12. W. Burgdorfer, in *CRC Handbook Series in Zoonoses*, J. H. Steele, Ed. (CRC Press, Boca Raton, Fla., 1980), pp. 279–300.
13. R. F. Harwood and N. T. James, *Entomology in Human and Animal Health* (Macmillan, New York, ed. 7, 1979).
14. R. Traub and C. L. Wisseman, Jr., *J. Med. Entomol.* **11**, 237 (1974).
15. A. Spielman, *Ann. N.Y. Acad. Sci.* **266**, 115 (1975).
16. L. Rosen, D. A. Shroyer, R. B. Tesh, J. E. Freier, J. C. Lien, *Am. J. Trop. Med. Hyg.* **32**, 1108 (1983).
17. H. E. Krampitz and V. Wongchari, in *Fleas*, R. Traub and H. Starcke, Eds. (Balkema, Rotterdam, 1980), pp. 349–358.
18. The bacterial agent of listeriosis has been reported as penetrating into the body cavity of *Nosopsyllus consimilis* fleas after developing in the midgut epithelium (*19*).
19. V. S. Vaschenok and P. A. Chirov, *Parazitologia* **10**, 61 (1976).
20. D. H. Molyneux, *Ann. Trop. Med. Parasitol.* **63**, 403 (1969).
21. Supported by NIH grants AI-17828 and AI-04242.

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Hypothalamic Reward Mechanism: Two First-Stage Fiber Populations with a Cholinergic Component

Abstract. *Refractory periods were estimated for fibers of the hypothalamic substrate of brain stimulation reward. Two nonoverlapping populations were evident: a homogeneous fast population and a more heterogeneous slow population. Cholinergic blockade eliminated the contribution of the fast but not the slow fibers, while dopaminergic blockade reduced responding without significantly influencing either directly activated fiber population. These data indicate that the hypothalamic reward substrate is more complex than has been widely appreciated; it contains two or more parallel subsystems, and one of these subsystems has a cholinergic link.*

Electrical stimulation of the medial forebrain bundle (MFB) can have rewarding effects stronger than those caused by natural biological rewards such as food, even under conditions of life-threatening deprivation. Since only a few of the approximately 50 fiber systems passing through this region are likely to play a role in the rewarding effects of stimulation, knowledge of which components of the MFB play causal roles in brain stimulation reward has been elusive (*1, 2*). Pharmacological studies suggest that dopaminergic fibers are involved (*3*). However, a variety of considerations make it clear that dopaminergic fibers are not usually directly depolarized by the rewarding currents used in brain stimulation reward experiments (*2*); rather, it seems likely that directly activated fibers, constituting a "first-stage" system, synapse on dopaminergic cells, which then serve as the second-stage system (*4*). Paired-pulse experiments suggest that the poststimulation excitability cycles and conduction velocities of the directly activated MFB fibers are faster than most monoaminergic fibers (*5*). Furthermore, double electrode experiments, in which propagation of the action potential from a depolarizing pulse at one end of the MFB is impeded by hyperpolarizing the other end of the bundle, indicate that most first-stage fibers carry the reward-relevant signal in a rostral-to-caudal direction—opposite to the direction of conduction of the dopaminergic fibers (*6*).

We now report that the first-stage reward fibers include at least two populations, one of which is cholinergic or is synaptically linked to cholinergic efferents. One first-stage population has short refractory periods that do not overlap the slower refractory periods of the remaining reward-relevant fibers; the contribution of the fast fibers is blocked by the muscarinic receptor antagonist atropine sulfate. The contribution of cholinergic fibers to MFB brain stimulation reward has not been seen in previous studies of this type, partly because refractory periods have been analyzed over a coarser sampling of test parameters and partly because pharmacological studies have usually been designed on the assumption that a single transmitter system carried the reward signal to the second stage neurons. Our data suggest that perhaps 25 percent of the reward-relevant signal is carried by cholinergic

