equation used for the coefficient of variation technique was $m = \bar{E}^2/\sigma_E^2$, where σ_E^2 is the variance of the amplitudes of the EPSP's and $\bar{E}$ is their mean amplitude. Our estimates of q were lower (5 to 15 μv) than those (20 to 90 μv) obtained when transmission was lower and $m < 10$. During the peak of facilitation, average estimates of q decreased by as much as 50 percent while m (≥ 25) increased by more than five- to tenfold. Under conditions of high release ($m \geq 25$), the probability of release P may be high and the coefficient of variation estimates based on a Poisson distribution may underestimate the true value of q [for example, see X. Wernig, *J. Physiol. (London)* **244**, 207 (1975)]. This underestimate would be more pronounced after facilitation.
17. This work was supported by NIH research career development award 5K04-NS-70346-02 to V.C., NIH research scientist award MH-18-588 to E.R.K. and NIH grant MH-262102-01. We thank J. H. Schwartz for his comments and criticism.

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Synaptic Facilitation and Behavioral Sensitization in *Aplysia*: Possible Role of Serotonin and Cyclic AMP

Abstract. *The neural changes accompanying sensitization of the gill-withdrawal reflex in* Aplysia *are associated with presynaptic facilitation at monosynaptic connections between sensory neurons and motor cells. To analyze the molecular mechanisms underlying the facilitation, the pharmacological actions of serotonin, octopamine, and dopamine were examined. Only serotonin enhanced synaptic transmission between the sensory and the motor neurons. A serotonin antagonist, cinanserin, reversibly blocked the synaptic facilitation. The action of serotonin may be mediated by adenosine 3',5'-monophosphate (cyclic AMP). Exposing the ganglion to dibutyryl cyclic AMP or injecting cyclic AMP into the cell body enhances the synaptic action of a sensory neuron. The mechanism of presynaptic facilitation, therefore, may include activation of one or more serotonergic neurons, which enhance the release of a neurotransmitter by increasing the intracellular concentration of cyclic AMP in the terminals of the sensory neurons.*

Sensitization of the gill-withdrawal reflex in *Aplysia* involves a presynaptic facilitation of neurotransmitter output at the monosynaptic connections made by an identified group of 24 sensory neurons innervating the siphon skin and six identified gill motor cells (*1*). We have found that facilitation can be simulated by serotonin and that this action may be mediated by adenosine 3',5'-monophosphate (cyclic AMP).

Facilitation associated with short-term sensitization is not affected by prolonged inhibition of protein synthesis (*2*). This finding suggested to us that facilitation might result from a small molecule within the presynaptic terminal, perhaps an intracellular second messenger, regulating the mobilization of the transmitter packets from one storage compartment to another. This idea was supported by the finding that strong and prolonged electrical stimulation of the connectives from the head (or other pathways that produce the facilitation) leads to a prolonged, synaptically mediated increase in cyclic AMP in the abdominal ganglion (*3*). This action can be simulated by three biogenic amines: dopamine, octopamine, and serotonin (*4*). Exposure to one of these substances for 5 minutes increases cyclic AMP in the ganglion. The duration of this increase (up to 45 minutes) resembles that of presynaptic facilitation (and

of short-term sensitization) produced by strong facilitating stimuli. This parallel suggested that presynaptic facilitation might be due to octopamine, dopamine, or serotonin and that the amine might mediate its effect on transmitter release by increasing the concentration of cyclic AMP in the presynaptic terminals of the sensory neurons.

To test this possibility, we examined the actions of serotonin, dopamine, and octopamine on the monosynaptic connection between the sensory neurons and the major gill motor neurons (usually cell L7). We found that only serotonin enhanced synaptic transmission (Fig. 1A). A sensory neuron was first stimulated intracellularly 15 times, once every 10 seconds, to produce synaptic depression (5). During the next 2.5 minutes, the sensory neuron was not stimulated, and the ganglion was exposed to serotonin ($1 \times 10^{-4}M$ or $1 \times 10^{-6}M$). At the end of the 2.5-minute period of rest, a second series of 15 stimuli was given. Serotonin in concentrations of $10^{-6}M$ produced facilitation, and the higher concentration ($10^{-4}M$) produced greater facilitation. A similar result in another system in *Aplysia* has been described (6).

