Beiträge zur Tabakforschung International · Volume 13 · No. 1 · January 1985 DOI: 10.2478/cttr-2013-0554

Chronic Inhalation Studies in Mice

I. Facilities and Equipment for "Nose-Only" Exposure to Cigarette Smoke *

by

C. J. Henry, D. R. Dansie, K. K. Kanagalingam and R. E. Kouri
Division of Toxicology and Molecular Biology, Microbiological Associates, Bethesda, Maryland, U.S.A.

T. Gayle, M. Guerin and R. Holmberg
Analytical Chemistry Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee, U.S.A.

and

L. Florant and J. Greenspan
Process and Instruments Corporation, Brooklyn, New York, U.S.A.

SUMMARY

Facilities and equipment are described for large-scale, long-term "nose-only" inhalation exposure of mice to whole cigarette smoke. Experimental procedures and equipment were designed to provide the mice with exposure conditions where [1] the lung was the major target organ for the smoke, [2] large quantities of fresh, whole cigarette smoke could be generated, [3] large numbers of animals could be exposed at one time, [4] routine, daily exposures could be given over a major portion of the lifetime of the animal, [5] monitoring and documentation of the quantity of smoke presented to the animals was provided during each exposure session, [6] safety systems were provided that assured exposure of the animals to smoke only under pre-set exposure conditions, and [7] cigarette smoke was generated under conditions where factors, such as cigarette type, smoke aerosol concentration and smoke particle size, were controlled.

ZUSAMMENFASSUNG

Die technische Ausstattung einer Berauchungsanlage wird beschrieben, mit der eine große Anzahl Mäuse über längere Zeit einer sich ausschließlich über die Nase vollziehenden Inhalation des gesamten Hauptstromrauches von Zigaretten ausgesetzt werden können („nose-only"-Exposition). Die Apparatur gewährleistet folgendes: [1] Das Zielorgan für den Rauch ist vor allem die Lunge. [2] Frischer Rauch wird in großen Mengen erzeugt. [3] Eine große Zahl Tiere kann gleichzeitig exponiert werden. [4] Die Tiere können dem Rauch routinemäßig, täglich über einen längeren Abschnitt ihrer Lebensspanne ausgesetzt werden. [5] Die Menge des den Tieren zugeführten Rauches wird während jeder Expositionsphase angezeigt und dokumentiert. [6] Kontrollsysteme gewährleisten, daß die Tiere dem Rauch nur unter den vorher festgelegten Bedingungen ausgesetzt werden. [7] Der Zigarettenrauch wird unter Bedingungen erzeugt, die eine Steuerung von Faktoren wie Zigarettensorte, Aerosolkonzentration im Rauch und Partikelgröße ermöglichen.

RÉSUMÉ

Ce travail porte sur la description des dispositifs techniques d'une installation de fumigation au moyen de laquelle il est possible de soumettre un grand nombre de souris pendant une durée prolongée à l'inhalation, exclusivement par le nez («nose-only»), de tout le flux principal de la fumée de cigarettes. L'appareillage est conçu de manière à garantir que: [1] La fumée atteigne essentiellement les poumons. [2] De la fumée fraîche soit produite en grande quantité. [3] Un grand nombre

* This work was supported by contracts from The Council for Tobacco Research — U.S.A., Inc.

Received: 30th April 1984 — accepted: 15th October 1984.

d'animaux puissent être exposés en même temps. [4] Les animaux puissent être soumis quotidiennement à l'action de la fumée pendant une longue période de leur existence. [5] La quantité de fumée absorbée par les souris soit connue et relevée pour chaque séance d'exposition. [6] Des systèmes de contrôle garantissent que les animaux ne sont soumis à la fumée que dans les conditions fixées au préalable. [7] La fumée de cigarette soit produite dans des conditions qui permettent de choisir et de régler des facteurs tels que le type de cigarette, la concentration d'aérosol dans la fumée et la dimension des particules de fumée.

INTRODUCTION

The experimental design of studies to evaluate the long-term effects of exposure to cigarette smoke in an animal model system must attempt to simulate human exposures. A smoke inhalation facility was designed and equipped to perform such studies. The facility resulted from a collaborative program between Microbiological Associates, the Analytical Chemistry Division at Oak Ridge National Laboratory, and Process and Instruments Corporation, Brooklyn, New York. We describe here these facilities and equipment.

The criteria used in designing the facilities and equipment were based on those factors that have been reported to be important in cigarette smoke-associated human diseases. These factors include high exposure to fresh mainstream cigarette smoke on a daily basis for a major portion of the lifetime of the individual (1, 2). Even under these conditions, however, the resultant smoke-associated diseases were observed in only a small fraction of exposed individuals. Thus, the conditions listed below were provided in order to approximate most closely the human situation:

1. an exposure system where the lung was the major target,
2. a smoke-generation system capable of providing large quantities of fresh cigarette smoke,
3. capacity to expose large numbers of animals,
4. daily exposures over a major portion of the lifetime of the animal,
5. monitoring and documentation of the quantity of smoke presented to the animal during each exposure session,
6. safety systems that assured exposure of animals to smoke only under pre-set exposure conditions, and
7. generation of cigarette smoke under conditions where factors were controlled, such as: cigarette type, smoke aerosol concentration and smoke particle size.

This communication will describe the operations, the facilities, the cigarette smoke generation equipment, and the animal exposure system used for large-scale, long-term cigarette smoke inhalation studies.

DESCRIPTION OF OPERATIONS

The equipment and procedures described here were designed for standardized, documented inhalation exposures of large numbers of mice to cigarette smoke. Mice were chosen as the animal model for these studies for the following: (a) the economy in animal husbandry operations, (b) availability of large numbers of genetically diverse inbred strains, (c) availability of strains of mice in which susceptibility to lung cancer following treatment with chemical carcinogens was genetically regulated (3, 4), and (d) availability of animal colonies that were well defined in terms of the types of biological adventitious agents normally present. It was especially important that the mouse colonies be free of *Mycoplasma pulmonis* and two respiratory viruses, Sendai virus and pneumonia virus of mice (5, 6). All mice used in these studies were vaccinated against Sendai virus (see References 3—6 for discussion).

Smoke was generated on the Smoke Exposure Machine, termed SEM II, a large capacity dynamic smoke exposure system (7, 8). The SEM II uses reverse smoking (positive puff) and automatically loads, lights, puffs and distributes the smoke from up to 30 cigarettes per cycle to the animal containment system in a flowing stream for nose-only exposure of the animals. Smoke and air flow monitoring devices provided documentation of exposure levels and provided safety for accidental overexposure or machine malfunction. Smoke levels were quantitated through the use of an optical sensor interfaced with a strip-chart recorder (9). A puff-by-puff profile of the smoke total particulate matter (TPM) and an integrated value of the total amount of smoke TPM delivered to the animals were documented for each exposure. During 4 years of use, the SEM II system proved to be a remarkably trouble-free precision instrument, utilized 6 to 8 hours per day, 5 days per week.