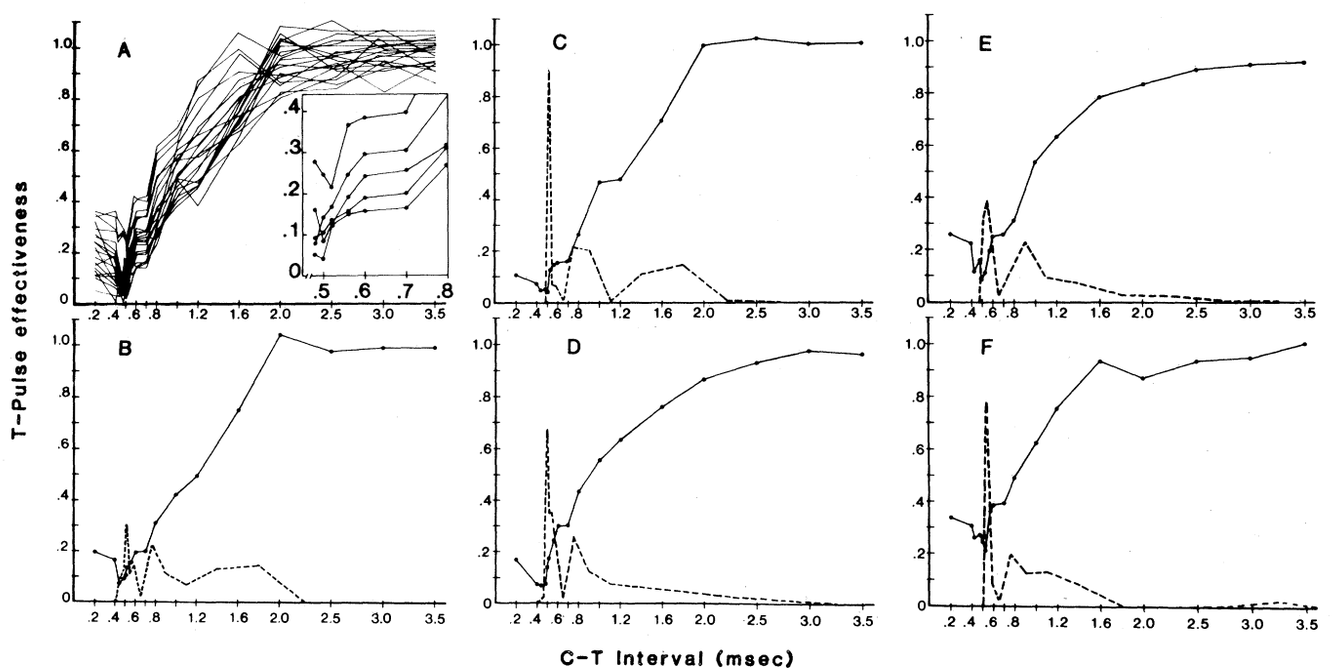

Fig. 1. Relation of T-pulse effectiveness to C-T interval. (A) Data for each of four replications in each of five rats. Note the horizontal step in each curve between 0.6 and 0.7 msec; the inset shows an expanded version of the horizontal step based on mean data for each animal. (B to F) Mean data for each animal (solid lines) along with relative slopes (dotted lines). These slopes (first derivatives) reflect the relative numbers of fibers that have refractory periods within each range of C-T intervals. The slopes fall to zero between 0.6 and 0.7 msec in each case.

fibers and that the entire range of refractory periods for these fibers or the portion of first-stage neurons that project to them is between 0.4 and 0.6 msec. Whether one or a mixture of more than one first-stage population carries the remaining portion of the signal is still an open question.

Refractory periods of the first-stage MFB neurons were inferred behaviorally using a paired-pulse technique. Each animal's responsiveness to 500-msec trains of paired pulses was compared with its responsiveness to trains of single pulses (*7*). The criterion of responsiveness was the reward threshold, defined as the lowest number of single or paired pulses (per train) that would maintain lever-pressing at 10 percent of the animal's maximal rate. By varying the delay (C-T interval) between the two equal-amplitude constituent pulses (C and T pulses) in the paired-pulse condition, a function indicating the range and relative frequency of refractory periods for the first-stage fibers was obtained. When T-pulses were as effective as C-pulses in adding to the rewarding impact of a stimulation train, it was assumed that the C-T interval was long enough to exceed the refractory period of all the first-stage neurons. The T-pulse effectiveness (TPE) was computed from Yeomans' (*5*) equation: TPE = (number of required single pulses/number of required paired pulses) − 1.