We next attempted to block the synaptic facilitation of the excitatory postsynaptic potential (EPSP) with cinanserin (Squibb), which blocks the action of serotonin in the *Aplysia* heart (7). Cinanserin did not affect the synaptic depression produced by repeated stimulation, but it reversibly blocked the facilitation produced by connective stimulation (Fig. 1B). Other serotonin antagonists (lysergic acid diethylamide, methysergide and curare) were not effective.

We next asked whether serotonin might act by increasing cyclic AMP. We exposed the ganglion to 2 mM N^6,$O^{2'}$-dibutyryl cyclic AMP, and found that it enhanced the evoked EPSP in the motor neurons; this action was specific. Two other excitatory synapses in the ganglion that do not undergo presynaptic facilitation [the one mediated by the right connective onto R15 (2) and the one made by L10 on R15 (5)] were not facilitated by dibutyryl cyclic AMP.

Because the site of action of cyclic AMP is primarily intracellular, we injected cyclic AMP into the cell body of the sensory neuron (Fig. 2, A and B) by an experimental procedure similar to the above (Fig. 1A).

Injection of cyclic AMP did not alter the amplitude or the duration of the spike in the cell body of the sensory neuron, but it enhanced the synaptic potential produced by each action potential. We compared these results with four types of controls. One group ($N = 5$) received 2.5 minutes of rest. A second group ($N = 6$) received a hyperpolarizing current pulse comparable to that used to inject cyclic AMP but from an electrode filled with $3M$ potassium citrate. Into a third group of cells ($N = 4$) we injected guanosine 3',5'-monophosphate (cyclic GMP, $1.5M$), which has been implicated in effects opposite to those of cyclic AMP in some hormonal systems (8). In the fourth control group ($N = 3$) we injected 5'-AMP, the breakdown product of cyclic AMP (Fig. 2B). After injection of cyclic AMP, the EPSP's in the experi-

