Pharmacology Biochemistry & Behavior, Vol. 7, pp. 167–172.

BRIEF COMMUNICATION

Combination Operant Conditioning – Liquid Nitrogen Immersion Chamber for Studying Neurotransmitter Systems and Behavior

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(Received 23 March 1977)

SMITH, J. E., W. R. LECKRONE AND C. T. CO. *A combination operant conditioning – liquid nitrogen immersion chamber for studying neurotransmitter systems and behavior.* PHARMAC. BIOCHEM. BEHAV. 7(2) 167–172, 1977. – Studies of neurochemical events associated with behavior require a method of tissue fixation that is rapid and does not itself produce neurochemical changes. An apparatus is described that permits immediate immersion of an unrestrained behaving animal into liquid nitrogen. This method of tissue fixation has the greatest versatility for studying multiple neurotransmitter systems. In addition to the measurement of neurotransmitter content and turnover, investigation of neurotransmitter receptors, enriched nerve ending fractions and enzyme activities are possible. The operant conditioning – liquid nitrogen immersion chamber described here can be used for studying these neurotransmitter systems as they relate to animal's responding on operant schedules of reinforcement.

Immersion chamber Liquid nitrogen Neurotransmitter systems Behavior

INVESTIGATIONS of brain neurotransmitters require methods of tissue fixation that will rapidly terminate the activity of enzymes that are responsible for the synthesis and degradation of these important compounds. Decapitation is not an adequate method for terminating the activity of these enzymes. Acetylcholine [20] (ACh), gamma-aminobutyric acid [1] (GABA), dopamine (DA) and norepinephrine (NE) (unpublished observations from this laboratory) content has been shown to be significantly lower in the brains of decapitated animals compared to animals sacrificed by other methods that terminate enzyme activity more rapidly. Freeze-blowing [22], microwave irradiation [18] and near- or total-freezing in liquid nitrogen [20] are more satisfactory methods of tissue fixation for studying most brain neurotransmitters. Each of these procedures has certain advantages and disadvantages that must be considered by the investigator and the best method for each application chosen.

This paper describes an apparatus that can be used for the near- or total-freezing method of tissue fixation that is specially designed for investigations of brain neurotransmitter systems and animal behavior. An operant conditioning chamber that doubles as a freezing chamber for rapid immersion of the behaving animal into liquid nitrogen (or other liquid fixation media) is described that permits tissue fixation within a few seconds of the emitted behavior without introducing additional handling, stressful restraint or a prolonged time delay.

METHOD

The major components of the operant conditioning-near-freezing chamber are shown in Fig. 1. The levers, stimulus lights, food magazines, etc., are in modular form and comprise two walls (a similar apparatus is commercially available from Coulbourn Instruments, Inc.). The floor and ceiling are the dipping components of the system. The entire apparatus is contained in a sound-proof chamber with a removable floor and sawdust pan which when removed exposes a container of liquid nitrogen.

Apparatus

Stationary operant conditioning subunit. The stationary walls of the chamber (shown in Fig. 1A) contain the