When the C-T interval was very short (<0.4 msec), the animal's responsiveness to paired pulses was somewhat better than that seen when the second pulse in each pair was omitted (Fig. 1A); this reflects a brief period of "local potential summation" when fibers at the fringe of the effective field of stimulation, only partially depolarized by the first pulse, are brought to firing threshold by the second pulse (*2*). When the C-T interval was somewhat longer (0.4 to 0.5 msec), responsiveness to paired pulses was equivalent to responsiveness to single pulses. In this case it was assumed that partial depolarization from the first pulse had time to dissipate and that all the axons fully depolarized by the first pulse were still refractory at the time of the second pulse. As the C-T interval was lengthened, the number of pulse pairs required for a constant behavioral output began to decrease, presumably because the neurons having the shortest refractory periods began responding to the second (T) pulses, thus contributing more to the net rewarding impact of a given pulse train. At longer and longer C-T intervals fewer and fewer pulse pairs per train were needed, presumably because more and more fibers were fired by both pulses in each pulse pair. When C-T intervals of 2.0 to 3.0 msec were reached, no further change was seen, indicating that all the reward-relevant axons depolarized by the first pulse had completely recovered from refractoriness in time to be fired by the second. Previous studies have shown that none of the reward-relevant fibers of the MFB recover from C-pulse stimulation until after 0.4 msec and that most fibers recover within 2.0 msec (*5*); our findings are generally consistent with these reports.

Unlike those of most previous studies, however, our refractory period curves showed a clear, if short, step function. The slope of the refractory period curve over a given range of C-T intervals is proportional to the number of fibers that