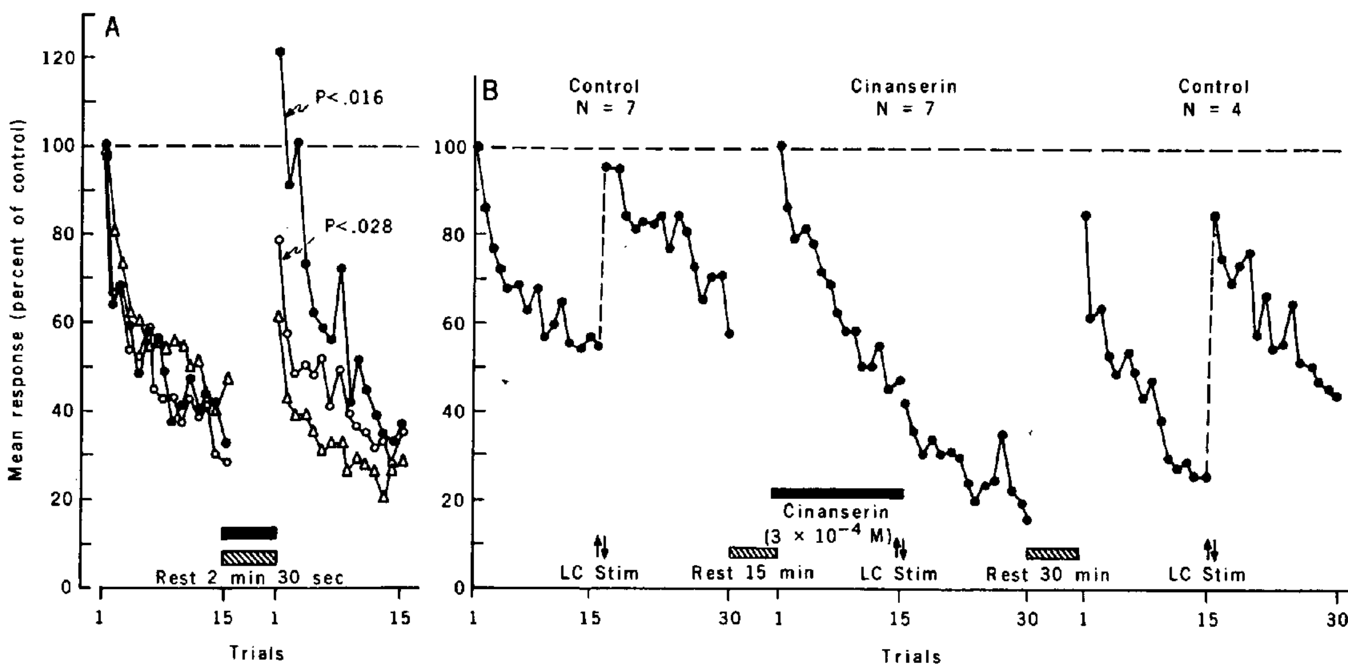

Fig. 1. (A) Effect of serotonin on monosynaptic EPSP. A sensory neuron was stimulated and produced a monosynaptic EPSP in a gill or siphon motor neuron; the interstimulus interval (ISI) was 10 seconds. In each experiment, the EPSP amplitudes were normalized to initial control EPSP ($N = 5$, in all cases). In the control group (open triangles), 15 stimuli produced synaptic depression. After a rest of 2½ minutes (hatched bar) (and a slight recovery of the EPSP), a second group of 15 stimuli was applied to the sensory neuron producing further depression. In the two experimental groups the 15 initial stimuli produced similar synaptic depression. During the 2½-minute rest period, serotonin [$2 \times 10^{-4}M$ (closed circles) or $2 \times 10^{-6}M$ (open circles)] was introduced into the perfusing solution. During the initial EPSP depression, the control and experimental curves overlapped completely. But after treatment with serotonin, both experimental curves were significantly higher than the control curve ($P < .016$ for $2 \times 10^{-4}M$ and $P < .028$ for $2 \times 10^{-6}M$; one-tailed Mann-Whitney U test). To determine the statistical differences between the experimental and the control curves, the amplitudes of the responses to each of the 15 stimuli of a run were summed to obtain a single score. (B) Time course of synaptic depression and facilitation in the presence and absence of cinanserin. Complex EPSP's were evoked by stimulating the siphon nerve (ISI, 10 seconds). Average EPSP's are plotted as percentages of initial EPSP amplitude. After 15 control stimuli had produced synaptic depression, a facilitating stimulus (6 hertz for 10 seconds) was applied to the left connective (*LC*) and 15 additional test stimuli were delivered; responses to these stimuli were facilitated compared to the preceding responses. After 13 minutes of rest, the ganglion was perfused with $3 \times 10^{-4}M$ cinanserin (dark bar); 2 minutes later, the experimental procedure was repeated. In the presence of cinanserin, synaptic depression was obtained as before, but stimulating the connective did not produce synaptic facilitation ($N = 7$). A final control run ($N = 4$), which was carried out 30 minutes after cinanserin was washed out of the chamber, produced facilitation.

mental group were significantly higher than those of either the first (rest) control group ($P < .002$) or the three other control groups (group 2, $P < .001$; group 3, $P < .014$; group 4, $P < .012$). Moreover, none of the last three controls produced significant facilitation when compared to the first (rest) control.

Thus, on the basis of these and earlier published data (*1*) we propose a specific and, it is to be hoped, testable model of the mechanisms underlying habituation and sensitization (Fig. 3). In the sensory neurons, the repeated activity that accompanies habituation reduces the release of transmitters, perhaps because of either (i) a progressive failure to mobilize transmitter from a storage compartment to one from which it can be released or (ii) a decrease in the probability of release.

Sensitization restores transmission as a result of the activation of one or more serotonergic neurons. These neurons enhance transmitter release by acting on the terminals of sensory neurons to increase their intracellular concentrations of cyclic AMP (*9*). The increase in cyclic AMP in turn enhances mobilization or the probability of release either (i) by increasing the free Ca^{2+} in the terminals or (ii) by acting on the Ca^{2+} conductance of the external membrane of the terminals or on subcellular organelles.