A unique feature of this inhalation system was that the animals were exposed to the smoke aerosol only via the nasal orifice for nose-only exposure. Groups of five animals were held in a "stock-like" holder using a combination of a neck slot and restraining spring. The nose of each animal passed through a dental rubber dam diaphragm forming a seal which prevented exposure of the body to the smoke aerosol. The animal containment system was efficient and easy to use. Mice were rapidly loaded and unloaded from the trays and the trays were placed on the module for the exposures. In such a manner, 480 mice were exposed in a nose-only fashion to smoke under the same conditions from a single SEM II.

DESCRIPTION OF FACILITIES

All smoke exposures at Microbiological Associates were carried out in a dedicated facility, with rooms arranged in 3 general areas: smoke generation areas, animal exposure and holding areas, and maintenance areas.

The exposure laboratory was operated as a barrier facility. Access was restricted to authorized personnel and policies were in force to prevent admission of disease agents that might compromise the health status of the animals. All personnel entering the animal-containment room were suitably dressed and all incoming materials were either sterilized or carefully cleaned. The facility was equipped with standard laboratory support services, including a non-recirculating, fresh-filtered air supply, high-efficiency particulate air (HEPA) and charcoal filtered exhaust systems, an oilless air supply, and a stand-by electrical generator. Maintenance areas provided cleaning capabilities for animal related equipment and for the smoke inhalation equipment.

DESCRIPTION OF EQUIPMENT

Smoke Generation

The SEM II was designed and manufactured by Process and Instruments Corporation, Brooklyn, N.Y., under contract to The Council for Tobacco Research - U.S.A., Inc. (Figure 1). The SEM II is an automatic smoking machine which generates a continuous stream of smoke and delivers it either whole or diluted for inhalation bioassay. Positive puff or reverse smoking was used in this design because it delivers the smoke aerosol without a vacuum generating device on the smoke line. Most reverse smoking systems use a movable puff chamber that seals over the cigarette and forces air through the cigarette generating the puff. A subsequent design (SEM III) has been developed using a negative puff smoking system (Process and Instruments Corporation, Brooklyn, N.Y.). In the SEM II, the entire smoking mechanism is located under a sealed clear plastic dome (Figure 2). The dome is pressurized and puff air is pushed through the cigarette by positioning the butt of the cigarette in front of a slider block where the smoke is diluted and sent to the smoke delivery system (Figure 3). The dome seals against the base plate of the machine and is hinged at the back so that it can be opened for access to the components inside.

The entire smoking sequence is automatic after loading a hopper with cigarettes, sealing the dome, and activating the control system. Cigarettes are taken from the hopper by a pneumatic loader and injected into cigarette holders fixed in a rotating drum (see Figure 2). The drum rotates stepwise and carries the cigarette to a puffing position at the slider block. The slider block seals against the inner surface of the drum, removing the butt of the cigarette from the dome atmosphere and positioning it in the smoke delivery system at nearly atmospheric pressure (see Figure 3). Dome pressure then forces air through the cigarette generating the puff. Puff volume is set by adjusting the dome pressure.

At the puffing position, the lighter, a high-intensity infrared lamp focused at the tip of the cigarette, is automatically actuated during the lighting step. This lamp may be manually operated to light or relight single cigarettes.

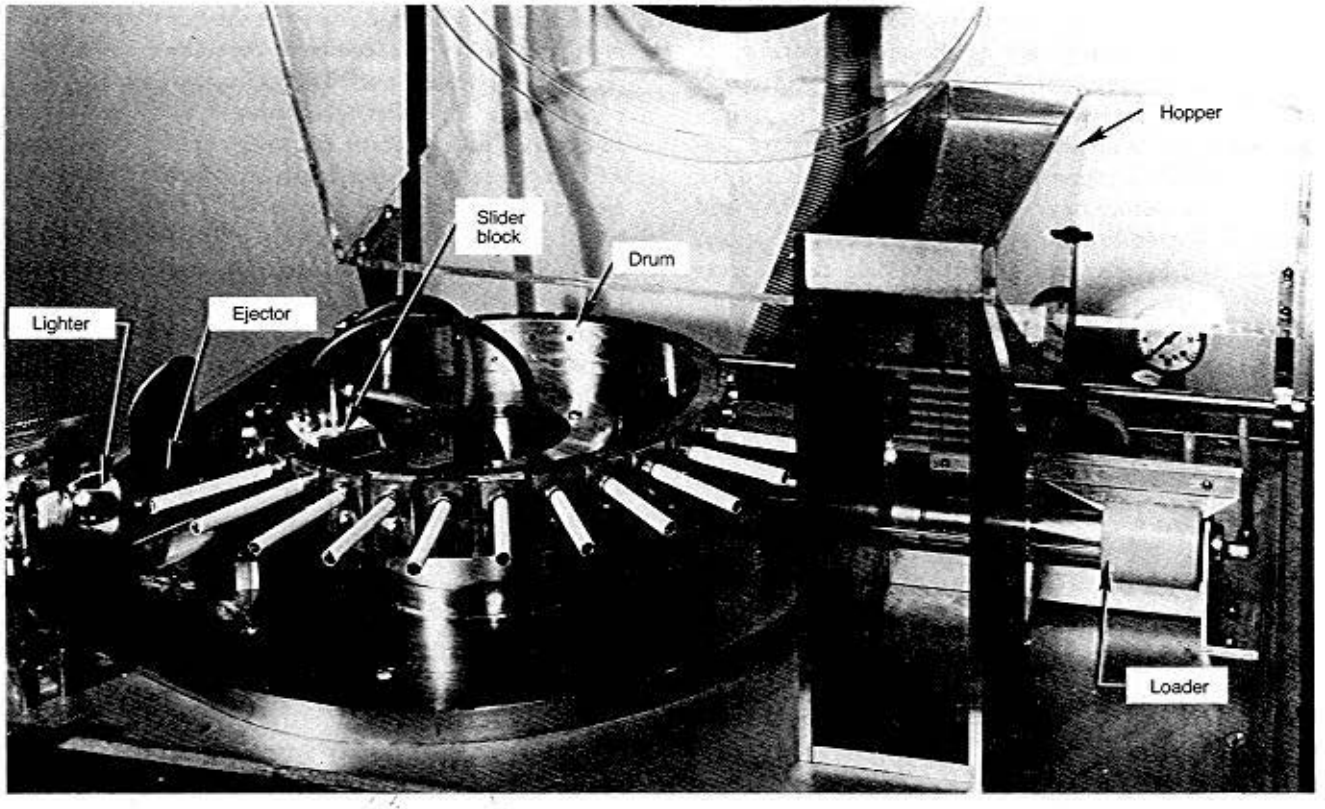
The drum rotates stepwise during the first 0.5 second of each step and is stationary for the remaining 1.5 seconds and makes one complete revolution per minute. As 30 cigarettes are puffed sequentially, each step is of two seconds' duration, generating a two-second puff and puffing each cigarette once per minute.