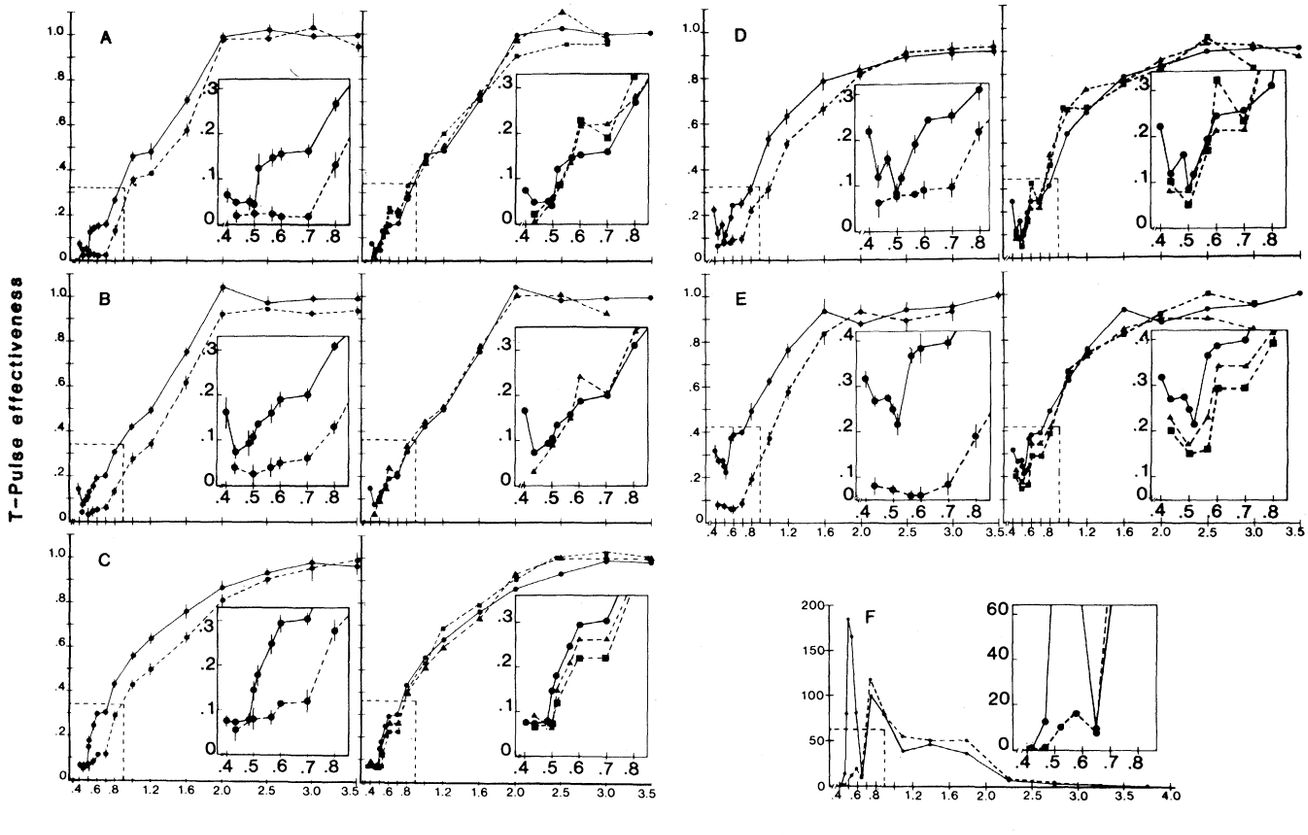

Fig. 2. Refractory period curves under drug conditions. Frames A to E represent the individual animals corresponding to frames B to F in Fig. 1. Within each frame, the panel on the left shows atropine sulfate data (dashed lines) with baseline data (solid lines). Panels on the right show baseline data (circles), atropine methyl nitrate data (triangles), and pimozide data (squares). The insets are expanded versions for C-T intervals between 0.4 and 0.8 msec, where the important comparisons are seen. (The field that is expanded is shown in dotted lines.) Frame F represents the slopes of the mean data for the five rats in baseline (solid line) and atropine sulfate (dashed line) conditions.

begin to respond to T-pulses within that range. Our curves invariably show a slope near zero at C-T intervals between 0.6 and 0.7 msec (Fig. 1). This result indicates that none of the fibers that have not already begun to fire in response to T-pulses begin to do so at C-T intervals between these values. If the reward fibers were homogeneous and if their refractory periods were normally distributed across the range of 0.4 to 2.0 msec, no such consistent steps should be seen across animals or across days of testing; the step at 0.6 to 0.7 msec, however, was consistent across both animals and days, and was seen in animals having MFB electrode placements that differed in placement by as much as 2.0 mm in the anterior-posterior plane. While this step was not noted in most previous studies, earlier studies have usually not examined multiple data points in the relevant range; in the one previous study that tested multiple points in this range, data consistent with ours were seen (*8*). Thus the lack of behavioral recovery when C-T intervals are increased from 0.6 to 0.7 msec seems to be reliable. This suggests at least two populations of reward-relevant first-stage fibers: a fast population that has completely recovered from C-pulse stimulation within 0.6 msec and a slow population (or mixture of populations) having no elements that recover from C-pulses in less than 0.7 msec.

One way to confirm such a hypothesis would be to show that the contributions of the fast and slow fibers could be differentially altered pharmacologically (*9*). Yeomans suggested that descending cholinergic fibers may represent the first-stage neurons in MFB self-stimulation (*10*). We thus examined the effects of a cholinergic blocker, atropine sulfate, on the refractory period functions. Dopaminergic fibers are also thought to be involved in brain stimulation reward, but they are thought to be efferent to the directly activated fibers. Since the refractory period distribution would shift only if there were differential interference with populations of first-stage neurons, a dopamine blocker would be expected to alter overall performance but not refractory period characteristics; thus we tested the effects of a dopamine blocker, pimozide, for purposes of comparison (*11*).

Cholinergic receptor blockade eliminated the contribution of the subset of fast fibers; no noticeable increase in T-pulse effectiveness could be detected at C-T intervals under 0.8 msec in four of the five animals (Fig. 2, A, B, D, and E), and only some suggestion of recovery was seen in the third (Fig. 2C). Since atropine would not have altered the cholinergic fibers' responsiveness to direct stimulation (the axons would still be depolarized by the same stimulating pulses or synaptic input), we must assume that the loss of T-pulse effectiveness at C-T intervals shorter than 0.7 msec reflects loss of the postsynaptic contribution of the fast first-stage fibers or their efferents. Neither atropine methyl nitrate (which does not cross the blood-brain barrier) nor pimozide altered the relative contributions of the fast and slow fiber populations. Although pimozide did increase the frequency of stimulation required for criterion-level responding under both single- and double-pulse conditions, neither pimozide nor atropine methyl nitrate altered the slope or initial points of recovery in the refractory period functions (Fig. 2). These facts fit with the view that dopamine plays a role at some stage synaptically removed from the directly activated fibers, and they rule out any significant contribution of peripheral atropine actions.