Our data provide direct evidence that serotonin and cyclic AMP can facilitate synaptic transmission between the sensory and motor neurons mediating the gill-withdrawal reflex (*10*). Cyclic AMP may, therefore, have a role in the short-term memory produced by behavioral sensitization. By extension, cyclic AMP may also have a role in the prolonged increase in responsiveness that accompanies behavioral arousal (*11*). However, the model (Fig. 3) is still speculative since the data we present for it are indirect and therefore not compelling. In particular, we have not yet identified the specific cell or cell group that mediates presynaptic facilitation and shown it to be serotonergic. Nor have we shown that the action of presumptive serotonergic cells actually increases endogenous cyclic AMP in the sensory neurons.

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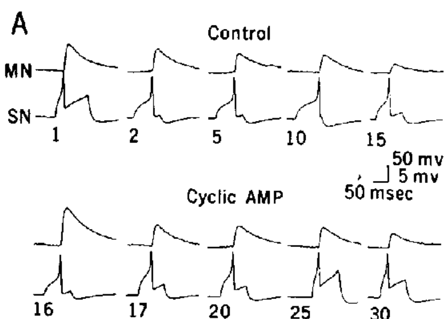

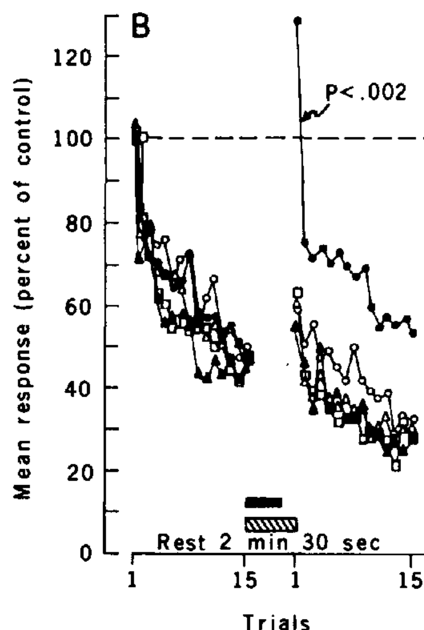

Fig. 2. Effect of cyclic AMP on monosynaptic EPSP. (A) The procedure was similar to that described for Fig. 1A. A sensory neuron was first stimulated once every 10 seconds for 15 stimuli (1 to 15). During the next 2½ minutes of rest, cyclic AMP was introduced by iontophoresis into the cell body of the sensory neuron. Hyperpolarizing current pulses (1 second long) of 3×10^{-7} to 4.5×10^{-7} ampere were applied every 2 seconds for 2 minutes from one barrel of a double electrode filled with 1.5*M* cyclic AMP; the second (recording) barrel was filled with 3*M* potassium citrate. Thirty seconds after the end of the injection a second series of 15 stimuli was given (16 to 30). The amplitudes of the evoked EPSP's of the second series are higher than the amplitudes of comparable EPSP's evoked in control experiments (below). (B) Summary of cyclic AMP effect and comparison with four control groups. The experimental group (closed circles) ($N = 8$) is compared with a first control group, in which the rest (2½ minutes) was simply followed by 15 additional stimuli without any current injection (open squares) ($N = 5$). In a second group, a hyperpolarizing current pulse, comparable to that used to inject cyclic AMP, was injected into the sensory neuron but the pulses were applied through an electrode filled with 3*M* potassium citrate (open triangles) ($N = 6$). In a third group, cyclic GMP (1.5*M*) was injected intracellularly (open circles) ($N = 4$). In a fourth group, 5′-AMP (closed triangles) ($N = 3$) was injected. The five curves generated during the initial EPSP depression produced by the first 15 stimuli overlap completely. But after injection of cyclic AMP the experimental group was significantly higher than either the first control group ($P < .002$, one-tailed Mann-Whitney U test) or the three other control groups (for potassium citrate, $P < .001$; for cyclic GMP, $P < .014$; for 5′-AMP, $P < .012$).