After a pre-set number of puffs are taken, an automatic eject system is actuated. The ejector, pneumatically operated and consisting of spring-loaded metal fingers, moves forward, grasps the cigarette butt, extracts the butt from the holder and drops it into a butt receptacle (chute). To prevent smoldering of butts that could add smoke to subsequently smoked cigarettes, a stream of CO_2 is released into the butt chute during the ejection cycle to extinguish the butts.

The machine can be operated in either a single cycle or a recycle mode. In the single cycle mode, only one batch of 30 cigarettes is smoked. At the end of the smoking cycle, each cigarette is sequentially ejected and

Figure 1.
The smoke exposure machine (SEM) series II.

Figure 2.
The smoking mechanism is housed under a sealed plastic dome. The dome is hinged and is shown here in the open position.



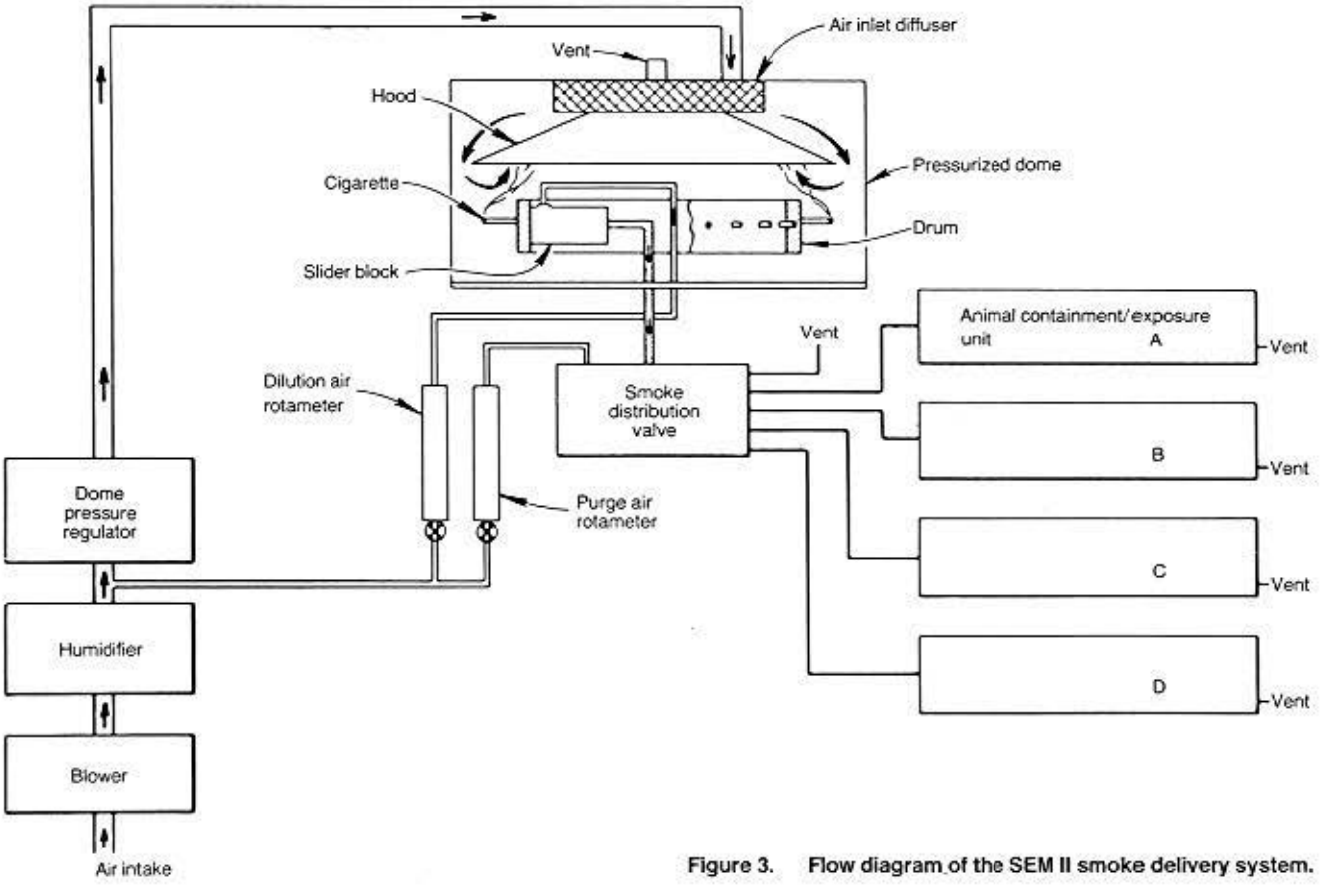
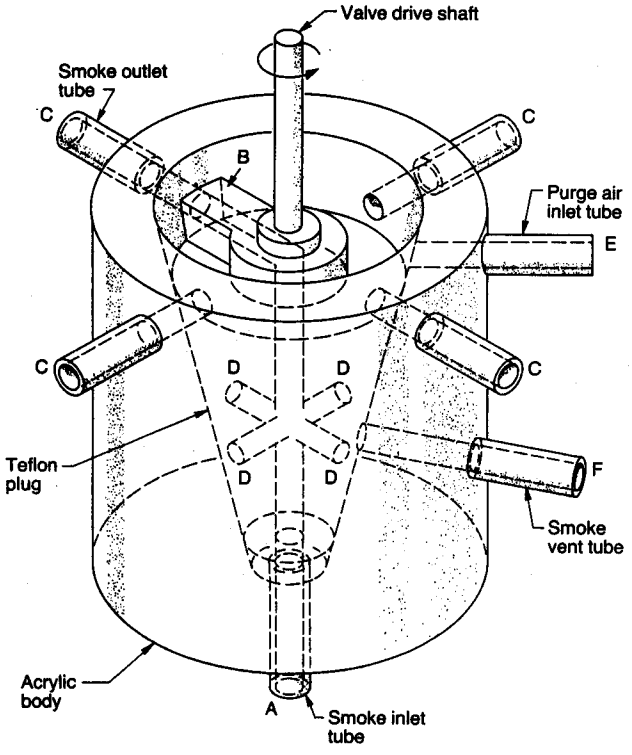


Figure 3. Flow diagram of the SEM II smoke delivery system.

Figure 4.
The smoke distribution valve (shown with top cover off). The inner cone is rotated in increments by a solenoid-driven stepping motor. Smoke enters this cone at A and exits at B through one of 4 smoke delivery tubes (C). Four smoke outlet tubes are connected to the four animal exposure units, but smoke is delivered to only one exposure unit at a time. Air entering the upper chamber (E) provides breathing air to the other three exposure units. The distribution valve may be positioned so that the exits from the delivery tube (D) are aligned with the smoke vent tube (F). Exit B will then be sealed and all the smoke will be vented. The distribution valve is programmable through a drum programmer. Any desired exposure sequence using all four smoke outlets and the vent may be programmed.



the machine is automatically turned off. In the recycle mode, instead of stopping after one run, cigarettes are automatically loaded onto the drum for a second run as cigarettes of the first run are being ejected. In this manner, the machine will continuously produce smoke for as long as desired or until all of the cigarettes in the hopper are used.