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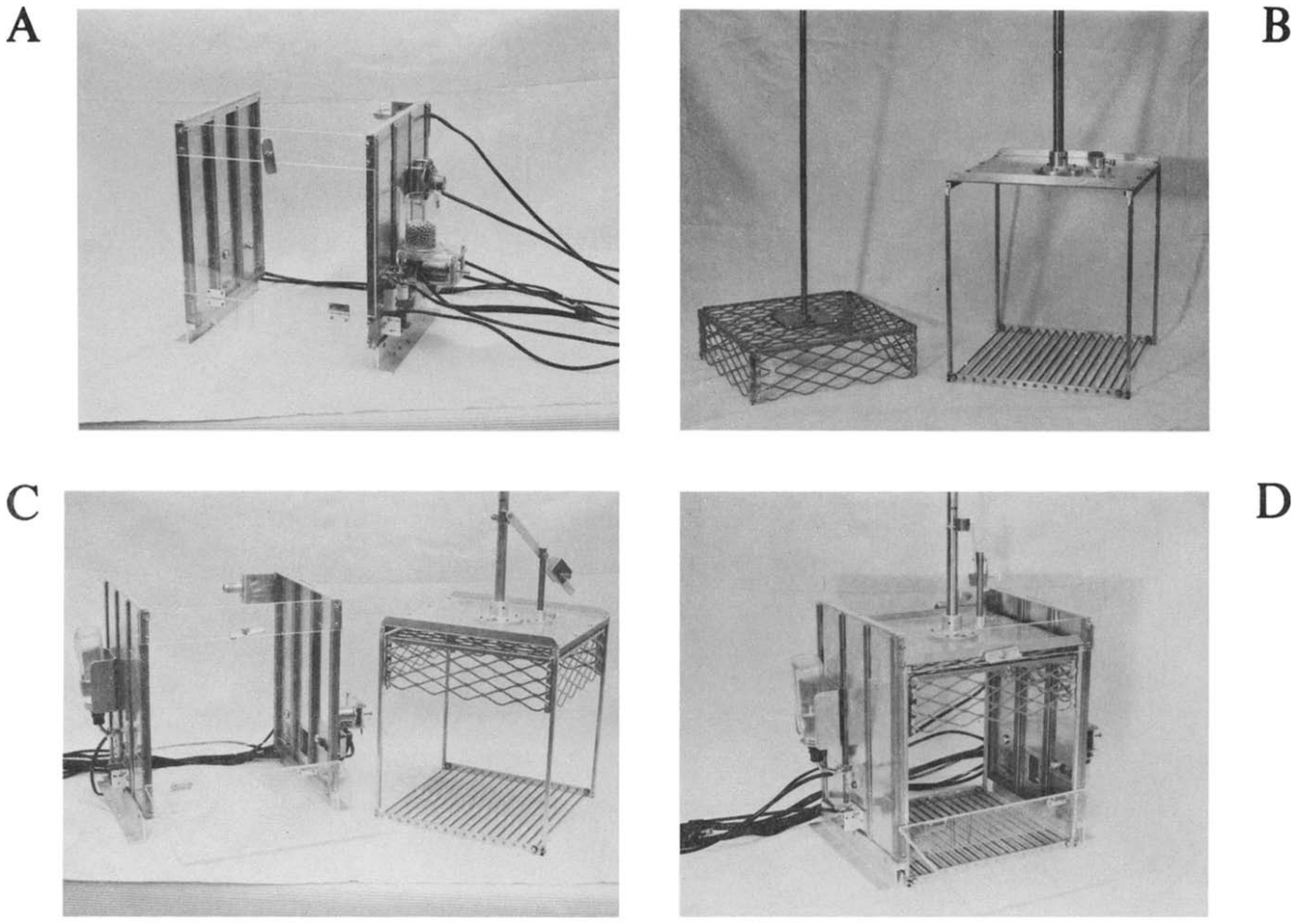

FIG. 1. Operant conditioning – liquid nitrogen immersion chamber. A. The stationary operant-conditioning subunit composed of two aluminum walls containing the operanda, stimulus and reinforcement modules and two Plexiglas walls with front hinged door. B. Movable dipping subunit consisting of the rigid floor and ceiling (on right) and movable hardware cloth ceiling (on left). C. Assembled dipping apparatus and stationary operant-conditioning subunit. D. Assembled operant-conditioning – liquid nitrogen immersion chamber.

operanda and stimulus modules (levers, stimulus lights, pellet magazine, etc.) and are constructed from light weight aluminum and Plexiglas with overall measurements of 29 × 27 × 30 cm. The two walls containing the modules are divided into three 8 cm wide sections by four aluminum posts (1.3 square × 30 cm) with grooves for the easy insertion of the operanda and stimulus modules. The front and back of the chamber are Plexiglas (0.6 × 29 × 30 cm) with a hinged door which allows easy access to the chamber from the front.