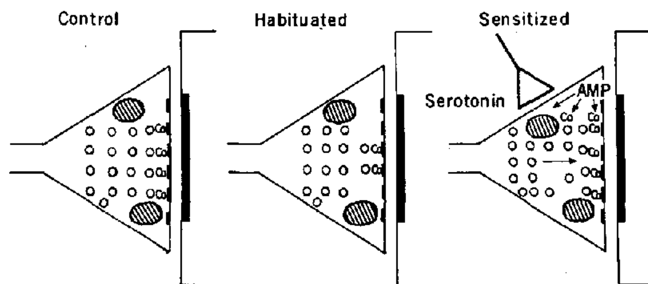

Fig. 3. A speculative model of habituation and sensitization. The presynaptic terminal of a sensory neuron is schematically illustrated under control, habituated, and sensitized conditions, with its complement of synaptic vesicles (small circles), mitochondria (shaded ovals), and active sites (thin black bars). The subsynaptic region of the postsynaptic neuron is also shown (thick black bar). The postulated presynaptic terminal that releases serotonin is marked with an asterisk.

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10. K. Weiss and I. Kupfermann (unpublished observations) have recently found that the sensitization of the biting response in *Aplysia* after

tization of the biting response in *Aplysia* after food is presented is produced by serotonergic neurons that act on the feeding muscle and is apparently also mediated by cyclic AMP (see also K. Weiss, J. Cohen, I. Kupfermann, *Brain Res.* 99, 381 (1975).

11. In *Aplysia*, as in some other invertebrates, serotonin sometimes produces actions that are mediated by norepinephrine in vertebrates, perhaps because these animals lack the enzyme dopamine-β-hydroxylase and cannot synthesize norepinephrine. An example is the innervation of the heart of *Aplysia*, where inhibition is mediated by acetylcholine (as it is in vertebrates), but where excitation is mediated by serotonin (rather than norepinephrine, as it is in vertebrates) (7). In both mollusks and vertebrates, the action of the biogenic amine may be mediated by cyclic AMP. In *Aplysia*, as in vertebrates, sensitization seems to be a component of behavioral arousal [P. M. Groves and R. F. Thompson, *Psychol. Rev.* 77, 419 (1970); T. J. Carew, V. F. Castellucci, E. R. Kandel, *Int. J. Neurosci.* 2, 79 (1971)]. Norepinephrine, which increases cyclic AMP in the cerebellum and facilitates its inputs, is thought to have a role in behavioral arousal [F. E. Bloom, *Rev. Physiol. Biochem. Pharmacol.* 74, 2 (1975); B. J. Hoffer, R. Freedman, D. Puro, D. J. Woodward, *Neurosci. Abstr.* 1, 204 (1975)]. It is possible that norepinephrine may have a role in the sensitizing component of arousal in vertebrates and that it mediates its action by cyclic AMP.
12. Supported by a fellowship from Centro Nazionale Ricerche Italiano and NATO to M.B.; NIH research career development award 5K04-NS-70346-03 to V.C.; NIH research scientist award MH 18-558 to E.R.K.; and NIH grant MH-262102-01. We thank J. H. Schwartz for his comments and criticism.

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Suppression by 1,3-Butanediol of the Ethanol Withdrawal Syndrome in Rats

Abstract. *1,3-Butanediol was tested for its ability to suppress an ethanol withdrawal syndrome. Male Sprague-Dawley rats were rendered physically dependent on ethanol by intragastric administration of ethanol at a dosage of 9 to 15 grams per kilogram per day over a 4-day period. A nonintoxicating oral dose of 1,3-butanediol at 4 grams per kilogram administered after elimination of ethanol from the blood was effective against the tremulous and convulsive components of the ethanol withdrawal syndrome in all animals for 1 to 5 hours. This period coincided with the time of maximum severity of the withdrawal syndrome, as seen in the control animals.*

The clinical management of ethanol withdrawal has to take into account both acute and chronic aspects of ethanol intake. During the early stages of the withdrawal period, a subject displays signs of intoxication similar to those observed during the first drinking episode. As detoxification proceeds and the blood ethanol concentrations decline to approximately 100 mg/dl, a gradual transition develops from depression to hyperexcitability. With the total elimination of blood ethanol, a series of neurological signs and reactions emerge which include general agitation, tremors, convulsions, hallucinations, and delirium tremens (*1*). In addition, a number of side effects resulting from long-term intoxication with heavy doses of ethanol are evident. These may include malnutrition, weight loss, a lessened resistance to infection, hepatitis, cirrhosis of the liver, various vitamin deficiencies, peripheral neuropathy, and general neurological, psychiatric, and clinical deterioration (*1*). Thus, an ethanol withdrawal syndrome can be a serious medical problem and may be fatal if not properly managed. Therefore, the primary aim of treatment is to reduce the neuromuscular and autonomic hyperactivity, thereby preventing exhaustion so that necessary clinical management and treatment can proceed (*1*).