The air flow scheme for the SEM II has one blower system, which uses room air. Air is provided for dome pressure, dilution air, and purge or breathing air (see Figure 3). The air passes through a humidification system and is then split between a dome pressure regulator and the purge and dilution flow meters. Dilution and purge air are regulated by needle valve adjustments. The dome pressure regulator is adjustable and actually maintains a differential pressure across the cigarettes, as measured between the dome atmosphere and the slider block. Air from the pressure regulator then enters the dome area and is diffused through an air filter (see Figure 3). Very little of the dome air is consumed by puff generation. The bulk of the air moves smoothly around the edges of the hood and efficiently carries the sidestream smoke out of the smoking area through the vent in the top of the dome (see Figure 3). Puff air passes through the cigarettes and the smoke aerosol enters the slider block where dilution air is then added. The diluted smoke passes to the smoke distribution valve (see Figure 4 for details) that directs the smoke to one of four animal exposure units or to the vent. The distribution system is programmable to establish exposures of various duration within the one-minute smoking cycle. If an animal exposure channel is not receiving smoke, it will receive breathing air.

Operating experience with the SEM II has shown that the smoke generated is quite similar to smoke produced by laboratory analytical smoking machines in particle size (10), gas composition, and in nicotine and TPM concentration (7).

Smoke Monitoring / Documentation / Safety System

The purpose of the smoke and flow monitoring and safety system is to deliver the smoke and purge air from the SEM II, to provide auxiliary breathing air to the test animal modules, to protect the animals from possible operator error or malfunctions of equipment, and to provide documentation of exposure levels. The instrumentation has also been designed to protect animals from over-exposure to abnormally high doses of smoke particulates and carbon monoxide, as well as to protect them from suffocation due to an inadequate volume of breathing air. Accurate measurements and recordings of the exposure doses of TPM have also been provided.

The output from the distribution valve of the SEM II consists of four separate smoke channels. Insight into how these channels are routed and utilized is vital to an overall understanding of the monitoring and safety system. In the exposure system each of these four channels is fed through a monitoring and safety network and into the animal exposure room where each channel is split into two parallel streams, one to the upper and one to the lower sections of the animal containment modules. As can be seen in Figure 5, the modules can be operated in tandem. Each module holds 30 mice in its upper section and 30 mice in its lower section. Mice are loaded onto each side of a module. One smoke channel can provide smoke or air for two modules containing 120 mice, and the four channels of a SEM II can provide exposure for 480 mice. The hinged modules, to be described in detail in the following section, contain the mice in a nose-only configuration such that the animals must breathe the air or smoke in the module channel when the module is closed. It is imperative therefore that a smoke/air mixture or fresh air alone be forced through the closed module at all times. The latter is provided by an auxiliary air supply independent of the SEM II (see Figure 5). The final safety devices in the smoke delivery system are the flow sensors, de-

Figure 5.
Piping diagram of one of the four-channel exposure systems. Unit is shown in normal operating mode with SEM smoke/air going to modules.

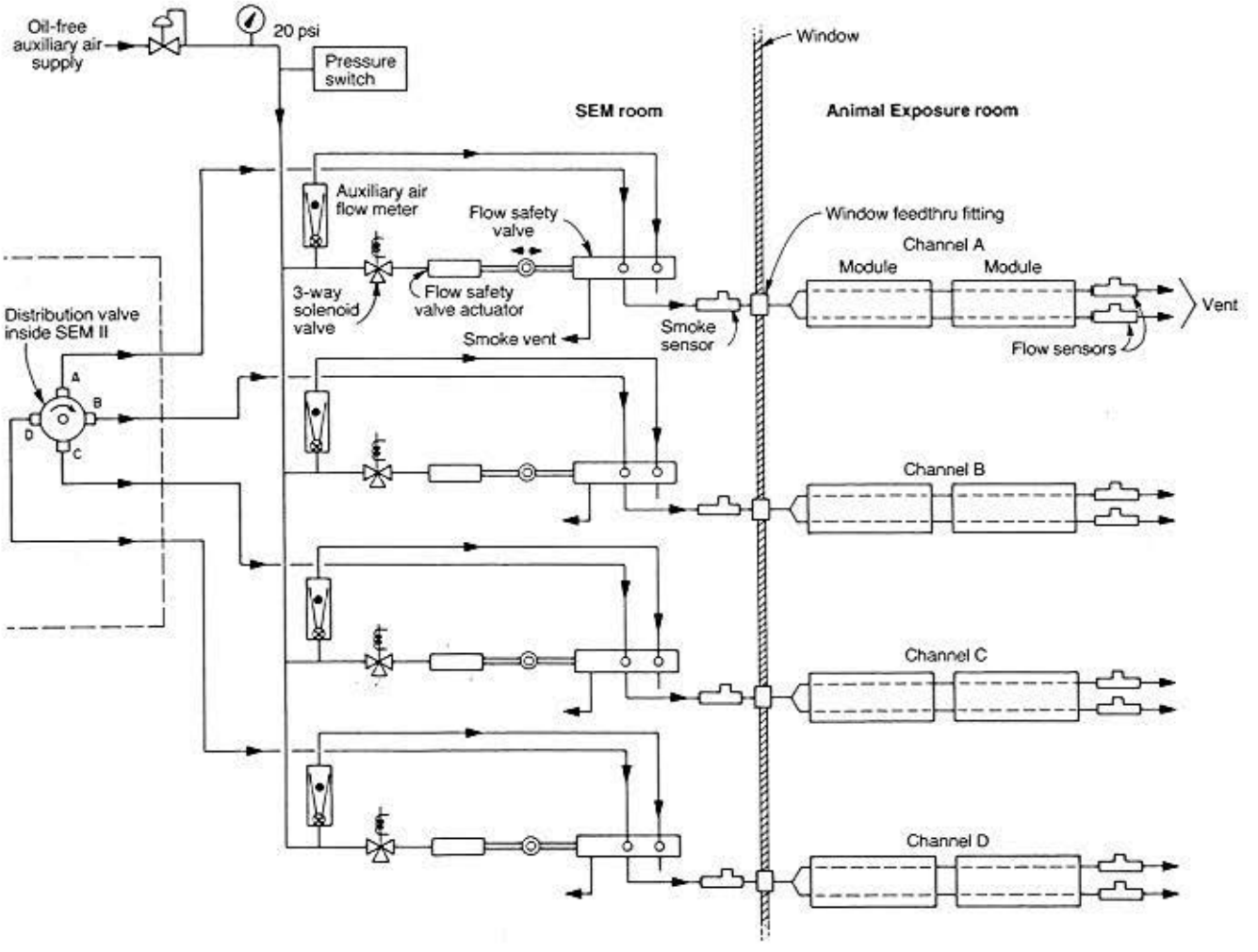


Figure 6.
A typical module flow sensor ready for use.

