the coast. Similar drums, however, with the same content, have been found in the North Sea.

The samples were purified by crystallization, distillation, or gas-liquid chromatography. The components were identified by their melting points, and ultraviolet, infrared, or mass spectra. In most cases, more than 90 percent of the contents could be identified. The remainder was tarry or highly polymerized material.

The acute toxicity to fish was estimated by exposing guppies to sequences of decreasing (in powers of ten) dilution of the samples. The results of the chemical and biological investigations are summarized in Table 1.

The samples can be grouped together into a limited number of subgroups, designated mixtures A to F, each of comparable chemical composition and toxicity. The drums containing mixture B can be recognized by their seal (Fig. 1); the drums containing mixture D, by their higher weight and typical screw cap (Fig. 2). None of the samples were measurably radioactive.

Sooner or later, the drums containing the wastes will become corroded and the contents will spill into the sea. The fate of the chemicals in the sea is largely unknown, but from general chemical considerations it can be anticipated that most of the chemicals identified are of a persistent nature. The history of endosulfan (present in mixture D) in fresh surface waters has been studied recently (*2*): its half-life was found to vary, according to circumstances, from 1 week to several months. Although the aqueous phase of the mixture is strongly alkaline, the hydrolysis of endosulfan, which is dissolved in the organic phase, proceeds very slowly. Mixture D, even when diluted to 1 part in 100,000,000, was found to be lethal to fish. Aside from the potential danger to the environment, the drums also damage the fishermen's nets and the fish caught in them. The number of drums dropped into the sea is not known exactly, but reasonable estimates run to several tens of thousands. Plans are currently being made to clear the drums from at least the intensely fished areas and to burn their contents (*3*).

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References and Notes

1. *Water Res.* 4, 259 (1970).
2. P. A. Greve and S. L. Wit, *J. Am. Water Works Assoc.*, in press.
3. The need for international agreements to control the dumping of industrial wastes in the sea was emphasized in a comment on this report by D. L. Kedde, public health officer of the Ministry of Social Affairs and Public Health, Leidschendam, the Netherlands. Appropriate legislation is lacking, he said, and, since the responsibility of industry ends when the drums are loaded on the ship, a loophole exists which permits an all too easy solution to the problem of disposing of chemical wastes. The prevention of this careless practice, in Kedde's opinion, will require international agreements and stringent enforcements. Dumping of drums in fished areas should be absolutely forbidden, he added, because the drums can damage fishing equipment.
4. I thank the departments of mass spectrometry of the Analytical Chemical Laboratory of the State University at Utrecht and the Central Institute for Nutrition and Food Research TNO at Zeist for their assistance.

3 February 1971; revised 21 May 1971

[^{3}H]Lysergic Acid Diethylamide: Cellular Autoradiographic Localization in Rat Brain

Abstract. *Intravenous administration of [^{3}H]lysergic acid diethylamide (LSD) to rats resulted in accumulation of the drug in the brain within 15 minutes. Autoradiographic methods were used to differentiate free and bound [^{3}H]LSD in brain tissue. Free [^{3}H]LSD was generally distributed in the pituitary and pineal glands, cerebellum, hippocampus, and choroid plexus. Bound [^{3}H]LSD was localized in neurons of the cortex, caudate nucleus, midbrain, and medulla, as well as in choroid plexus epithelium.*

D-Lysergic acid diethylamide (LSD), the most potent hallucinogenic compound known, produces a multiplicity of pharmacological actions in the central nervous system, which have been postulated to result from stimulation or inhibition of serotonin receptors and possibly to involve neurons containing norepinephrine (*1*). Distribution and localization of LSD in various organs and tissues has been measured in a number of laboratories (*2*). However, most often tissue homogenates of whole organs or specific regions of organs have been used. The results so obtained reflect the total concentration of the compound present in the homogenized fraction but do not permit localization of the compound to individual cells in the regions reported. Metabolic studies of brain tissue in vitro have shown that the brain does not metabolize LSD (*3*); we assume in this report that the cellular localization demonstrated by means of autoradiography is that of LSD. However, the presence of a metabolite circulating from the liver to the brain is not excluded.