Movable dipping subunit. The dipping portion of the chamber consists of two components – a rigid floor and ceiling and a movable expanded metal ceiling (Fig. 1B). The rigid floor assembly consists of a grid floor made of brass rods (0.3 × 28 cm) inserted into Teflon bushings which are themselves pressed into an aluminum support bar (0.3 × 1.6 × 28 cm). With this construction the floor can be used for grid shock presentation. The corners of the dipping apparatus are four aluminum posts (0.6 square × 30 cm) which connect the grid floor to the rigid ceiling made of 20 gauge aluminum 28 × 26 cm. A 52 cm piece of 1.6 cm stainless steel pipe is attached to the center of the rigid ceiling. The movable ceiling made of stainless steel expanded metal is attached to a brass 1.0 cm rod, 76 cm long. In the assembled apparatus this rod is inside the metal pipe which is attached to the rigid aluminum ceiling.

Dipping Procedure

In the dipping process the mobile ceiling is lowered until it meets the grid floor (Fig. 2B) forming a compact closed dipping chamber. The sawdust pan floor is removed earlier and replaced by a piece of stiff aluminum foil. Another piece also covers the liquid nitrogen container to decrease evaporation. The pipe attached to the stationary ceiling is then lowered until the dipping chamber moves out the bottom of the sound-proof chamber and into a container of liquid nitrogen (Fig. 2C and D). The chamber is under positive pressure from the blower ventilation system (visible in the upper left corner of Fig. 2D) so that the liquid nitrogen does not produce a