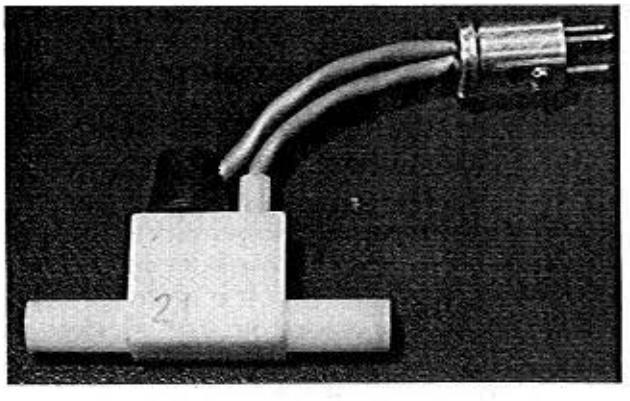
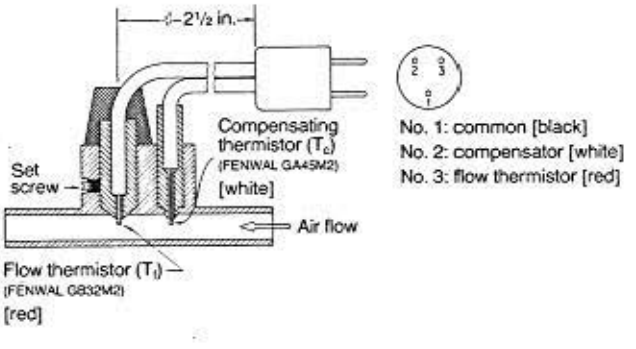


Figure 7.
A cutaway drawing of the flow sensor showing internal constructions.



signed to automatically open the modules to room air if necessary (see following section and Figures 6 and 7). A solid state sonic alarm device (Sonalert (Figure 8)) provides an audible alarm should the flow thermistor sensing elements malfunction.

Figure 8 is a photograph of the smoke monitoring recorder bank console. Contained in this console are eight monitoring and recording units sufficient to monitor the smoke generation and delivery from two SEM II systems. This console is located in the smoke generation room, but projects (inside a metal housing) into the animal exposure room. The recorders and console drawers may be opened into the smoke generation room for service.

Figure 9 is a view of one of the two auxiliary air safety system racks with associated components for four channels. A module rack in the animal exposure room can be seen through the window. The major components in the smoke and flow monitoring and safety system are briefly described as follows:

a. a smoke particulate sensor in each channel to measure continuously the smoke concentration (Figures 10 and 11),

b. an amplifier-integrator network to condition and transform the sensor response into a high-level electrical signal linear with instantaneous smoke concentration and also to provide a signal proportional to the integral of smoke concentration,

c. a three-pen strip chart recorder for each channel to record the instantaneous TPM concentration and the integrated values for the TPM with respect to time; the third recorder pen is provided as a spare (Figure 12),

d. an alarm (on each channel) that actuates if the instantaneous smoke concentration exceeds a pre-set level,

e. a time-sequenced alarm to actuate the flow safety valve if duration of smoke in the smoke/air cycle exceeds a pre-set limit,

Figure 8.
Overall view of smoke monitoring console.

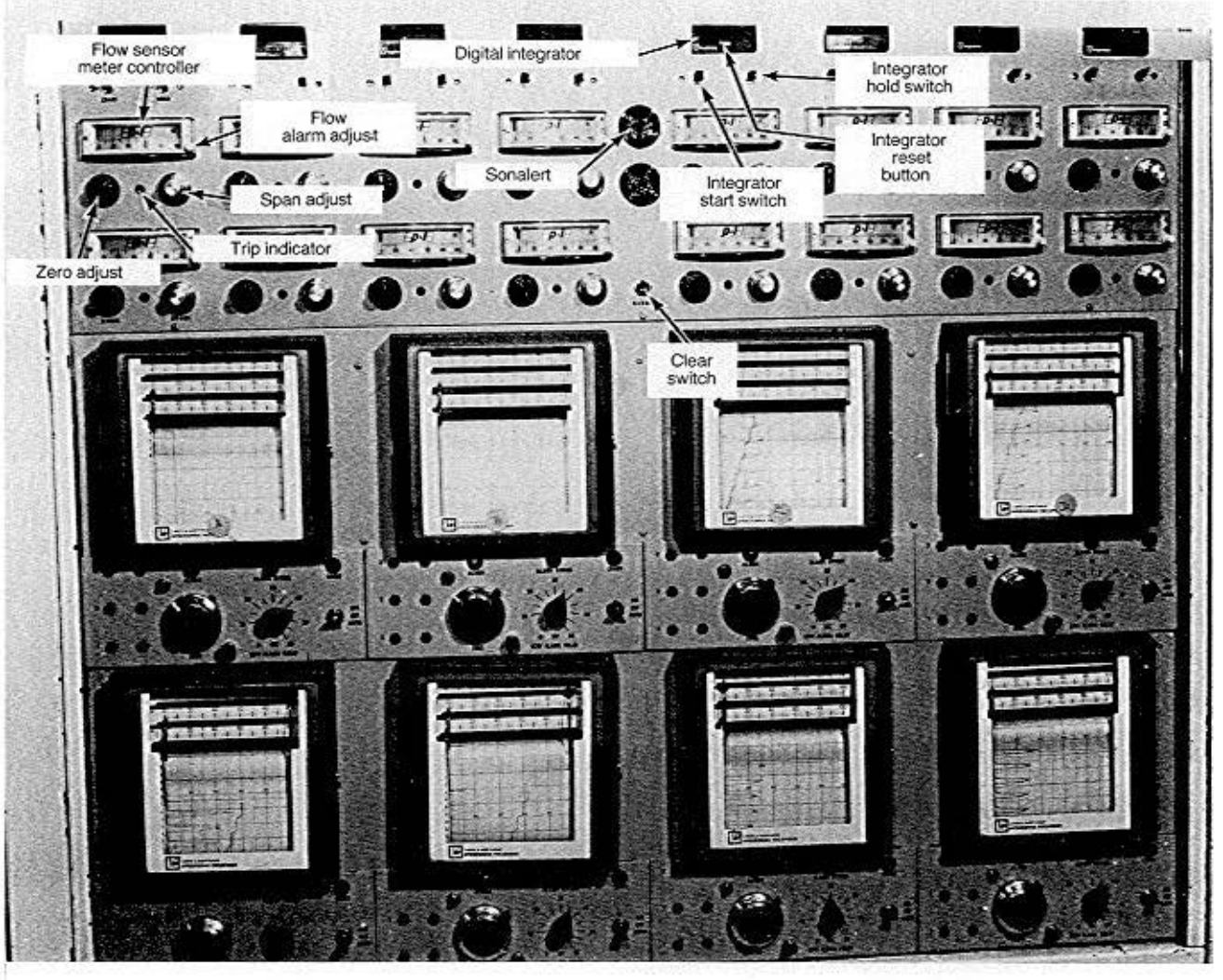


Figure 9. One of the two auxiliary air safety system racks.