The availability of [^{3}H]LSD with high specific activity makes it possible to study cellular localization with high-resolution autoradiography. Because [^{3}H]LSD may exist in tissue in free and bound forms, attempts to localize this compound in tissues by means of conventional histological procedures alone may lead to false results (*4*). The use of solvents for fixation, dehydration, clearing, embedding, and removal of embedding materials should be avoided as should wet photographic tissue mounting for autoradiography. The artifacts likely to occur in a procedure using such compounds result from extraction, leaching, and translocation of the labeled compound during tissue processing (*5*). Therefore, we used the technique of applying freeze-dried frozen sections to dried photographic emulsion developed for the study of diffusible substances (*4–7*); in conjunction with this technique we used a conventional histological method for cellular autoradiographic localization to differentiate between the firmly bound and free or loosely bound forms of [^{3}H]LSD as defined previously (*7*). Autoradiography is a valuable technique used for studying cells within a heterogeneous population (*8*).

D-Lysergic acid diethylamide, randomly labeled with ^{3}H in the diethyl side chain, was obtained in 70 percent ethanol solution with a specific activity of 520 μc/mg from the New England Nuclear Corporation. Upon arrival the compound was subjected to thin-layer chromatography with use of two solvent systems [chloroform, ethanol, and acetic acid (18 : 10 : 2) and trichloromethane and methanol (9 : 1)]. The chromatogram showed three distinct spots in both systems, with approximately 60 percent of the activity corresponding to authentic LSD. The compound was purified on silica gel columns 15 cm long (Quantum Industries) and eluted with methanol. The eluent corresponding to LSD was again subjected to thin-layer chro-

matography, yielding a single spot with the same R_F value as that of pure unlabeled LSD. The purified [^{3}H]LSD was evaporated to dryness and then diluted with tartaric acid in saline solution.

For high-resolution cellular autoradiography, male rats (Sprague-Dawley, 100 to 125 g) were anesthesized with ether; the femoral vein was cannulated, and the animals were injected with heparin. The animals were allowed to recover from anesthesia for 2 hours; then they were injected with freshly purified [^{3}H]LSD tartarate (1 μc/g, 1.7 μg/g) and killed 5, 15, or 30 minutes, or 1 hour later by decapitation. We used a high dose of [^{3}H]LSD (1.7 μg/g) to reduce the necessary time of exposure. To determine whether localization of [^{3}H]LSD is dependent on dosage, lower doses would need to be used. The brains were removed (in the cold room, -10°C) as quickly as possible and bisected sagittally. The halves were cut coronally into 4- to 5-μm thick sections rostrocaudally. The resulting tissue blocks from one half of each brain were mounted on brass holders with a thin layer of Tissue Tek (Ames Co.), immediately frozen in liquid nitrogen at -220°C (*7*), and stored in a liquid nitrogen refrigerator. For sectioning, the holder with attached tissue was mounted on an International Minot microtome and allowed to equilibrate for 1 hour at -60°C. Sections (2 μm) were cut and then freeze-dried in a cryosorption pump (Delmar Scientific Glass Products) at the temperature of Dry Ice and at a vacuum of 10^{-5} mm-Hg for 16 to 20 hours. The freeze-dried sections were stored in a desiccator over Drierite and later mounted (in a room with low humidity, 10 to 20 percent relative humidity) on slides coated with Kodak NTB-3 photographic emulsion which had been previously dried for at least 48 hours over Drierite. For binding studies, tissues from the contralateral halves of brains were processed for autoradiography by a conventional histological procedure; they were fixed in 10 percent unbuffered formalin, dehydrated with graded concentrations of ethanol, cleared with xylene, and embedded in paraffin. Sections (2 μm) were cut on an American Optical rotary microtome at room temperature, mounted on slides, deparaffinized with xylene, and rehydrated through alcohol to water before being dipped into Kodak NTB-3 photographic emulsion. After photographic exposure for 7 to 21 days at -15°C all slides obtained with both methods were developed in D-19 Kodak developer for 3.5 minutes, rinsed in tap water, fixed with Kodak acid fixer for 5 minutes, and washed for 15 minutes. The slides were air-dried, stained with methyl-green-pyronin for 5 minutes, rinsed in water for 1 minute, covered, and examined under the microscope. To determine the time at which the highest concentration of [^{3}H]LSD appears in the brain, and therefore the proper time to take tissues for autoradiography, we made time course studies by means of whole-body autoradiography (*9*). The results showed that [^{3}H]LSD penetrates the brain within the first 5 minutes, reaches the highest concentration in 15 minutes, and then disappears 1 hour after injection. These results are in agreement with biochemical studies on uptake and elimination of LSD in rat brain (*10*).