The data support two relatively new conclusions about the mechanisms of MFB brain stimulation reward. First, the reward substrate of the MFB comprises more than one population of axons, with more than one neurotransmitter (*12*). The fast population contains a cholinergic link having a muscarinic synapse. The cholinergic link may be at the first stage (although it could be later); the muscarinic synapse may be located in the ventral tegmental area (*10*). It is not yet clear how many other transmitter systems are activated by MFB stimulation and which are involved causally in the rewarding impact of the stimulation. Second, cholinergic fibers make a positive contribution to MFB reward; their making only a partial contribution may account in part for the fact that their contribution has not been previously identified. Earlier work suggested only an antagonistic role for muscarinic systems in reward (*13*). Although there may also be muscarinic actions that antagonize such function, our data indicate an important positive contribution of cholinergic fibers to MFB reward.

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References and Notes

1. D. C. German and D. M. Bowden, *Brain Res.* **73**, 381 (1974); R. A. Wise, *ibid.* **152**, 215 (1978); H. C. Fibiger, *Annu. Rev. Pharmacol. Toxicol.* **18**, 37 (1978).
2. C. R. Gallistel, P. Shizgal, J. S. Yeomans, *Psychol. Rev.* **88**, 228 (1981).
3. G. Fouriezos and R. A. Wise, *Brain Res.* **103**, 377 (1976); K. B. J. Franklin and S. N. McCoy, *Pharmacol. Biochem. Behav.* **11**, 71 (1979); R. U. Esposito, W. Faulkner, C. Kornetsky, *ibid.* **10**, 937 (1979); C. R. Gallistel, M. Boytim, Y. Gomita, L. Klebanoff, *ibid.* **17**, 769 (1982); J. R. Stellar, A. E. Kelley, D. Corbett, *ibid.* **18**, 433 (1983); P. Zarevics and P. E. Setler, *Brain Res.* **169**, 499 (1979).
4. R. A. Wise, *Pharmacol. Biochem. Behav.* **13** (Suppl. 1), 213 (1980); P. Shizgal *et al.*, *J. Comp. Physiol. Psychol.* **94**, 227 (1980); J. S. Yeomans, in *Neural Basis of Feeding and Reward*, B. G. Hoebel and D. Novin, Eds. (Haer Institute, Brunswick, Maine, 1982), p. 405.
5. J. S. Yeomans, *Physiol. Behav.* **22**, 911 (1979); C. Bielajew and P. Shizgal, *Brain Res.* **237**, 107 (1982).
6. P. Shizgal, I. Kiss, C. Bielajew, in *Neural Basis of Feeding and Reward*, B. G. Hoebel and D. Novin, Eds. (Haer Institute, Brunswick, Maine, 1982), p. 419.
7. Electrodes were implanted in the lateral hypothalamus of four animals and the ventral tegmental area of one animal. These two sites, separated by approximately 3 mm in the anterior-posterior plane, are known from work of Shizgal *et al.* (*4*) to lie at two sites traversed by the same first-stage axons. The animals were trained to press a lever on a continuous reinforcement schedule for 0.5-second trains of single 0.1-msec pulses. Intensity was adjusted to produce frequency thresholds in the range of 40 to 50 Hz. Testing sessions consisted of six blocks of three paired-pulse and seven single-pulse threshold determinations. Within each block, one short, one medium, and one long C-T interval were tested. A single-pulse threshold determination preceded and followed each paired-pulse determination; the averaged frequency thresholds for the two single-pulse conditions was used to estimate the T-pulse effectiveness under the intervening paired-pulse condition.
8. To our knowledge only P. P. Rompre and E. Miliaressis (in preparation) have tested multiple points in this range of C-T intervals; they found a flattening of the refractory period curve at approximately the same C-T intervals. On the basis of their data and their analysis using the first derivative of the refractory period curves [P. P. Rompre, thesis, University of Ottawa (1983)] these authors have also hypothesized the existence of two distinct populations of mesencephalic fibers with relevance for reward.
9. E. E. Coons, N. Schupf, L. G. Ungerleider, *J. Comp. Physiol. Psychol.* **90**, 317 (1976); P. Milner, in *Brain Stimulation Reward*, A. Wauquier and E. T. Rolls, Eds. (North-Holland, Amsterdam, 1976), p. 543.
10. J. S. Yeomans, O. Kofman, and V. McFarlane [*Brain Res.*, in press] showed that infusions of the muscarinic agonist, carbachol, into the ventral tegmental area (VTA) has reinforcing properties as measured by the place preference paradigm and that intra-VTA infusions of atropine sulfate attenuated MFB self-stimulation.
11. Atropine sulfate was administered intraperitoneally at a dose of 0.6 mg/kg, 15 minutes before testing; atropine methyl nitrate was administered at the same dose in the same way. Atropine methyl nitrate does not readily cross the blood-brain barrier and thus provides the same peripheral cholinergic blockade with little central cholinergic blockade [I. R. Innes and M. Nickerson, in *The Pharmacological Basis of Therapeutics*, L. S. Goodman and A. Gilman, Eds. (Macmillan, New York, ed. 5, 1975), p. 514]. Pimozide (0.25 mg/kg) was injected intraperitoneally 4 hours before testing (the latency for approximate peak behavioral effectiveness) [J. Atalay and R. A. Wise, *Pharmacol. Biochem. Behav.* **18**, 655 (1983)].
12. The conclusion that two or more populations are involved was first advanced two decades ago by J. A. Deutsch [*J. Comp. Physiol. Psychol.* **58**, 1 (1964)], but was subsequently rejected on methodological grounds; it was argued to have been based on a measurement artifact [J. S. Yeomans, *Physiol. Behav.* **15**, 593 (1975)]. Deutsch's proposed fast population had estimated refractory periods remarkably close to those estimated in the present study, which is not subject to Yeomans' methodological criticism. Deutsch proposed two components of brain stimulation "reward," a "drive" component and a "reinforcement" component; he argued that the fast population was responsible for the reinforcement effect. Our task does not distinguish between the two components; thus we cannot comment on the possibility that Deutsch was correct, despite the problems with his procedure.
13. The early literature suggests that muscarinic systems antagonize rather than contribute to