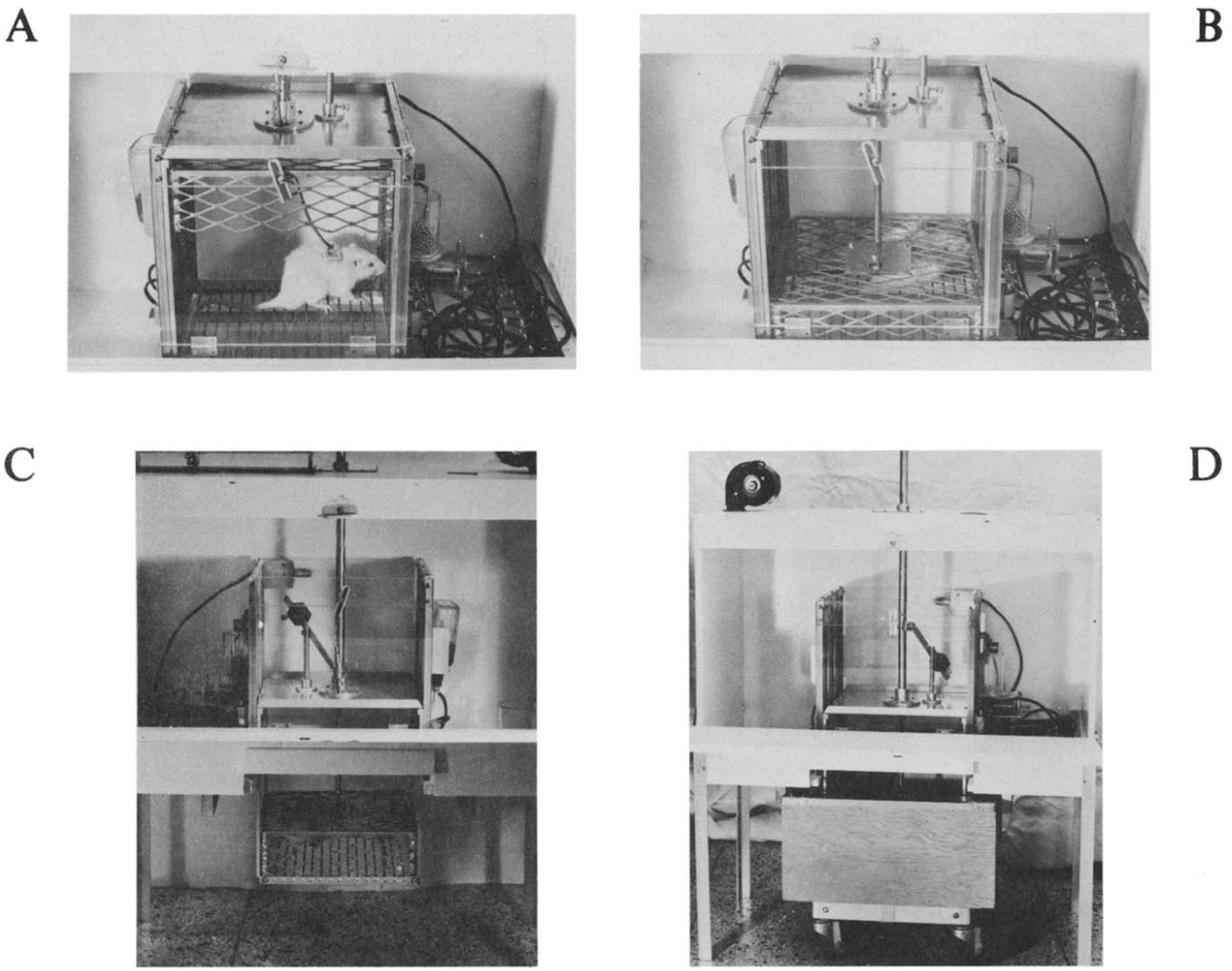

FIG. 2. Liquid nitrogen dipping procedure. A. Rat with chronic indwelling jugular catheter, harness and swivel in chamber prior to dipping. B. Movable expanded metal ceiling has been lowered to form compact dipping chamber. C. The rigid floor-ceiling subunit of the dipping apparatus is lowered out the bottom of the sound-proof chamber. Compact dipping chamber containing the rat is clearly visible. D. Liquid nitrogen container in place with rat in compact dipping chamber submerged in liquid nitrogen.

decrease in temperature that is detectable in the conditioning unit. A small hole with wide angle magnifying optics is installed in the door of the soundproof outer chamber so that the whole dipping process can be completed without opening the chamber in one smooth motion that takes about 2 sec.

Neurochemical Procedure

Fifteen naive adult male Wistar rats (90–120 days old) were used for brain neurotransmitter measurements with the near-freezing tissue fixation procedure. With this method the rats are sacrificed by being immersed in liquid nitrogen for approximately 9 sec with deep brain structures reaching 0°C by this time [20]. The brains were dissected at −18°C in a cold box into cerebral cortex, striatum, hippocampus, diencephalon (thalamus plus hypothalamus), mesencephalon and pons-medulla oblongata. The brain parts were immersed in liquid nitrogen and stored at −70°C until assayed. The tissue samples were individually pulverized in liquid nitrogen in a stainless steel mortar on dry ice. In one study where only DA, NE and serotonin (5-HT) were measured (N = 6), the tissue powder was extracted with 20 volumes of ice-cold 1N formic acid/acetone (v/v-15:85) (FA/A). Lipids were removed with a three volume heptane/chloroform (v/v-8:1) wash. The organic phase was aspirated and discarded and the aqueous phase taken to dryness under nitrogen at 37°C. DA, NE and 5-HT were extracted and concurrently assayed in the individual brain regions by a previously described procedure [17].

In another study (N = 9) part of the tissue powder from the cerebral cortex and diencephalon was extracted with FA/A and ACh, DA, NE and 5-HT were concurrently measured with the method just described [17]. Another portion of the tissue powder was extracted with 5% TCA

TABLE 1

CONTENT OF SEROTONIN, NOREPINEPHRINE AND DOPAMINE IN THE CEREBRAL CORTEX, DIENCEPHALON, MESENCEPHALON, STRIATUM, HIPPOCAMPUS AND PONS-MEDULLA OF NAIVE RATS SACRIFICED BY THE NEAR-FREEZING METHOD

	Content(nmoles/g)					
	Cerebral Cortex	Diencephalon	Mesencephalon	Striatum	Hippocampus	Pons-Medulla
5-HT	2.62 ± 0.51	4.74 ± 0.68	4.33 ± 1.33	2.41 ± 1.25	2.12 ± 0.26	5.13 ± 1.37
NE	2.00 ± 0.39	4.42 ± 0.77	2.85 ± 0.78	2.03 ± 0.67*	2.06 ± 0.63*	4.15 ± 0.47
DA	2.97 ± 0.96	1.59 ± 0.62	1.09 ± 0.43*	27.91 ± 1.27	0.33 ± 0.28*	1.10 ± 0.33*