Table 1. Summary of autoradiographic localization of [^{3}H]LSD in rat brain; + indicates presence of cellular localization.

Structure	Free	Bound
Choroid plexus	+	+
Pineal	+	
Pituitary	+	
Hippocampus	+	
Cerebellum granular layer	+	+
Cerebellum Purkinje cells		+
Cerebral cortex		+
Caudate nucleus		+
Colliculus nuclei		+
Olivary nuclei		+
Midbrain raphe		+
Tegmental nucleus		+
Medial reticular gray		+

The rat choroid plexus accumulated the highest concentration of [^{3}H]LSD (Fig. 1A). The concentration in the choroid persisted even after 1 hour, at

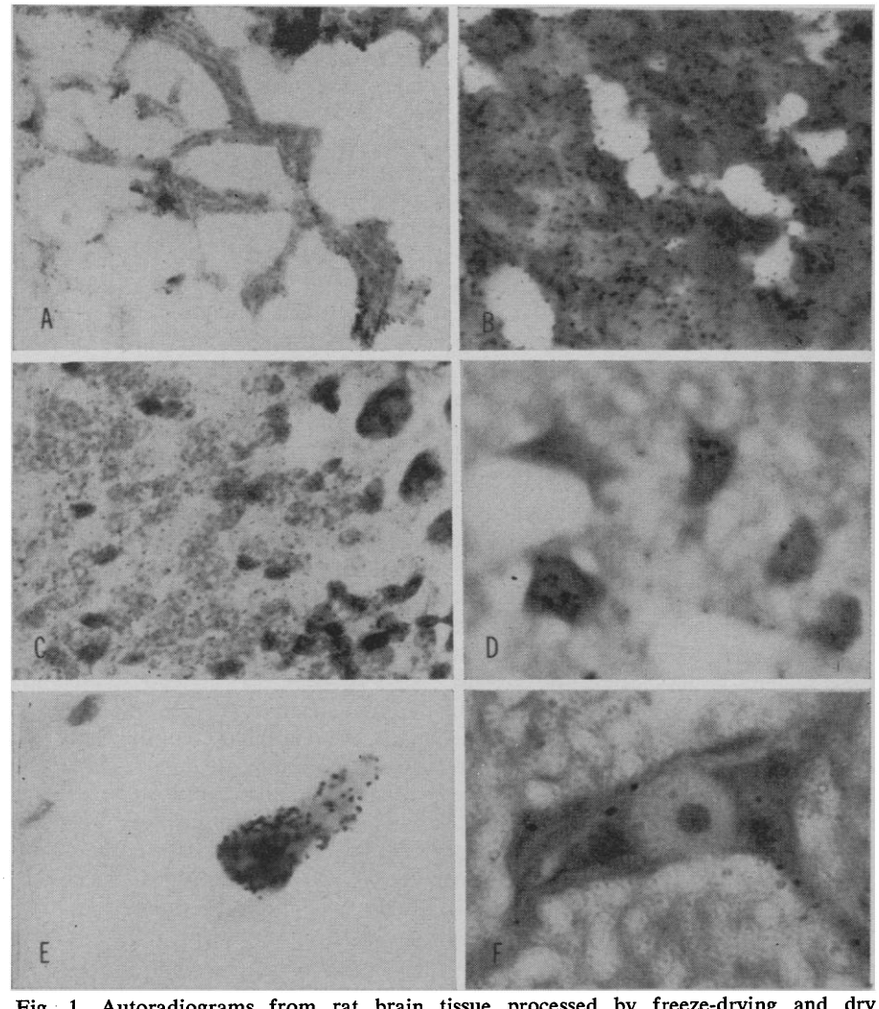

Fig. 1. Autoradiograms from rat brain tissue processed by freeze-drying and dry mounting and exposed for 17 days. The rat was injected with [^{3}H]LSD (1.7 μc/g, 1.7 μg/g) 15 minutes before being killed. (A) Choroid plexus ($\times$ 256); (B) pineal gland ($\times$ 480); (C) cerebellum granular layer and Purkinje cells ($\times$ 400); (D) frontal cortex ($\times$ 480); (E) caudate nucleus neuron ($\times$ 930); (F) midbrain neuron in superior colliculus ($\times$ 1200).

which time the brain tissue was practically devoid of [^{3}H]LSD activity. The autoradiographs of freeze-dried sections of the choroid plexus exposed under the same conditions were more dense than similar sections prepared by the conventional histological method while the bound [^{3}H]LSD remained in the choroid epithelium.