reward function [D. L. Margules and L. Stein, *Am. J. Physiol.* **217**, 475 (1969); E. F. Domino and M. E. Olds, *J. Pharmacol. Exp. Ther.* **164**, 202 (1968); P. Stark and E. S. Boyd, *J. Pharmacol.* **205**, 745 (1963); L. M. Newman, *J. Comp. Physiol. Psychol.* **79**, 394 (1972)]. Our data do not contradict this conclusion; measures of refractory period distributions are based on relative comparisons between single-pulse and paired-pulse effectiveness, whereas evidence of overall suppressive effects are based on analysis of absolute measures of stimulation effectiveness. It is possible that other muscarinic systems, independent of and antagonistic to the one identified here, usually mask the positive contribution revealed here.

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Multiple Organ Carcinogenicity of 1,3-Butadiene in B6C3F$_1$ Mice After 60 Weeks of Inhalation Exposure

Abstract. *Groups of 50 male and 50 female B6C3F$_1$ mice were exposed 6 hours per day, 5 days per week, for 60 to 61 weeks to air containing 0, 625, or 1250 parts per million 1,3-butadiene. These concentrations are somewhat below and slightly above the Occupational Safety and Health Administration standard of 1000 parts per million for butadiene. The study was designed for 104-week exposures but had to be ended early due to cancer-related mortality in both sexes at both exposure concentrations. There were early induction and significantly increased incidences of hemangiosarcomas of the heart, malignant lymphomas, alveolar-bronchiolar neoplasms, squamous cell neoplasms of the forestomach in males and females and acinar cell carcinomas of the mammary gland, granulosa cell neoplasms of the ovary, and hepatocellular neoplasms in females. Current workplace standards for exposure to butadiene should be reexamined in view of these findings.*

1,3-Butadiene, in production volume one of the top 20 organic chemicals manufactured in the United States (*1*), is a colorless gas used mainly to make synthetic rubber (styrene-butadiene rubber and polybutadiene rubber) and thermoplastic resins (acrylonitrile-butadiene-styrene) (*2*). In 1983, 2.3 billion pounds of butadiene was produced in the United States (*1*). The maximum 8-hour time-weighted average workroom exposure concentration promulgated by the Occupational Safety and Health Administration (OSHA) is 1000 ppm (*3*).