Values are means ± standard deviations for N = 6.
*Fluorescence readings were less than twice the blank value.

and the levels of aspartate (Asp), β-alanine (β-Ala), gamma-aminobutyric acid (GABA), glutamine (Glu-NH_2), glutamate (Glu), glycine (Gly) and serine (Ser) measured with a modification of a previously described procedure [11].

RESULTS

In Table 1 content values for DA, NE and 5-HT from six brain areas of naive rats sacrificed by the near-freezing method are shown. The levels of the biogenic monoamines reported in this study are in agreement with other reported values when similar brain areas have been assayed. The content of NE is consistent with previously reported values for the cortex (14,23], hippocampus [3, 19, 23, 24], striatum [3, 14, 19, 23, 24] and mesencephalon [3]. The content of DA is consistent with previously reported values for the striatum [3, 5, 14, 19, 23] and hippocampus [19, 23]. The levels of 5-HT are in agreement with previously reported values for the cortex, striatum [3,14], hippocampus [3,9], mesencephalon [3] and pons-medulla oblongata [14].

Table 2 shows content values for ACh, DA, NE, 5-HT, Asp, Glu, Gly, GABA, β-Ala, Glu-NH_2 and Ser concurrently measured in the cerebral cortex and the diencephalon (hypothalamus and thalamus) of naive rats sacrificed by the near-freezing method. The ACh content in the cortex and diencephalon is similar to previously reported values for these brain regions [12].

TABLE 2

CONTENT OF ACETYLCHOLINE, DOPAMINE, NOREPINEPHRINE, SEROTONIN, ASPARTATE, GLUTAMATE, GLYCINE, GABA, β-ALANINE, GLUTAMINE AND SERINE CONCURRENTLY MEASURED IN THE CEREBRAL CORTEX AND DIENCEPHALON OF NAIVE RATS SACRIFICED BY THE NEAR-FREEZING METHOD

	Cerebral Cortex	Diencephalon
	nmoles/g	
ACh	14.4 ± 0.9	16.4 ± 2.9
DA	4.5 ± 0.6	1.9 ± 0.6
NE	2.1 ± 0.3	4.0 ± 0.8
5-HT	2.0 ± 0.3	3.1 ± 0.6
	μmoles/g	
Asp	2.7 ± 0.2	2.1 ± 0.3
Glu	4.4 ± 1.0	4.4 ± 0.5
Gly	4.3 ± 1.1	0.6 ± 0.2
GABA	1.2 ± 0.2	2.1 ± 0.4
β-Ala	0.14 ± 0.12	0.15 ± 0.05
Glu-NH_2	10.1 ± 1.1	6.8 ± 1.0
Ser	0.9 ± 0.2	0.6 ± 0.1

Values are means ± standard deviation for N = 9.

DISCUSSION

Accurate assessment of some neurotransmitter systems in brain requires a method of tissue fixation that will rapidly terminate all neurochemical events. Decapitation is not an adequate fixation method. ACh [20] and GABA [1] have been shown to be significantly lower in decapitated animals than in animals sacrificed by methods that terminate enzyme activity more rapidly. We recently found that DA and NE were significantly lower in whole brains of decapitated rats than in rats sacrificed by the near-freezing procedure (NE-decapitated, 1.97 ± 0.27 nmoles/g; near-freezing, 2.19 ± 0.22 nmoles/g: t = 2.62, 32 df; DA-decapitated, 3.70 ± 0.51 nmoles/g; near-freezing, 4.25 ± 0.73 nmoles/g: t = 2.60, 33 df). More rapid procedures for terminating enzyme activity are required for accurate assessment of brain neurotransmitters. Microwave fixation [18], freeze-blowing [22] and near- or total-freezing by immersion in liquid nitrogen [20] are three such methods that are currently being used. Microwave fixation is the most rapid means of terminating enzyme activity in brain tissue. For studies of brain neurotransmitter content or turnover, microwave fixation is probably the method of choice. However, because of the denaturation of protein and the fracturing of membranes this method cannot be used in studies using subcellular fractionation, studies of receptor binding or studies of enzyme activities. This method may also not be useful for studies of neurotransmitter micro localization since an artifactual redistribution of NE and DA was recently shown to occur as a result of microwave fixation [7]. Microwave fixation may also have limitations in studies of neurotransmitter changes correlated with animal behavior since that method requires placement of the