Empirically, any drug that will suppress nervous excitability may ameliorate the severity of the withdrawal reaction. To date, a variety of compounds that are either structurally or pharmacologically similar to ethanol have been effective in treatment (*2*). Among these are aliphatic alcohols and their corresponding aldehydes, paraldehyde, chloral hydrate, barbiturates, phenothiazines, and benzodiazepines (*2*). However, the optimal drug is not only efficacious in controlling the withdrawal syndrome, but is devoid of major side effects. Of the drugs just listed, some are either more toxic than ethanol or have severe side effects, including their ability to induce dependence (*2*).

The availability of a number of animal models of ethanol dependence (*3*) now allows the testing of a variety of potential therapeutic agents for their ability to suppress the signs and responses of the ethanol withdrawal syndrome. Using our model of ethanol dependence in the rat (*4*), we screened a number of compounds listed above and found some effective. In addition, 1,3-butanediol (BD), a compound of low toxicity (*5–7*) suppresses a variety of signs and responses in rats characteristic of the ethanol withdrawal syndrome.

Male Sprague-Dawley rats (200 to 300 g) were rendered ethanol dependent by intubation of a 20 percent solution of ethanol at a dose of 9 to 15 g/kg daily in up to six fractions over a 4-day period (*4*). Since we used the maximum tolerable doses for the induction of ethanol dependence, about one-fifth of the animals usually died as a result of overdosage. On the day of withdrawal, the animals were observed at hourly intervals, initially for the disappearance of ethanol intoxication during the prodromal detoxication phase and then for the onset of a withdrawal syndrome during the ethanol dependence phase. With the onset of signs of the ethanol withdrawal syndrome, blood samples were taken also at hourly intervals from the tail vein; this continued until the complete clearance of ethanol from the blood. Blood ethanol levels were determined by means of an automated adaptation (*8*) of a gas chromatographic method of Roach and Creaven (*9*). At this point, the animals were

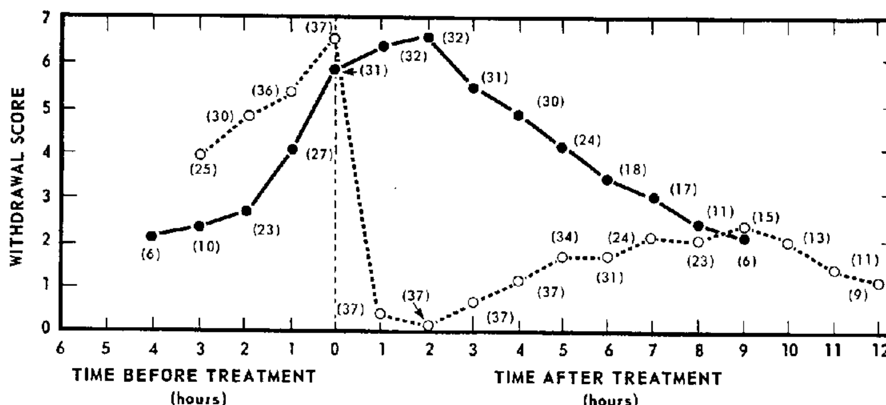

Fig. 1. Effect of 1,3-butanediol (BD) (4 g/kg) on the withdrawal score (as defined in the text). (——) Untreated ethanol-dependent rats; (-----) BD-treated, ethanol-dependent rats. Time zero corresponds to the point at which all ethanol has been eliminated from the blood, and for BD-treated animals denotes the point at which BD was administered. Numbers in parentheses denote the number of animals observed, while each point refers to the mean of the withdrawal scores. Statistical differences were found for the period 1 to 5 hours after BD treatment using the median test ($P < .05$).