Autoradiographs of the pituitary and pineal glands showed generalized nonspecific distribution of [^{3}H]LSD over all the tissue in the freeze-dried sections (Fig. 1B). The hippocampus also appeared to have generalized distribution of [^{3}H]LSD particularly in the granular cell layer of tissues processed by the freeze-dried method only. However, sections of pituitary, pineal glands, and hippocampus prepared by the conventional method were devoid of activity. This loss of activity indicates that [^{3}H]LSD is present in the pituitary, pineal gland, and hippocampus in a free form which was extracted in subsequent tissue processing by the various solvents involved. In the cerebellum, [^{3}H]LSD activity was present in the granular layer and in Purkinje cells of tissues prepared by the freeze-drying method (Fig. 1C). The activity in cerebellar tissue processed by the conventional histological method was absent from the granular layer and present only in the Purkinje cells, indicating strong binding of [^{3}H]LSD to these cells.

The [^{3}H]LSD was distributed in various regions of the brain (Table 1) and localized and bound to certain cells within those regions. Activity was present in some neurons of the frontal cortex and olfactory bulb (Fig. 1D). The specific neuronal localization in the cortex was demonstrable with both methods and indicated binding of [^{3}H]-LSD to the cell bodies of these neurons. Likewise, certain neurons in the caudate nucleus had a preferential accumulation and binding of [^{3}H]LSD (Fig. 1E) as did the midbrain and medulla in which [^{3}H]LSD was localized in various large neurons. Also, [^{3}H]LSD was localized in cells of the superior and inferior colliculi, the dorsal nucleus of the midbrain raphe, the dorsal tegmental nuclei, olivary nuclei, and medial reticular gray regions. The activity in the multipolar cells in the midbrain and medulla appeared to be concentrated on the periphery of the neurons and their processes, but it was absent from cell nuclei (Fig. 1F); this suggests that [^{3}H]LSD binds to the membranes of these neurons or to the postsynaptic neuronal sites.

Our studies demonstrate that [^{3}H]-LSD is localized to only a few neurons within a given region. For example, the majority of the neurons within the caudate nucleus were not labeled. Other investigators, using biochemical techniques on brain homogenates, have shown that LSD accumulates rapidly in brain, the concentration varying with the region of the brain. These studies give the sum total of LSD in the brain or regional fraction assayed without regard to the actual distribution in the specific area or cell type within these areas (*2*). By using both the freeze-drying and the conventional histological methods, we could differentiate between free and bound LSD in the brain tissue. Free [^{3}H]LSD was present in pituitary, pineal gland, granular layers of the cerebellum, hippocampus, and choroid plexus. Firmly bound [^{3}H]LSD was present in specific neurons in the cortex, caudate nucleus, midbrain, and medulla, as well as in the choroid plexus epithelium. The high accumulation and binding of [^{3}H]LSD in the choroid plexus suggests that the choroid may be involved in the active transport of LSD to and from blood, brain, and cerebrospinal fluid. Also, the choroid plexus may act as a storage site for LSD to be distributed back to the brain at a later time.

The action of LSD has been linked to serotonin by a number of investigators (*1*). The amount of serotonin in the brain increases after administration of LSD (*11*), and LSD affects the spontaneous firing of certain neurons resulting in both excitatory and inhibitory actions. Electrophysiological and biochemical studies on the brain suggest that [^{3}H]LSD may act on the postsynaptic receptors as serotonin does, and may specifically inhibit serotonergic fibers perhaps through a neuronal feedback mechanism (*12*).