Butadiene is mutagenic to *Salmonella typhimurium* strains (TA1530 and TA1535), which are sensitive to base-pair substitution mutagens (*4*). Mutagenicity of butadiene apparently requires metabolic activation (*4*) and may be due to the epoxide intermediates butadiene monoxide (1,2-epoxybutene-3) and diepoxybutane. Butadiene monoxide is the primary metabolite of butadiene biotransformation by rat liver microsomal monooxygenase (*5*) and may be conjugated with glutathione or further metabolized to diepoxybutane or 3,4-epoxy-1,2-butanediol (*6*).

Butadiene monoxide and diepoxybutane have been found to induce local neoplasms when applied to the skin of mice or when administered to mice or rats by subcutaneous injection (*7*). In the study reported here malignant neoplasms were observed at multiple sites in B6C3F$_1$ mice exposed to butadiene vapors for only 60 to 61 weeks.

Groups of 50 male and 50 female B6C3F$_1$ mice (Charles River) were exposed 6 hours per day, 5 days per week to air containing butadiene at target concentrations of 0 (chamber control), 625, or 1250 ppm (*8*). The animals, which were housed individually, were 8 to 9 weeks of age when first exposed to the butadiene vapors. The study was intended to last 103 weeks, but was ended after 60 weeks (for males) or 61 weeks (for females) because of reduced survival due to fatal tumors. Tap water and food (NIH-07 diet) were freely available except during exposure periods, when only water was available.

All animals that died during the study or that were killed at the end of the exposure period were subjected to a gross necropsy and a complete histopathologic examination (*9*). Differences in survival were analyzed by life table methods (*10*). Incidences of neoplastic lesions were analyzed by life table methods and by the Fisher exact test for pairwise comparisons of high-dose or low-dose groups with controls and the Cochran-Armitage test for dose-response trends (*10*).

Mean body weights of male or female mice did not appear to be affected by exposure to butadiene. However, survival was significantly ($P < 0.01$) reduced in all exposure groups. Survival rates were as follows: for males at 60 weeks, 49 of 50 (controls), 11 of 50 (625 ppm), and 7 of 50 (1250 ppm); for females at 61 weeks, 46 of 50 (controls), 15 of 50 (625 ppm), and 30 of 50 (1250 ppm).

Early deaths were due primarily to malignant neoplasms involving multiple organs. Because the study was stopped while background tumor incidences were low (*11*), it was possible to examine the effect of butadiene on total tumor rates. At the end of the study there were tumors in 20 percent of the control males and 12 percent of the control females, compared to 80 to 94 percent of the exposed mice. The total number of primary malignant and benign neoplasms per animal was also much greater ($P < 0.01$) in the butadiene-exposed groups.

Primary tumors caused by exposure to butadiene are listed in Table 1. Malig-

Table 1. Incidence of primary tumors in B6C3F$_1$ mice exposed to butadiene by inhalation for 60 to 61 weeks. Values are numbers of animals and incidence (percent).

Tumor	Males			Females		
	Control	625 ppm	1250 ppm	Control	625 ppm	1250 ppm
Malignant lymphoma	0 of 50 (0)*	23 of 50 (46)†	29 of 50 (58)†	1 of 50 (2)*	10 of 49 (20)†	10 of 49 (20)†
Hemangiosarcoma of heart	0 of 50 (0)‡	16 of 49 (33)†	7 of 49 (14)†	0 of 50 (0)*	11 of 48 (23)†	18 of 49 (37)†
Alveolar-bronchiolar neoplasms	2 of 50 (4)*	14 of 49 (29)†	15 of 49 (31)†	3 of 49 (6)*	12 of 48 (25)†	23 of 49 (47)†
Squamous cell neoplasm of forestomach	0 of 49 (0)	7 of 40 (18)†	1 of 44 (2)	0 of 49 (0)*	5 of 42 (12)§	10 of 49 (20)†
Acinar cell carcinoma of mammary gland	0 of 50 (0)	0 of 50 (0)	0 of 50 (0)	0 of 50 (0)*	2 of 49 (4)	6 of 49 (12)§
Granulosa cell neoplasm of ovary				0 of 49 (0)*	6 of 45 (13)†	12 of 48 (25)†
Hepatocellular neoplasms	8 of 50 (16)	6 of 49 (12)	2 of 49 (4)	0 of 50 (0)‡	2 of 47 (4)	5 of 49 (10)§

*Increasing trend ($P < 0.01$). †Increased compared to control ($P < 0.01$). ‡Increasing trend ($P < 0.05$). §Increased compared to control ($P < 0.05$).