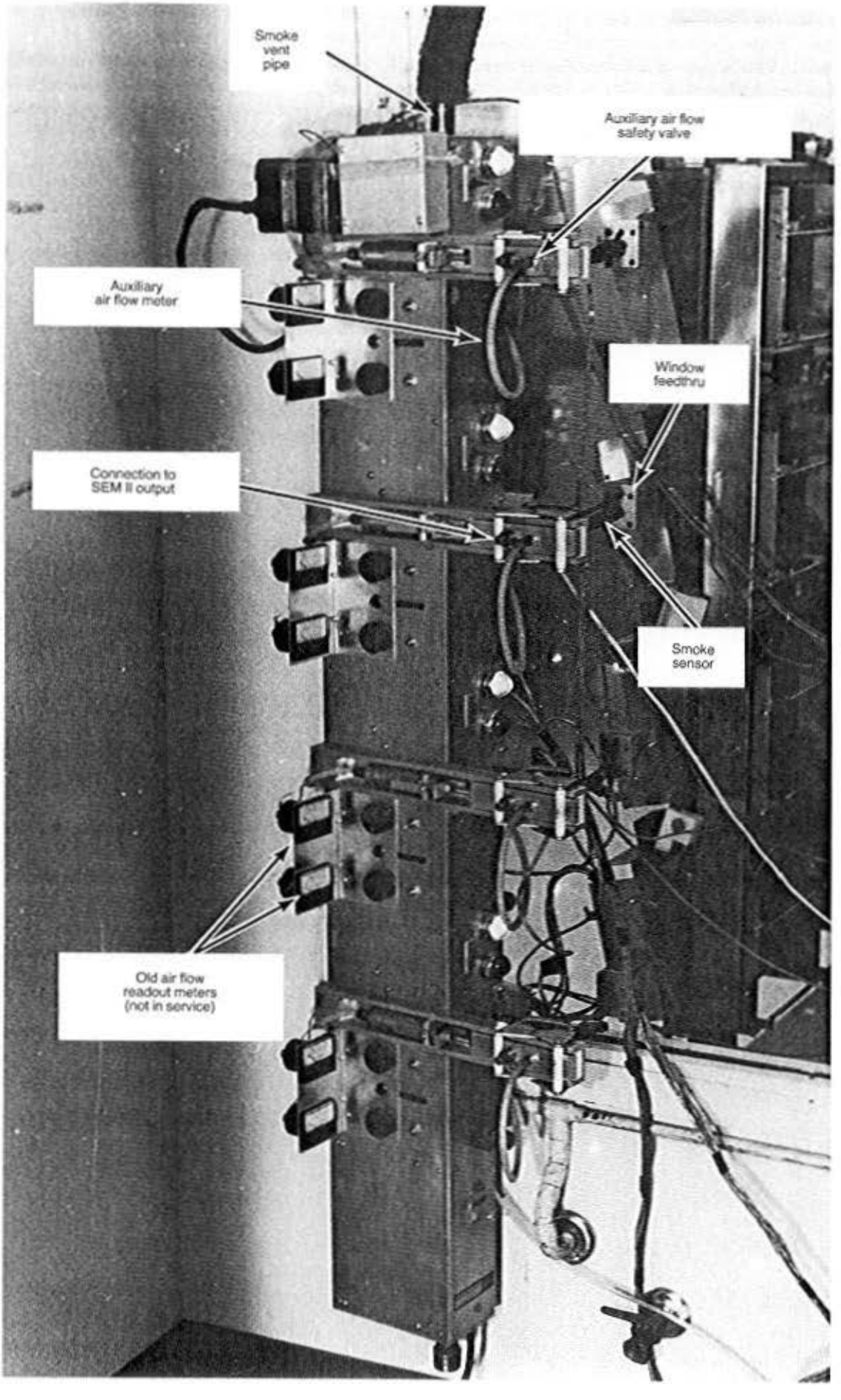


Figure 10.
The smoke sensor consists of the light emitting diode - phototransistor combination mounted flush along the inside wall of a rigid plastic tube through which smoke passes.

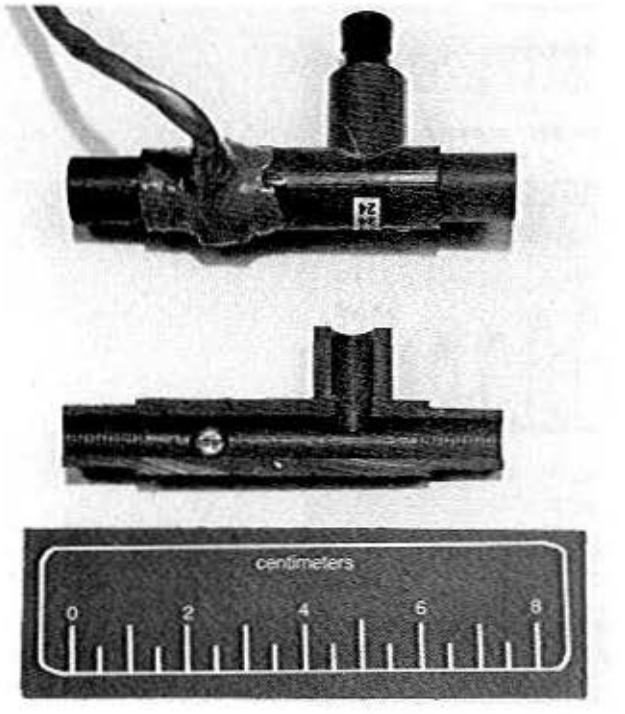
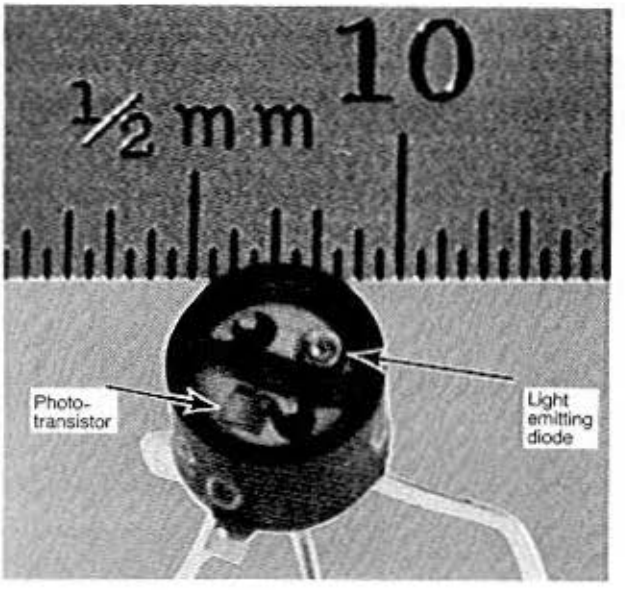


Figure 11.
The light emitting diode - phototransistor unit.



f. an auxiliary air flow safety valve actuated by the smoke alarms and by cessation of smoke or purge air flow (see Figure 9),

g. a flow sensor located at the outlet of the exposure system on the exit of the two streams of each channel (see Figures 6 and 7); when smoke or air flow falls below a pre-set value, the flow sensor causes the flow safety valve to switch automatically to auxiliary air,

h. a time-delay circuit to actuate a quick release mechanism which opens the exposure modules to room air if auxiliary air does not flow through the system within 8 seconds after the flow safety valve has switched to auxiliary air, and

i. an air pressure switch in the auxiliary air line will cause the exposure modules to open to room air if auxiliary air pressure falls below pre-set level.