animal into a restraining device that could possibly introduce stress artifacts or result in a time delay between the emitted operant and tissue fixation.

Freeze-blowing [22] is the second most rapid method of terminating enzyme activity in brain tissue. This method also requires a restraining device but its greatest drawback is that studies of defined neuroanatomical brain regions or subcellular fractionation are not possible.

The near- or total-freezing method is the next most rapid method for terminating brain enzyme activity. However, in the past it was necessary to transfer the animal from the operant conditioning chamber to a special dipping apparatus resulting in a time delay and exposure to a different environment between the emitted behavior being studied and tissue fixation [3, 13, 16]. With this fixation method studies using subcellular fractionation, receptor binding as well as analysis of enzyme activities are possible in defined brain regions. However, ACh content in deep brain structures such as the striatum appear to be lower in animals sacrificed by this procedure than in animals sacrificed by microwave irradiation. These higher levels of ACh in deep structures found after microwave fixation are probably the result of the increased time required for enzymes in these structures to be inactivated (reach 0°) by the near-freezing method. However, it is also possible that these increased levels may be the result of the restraint stress induced by the holding apparatus required for microwave fixation.

Even though ACh content in deep structures may be lower with near-freezing, other neurotransmitters do not seem to be affected. The levels of DA in the striatum obtained after near-freezing (Table 1) are the same as that reported with microwave irradiation [5]. We have recently completed a study of DA turnover in the striatum [15] and obtained turnover rates with near-freezing (24.3 ± $1.1_{S.D.}$ nmoles g^{-1} hr^{-1}) which are the same as those reporting using microwave fixation (23 ± 6.5_{SEM} nmoles g^{-1} hr^{-1} [25]. Serotonin levels in the hippocampus (Table 1) with near-freezing are also the same as those obtained with microwave irradiation [9]. It would appear that the advantages of microwave fixation may be in the measurement of ACh content, and the higher levels seen with that method may be partially due to the restraint stress produced by that procedure. It is also possible that microwave irradiation may produce an artifactual diffusion of neurotransmitters into surrounding areas [7]. Near-freezing allows greater latitude in the types of neurochemical investigations that are possible (subcellular fractionation studies, receptor binding studies, and enzyme activity studies). However, the disadvantages of each method must be considered with the advantages and the best method for each application chosen.

The combination operant conditioning near-freezing chamber described here permits the rapid immersion into liquid nitrogen of an animal responding on an operant schedule without introducing a time delay or exposure to a new environment. In our laboratory chronic indwelling jugular catheters are implanted into rats for intravenous morphine self-administration. These same catheters can be used for the intravenous injection of radioactive precursors of brain neurotransmitters into unrestrained-behaving animals producing a minimal disruption of behavior. The rat shown in Fig. 2 has such a chronic indwelling jugular catheter with harness and swivel assemblies which are attached to a counter-balance on the aluminum ceiling. A polyvinylchloride tubing exits through the top of the chamber and is attached to an infusion pump. With the jugular catheter for drug or radioactive precursor administration and the operant conditioning – liquid nitrogen dipping apparatus, sophisticated studies of neurotransmitter systems and behavior are possible.

ACKNOWLEDGEMENTS

This project was supported in part by USPHS Grant DA 01519–01. We gratefully acknowledge the assistance of R. Plass in wiring this apparatus, L. Tomey for the photography and J. Wilson for preparing the manuscript.

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