The presence of relatively high numbers of cells that contain serotonin in various regions of the brain suggests that serotonin has neuroregulatory or neurotransmitter functions. Direct evidence for the existence of serotonergic neurons in the central nervous system by histochemical fluorescence and autoradiographic studies of ^{3}H-labeled serotonin support the neurotransmitter function of serotonin (*13*). The autoradiographic localization of [^{3}H]LSD occurs in neurons within regions reported to contain serotonin, suggesting the possibility that LSD is present in serotonergic neurons. The accumulation of [^{3}H]LSD on the periphery of neuronal cells in the midbrain and medulla suggest postsynaptic localization in granules containing serotonin. Our studies strengthen the hypothesis that the action of LSD in the brain is linked in someway to serotonergic neurons. Such cellular autoradiographic localization of [^{3}H]LSD has yet to be correlated with histochemical fluorescence studies for biogenic amines to determine the relationship of cellular localization and binding of LSD to subcellular structures in serotonin-containing neurons.

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References and Notes

1. D. W. Woolley and E. A. Shaw, *Proc. Nat. Acad. Sci. U.S.* **40**, 228 (1954); D. X. Freedman and G. K. Aghajanian, *Lloydia (Cincinnati)* **29**, 309 (1966); F. E. Bloom, E. Costa, G. C. Salmoiraghi, *J. Pharmacol. Exp. Ther.* **146**, 16 (1964); R. G. Boakes, P. B. Bradley, I. Briggs, A. Dray, *Br. J. Pharmacol.* **40**, 202 (1970).
2. A. H. Amin, T. B. Crawford, S. H. Gaddum, *J. Physiol. (London)* **126**, 596 (1954); D. M. Twarog and I. H. Page, *Am. J. Physiol.* **175**, 157 (1953); A. Stoll, E. Rothlin, J. Rutschmann, W. R. Schalch, *Experentia* **11**, 396 (1955); D. X. Freedman and N. J. Giarman, *Ann. N.Y. Acad. Sci.* **96**, 98 (1962).
3. J. Axelrod, R. O. Brady, B. Witkop, E. V. Evarts, *Ann. N.Y. Acad. Sci.* **66**, 435 (1957).
4. L. J. Roth, A. Hanngren, W. P. Norris, *Argonne National Laboratory Semiannual Report (ANL6906)* (July-December 1963), p. 61; L. J. Roth and W. E. Stumpf, Eds. *Autoradiography of Diffusible Substances* (Academic Press, New York, 1969).
5. W. E. Stumpf and L. J. Roth, *Advan. Tracer Methodol.* **4**, 113 (1968).
6. ———, *J. Histochem. Cytochem.* **14**, 274 (1966); *ibid.* **15**, 243 (1967).
7. I. M. Diab and L. J. Roth, *Stain Technol.* **45**, 285 (1970).
8. L. J. Roth, *Handbuch der Experimentellen Pharmakologies*, B. B. Brodie and J. R. Gillette, Eds. (Springer-Verlag, Heidelberg, 1971), vol. 28, p. 286.
9. Unpublished whole-body autoradiographic studies were performed in our laboratory on 20- to 30-g mice injected with [^{3}H]LSD (1 μc/g) and killed 5, 15, or 30 minutes or 1, 4, or 8 hours after injection. The autoradiograms obtained were essentially similar to those reported by J. E. Idanpaan-Heikkila and J. Schoolar [*Science* **164**, 1295 (1969)].
10. J. Rosencrans, R. A. Lovell, D. X. Freedman, *Biochem. Pharmacol.* **16**, 2011 (1967).
11. D. X. Freedman, *J. Pharmacol. Exp. Ther.* **134**, 160 (1961); ———, R. Gottlieb, R. A. Lovell, *Biochem. Pharmacol.* **19**, 118 (1970); N. E. Anden, H. Corrodi, K. Fuxe, T. Hokfelt, *Br. J. Pharmacol.* **34**, 1 (1968).
12. G. K. Aghajanian, W. E. Foote, M. H. Sheard, *Science* **161**, 706 (1968); *J. Pharmacol. Exp. Ther.* **171**, 178 (1970); R. M. Marchbanks, *Biochem. Pharmacol.* **16**, 1971 (1967); H. M. Greschenfeld, *Science* **171**, 1254 (1971).
13. A. Dahlstrom and K. Fuxe, *Acta Physiol. Scand.* **62** (suppl. 232), 1 (1965); G. K. Aghajanian and F. E. Bloom, *J. Pharmacol. Exp. Ther.* **156**, 23 (1967).
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