Animal Containment System

The primary components of the nose-only animal containment system are presented in Figures 13 to 20. A description of the important aspects of this system follows.

Mouse Trays: The mouse trays restrain a group of five mice by confining their necks within slots just wide enough to hold the neck but narrower than the head or body of the animal. Mice are held in these stock-like holders using a combination of the neck slot, restraining spring, head clip, and chin rest (Figure 13). Each tray has 5 slots, 11/32 inches wide to comfortably hold average size inbred mice. The design will accommodate a reasonable variation in neck size. Special trays with adjustable slots are available.

A tray with mice ready for loading onto an exposure module is shown in Figure 14. As each mouse is placed onto the tray, the neck spring is lowered onto the top of the neck and secured by the neck spring catch. A head clip attached to each spring helps immobilize the head. The chin rest at the bottom of each neck slot acts as an additional restraint and insures that the nose of the animal is aligned with the cone-shaped opening on the exposure module. A removable stainless steel screen platform fits within the recessed bottom of the tray to support the mice. Excrement from the mice passes through the screen and is contained within the tray recess (see Figure 13).

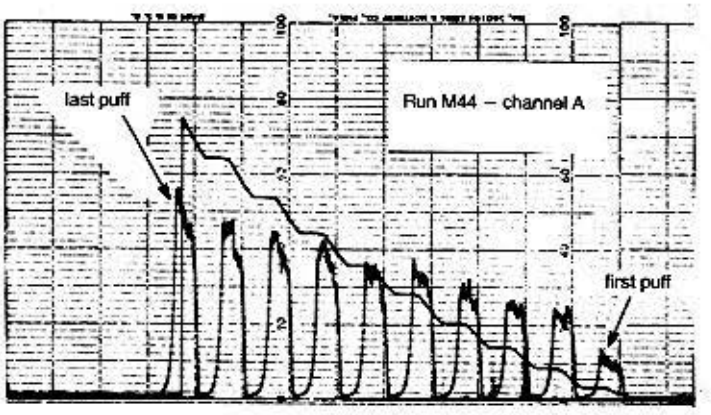
The tray supports, shown in Figure 13, mount on the module and the mouse trays fit on the tray supports. Raised back edges (tray stops) on the ends of the arms of the tray supports hold the trays in place.

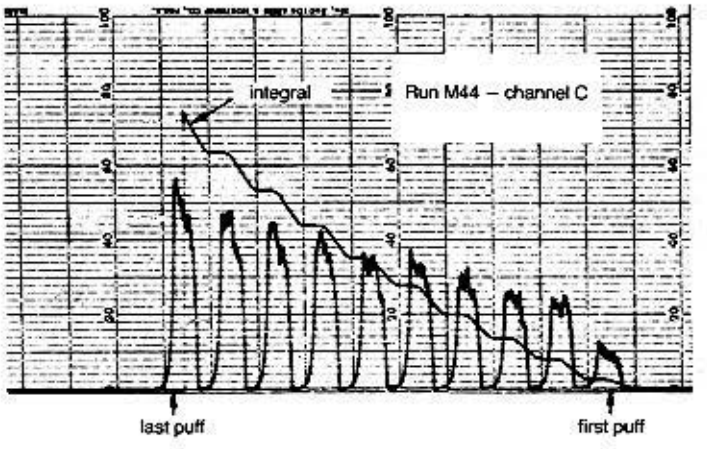
Exposure Modules: The exposure modules accommodate 60 mice and are designed so that only the nose of each mouse protrudes into the smoke/air channel. Figure 15 is a photograph of an assembled module in the opened position with one mouse tray installed.

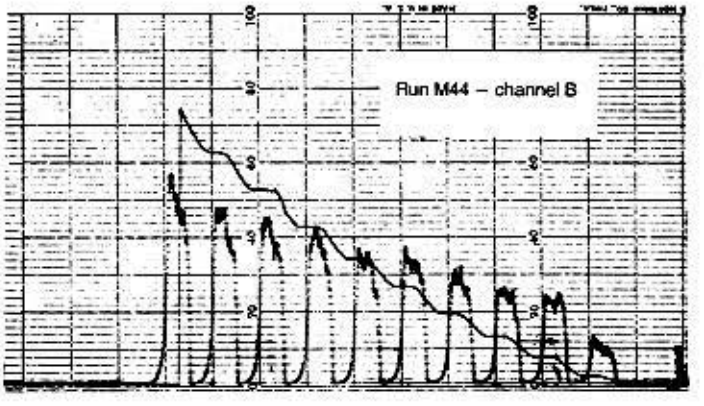
Smoke enters the module through the two smoke tubes shown (Figure 15), flows through the smoke passageway or channel (upper and lower) and exits through the smoke tubes on the opposite end. The mice to be exposed are restrained in the stock-like holders that fit on

Figure 12.

Total particulate matter (TPM) profiles from all four channels of the SEM II. These profiles are graphical readouts of the electrical signal from the optical sensor (Figures 10 and 11) displayed as a function of time. Each peak is the response of the sensor to smoke from one puff. Puffs were taken at 1-minute intervals with an exposure time of 30 seconds.







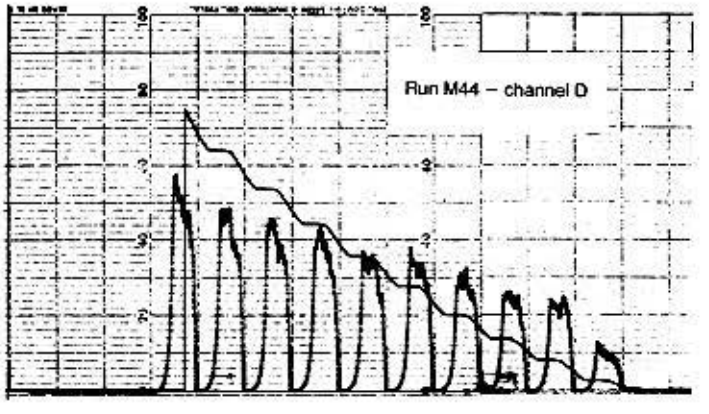


Figure 13. Mouse tray and tray support.

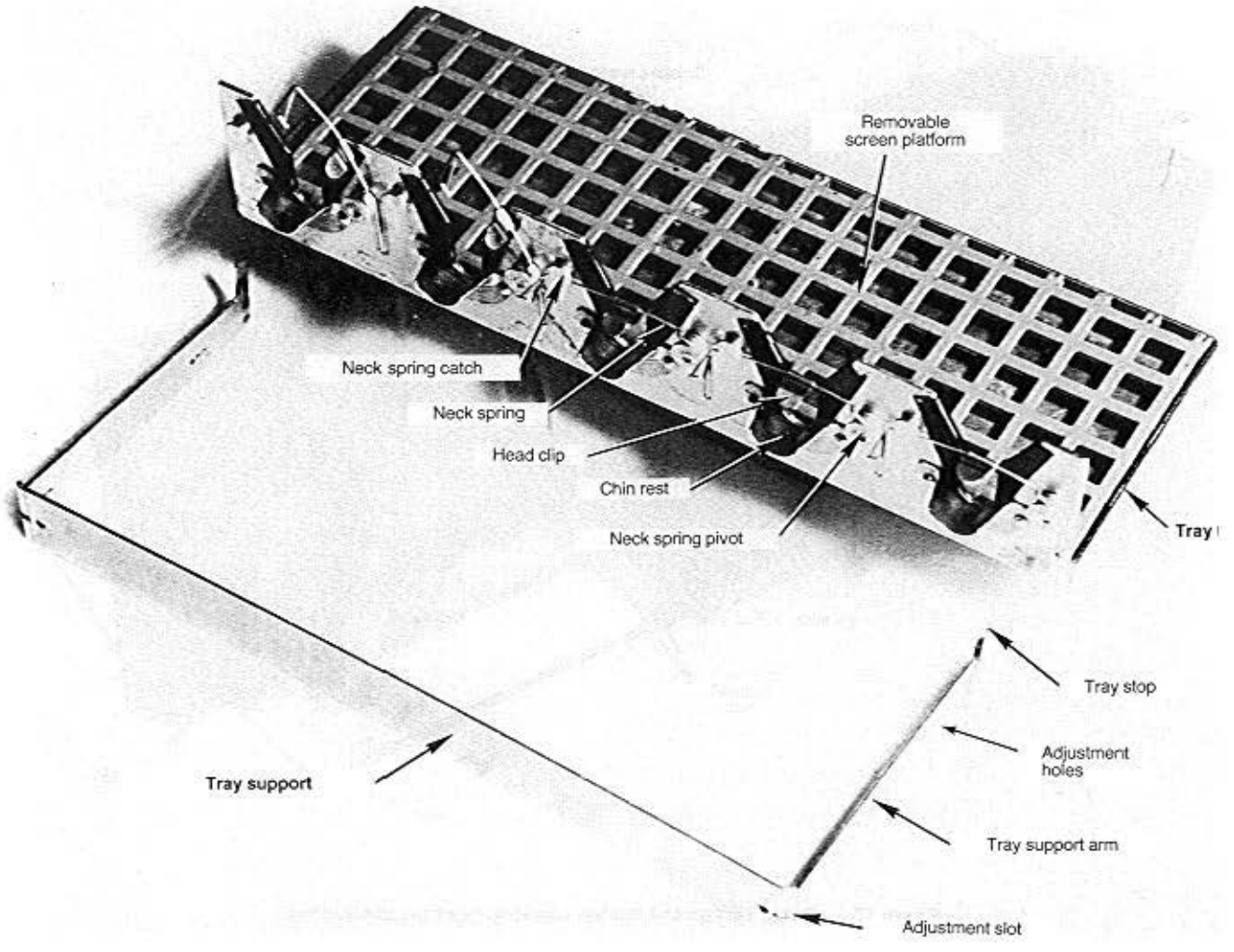


Figure 14. A loaded mouse tray ready for installation on module.

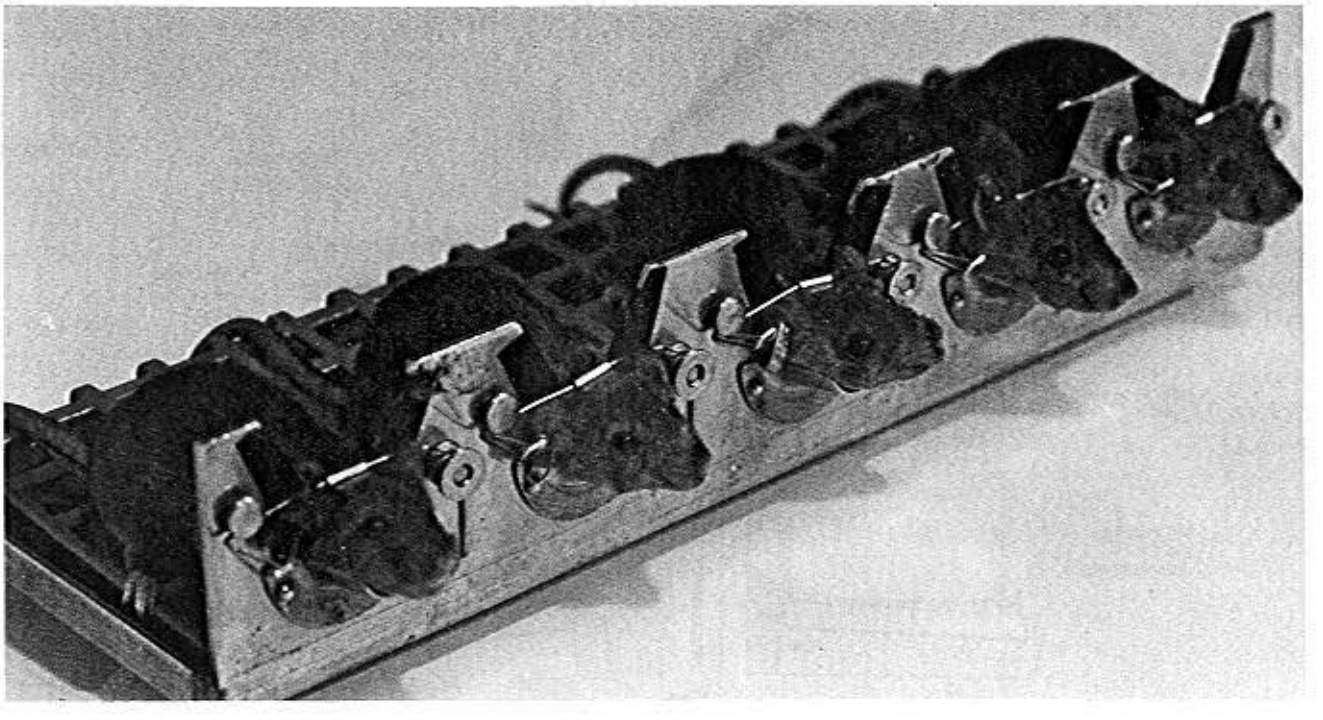


Figure 15. An opened module with one tray installed.

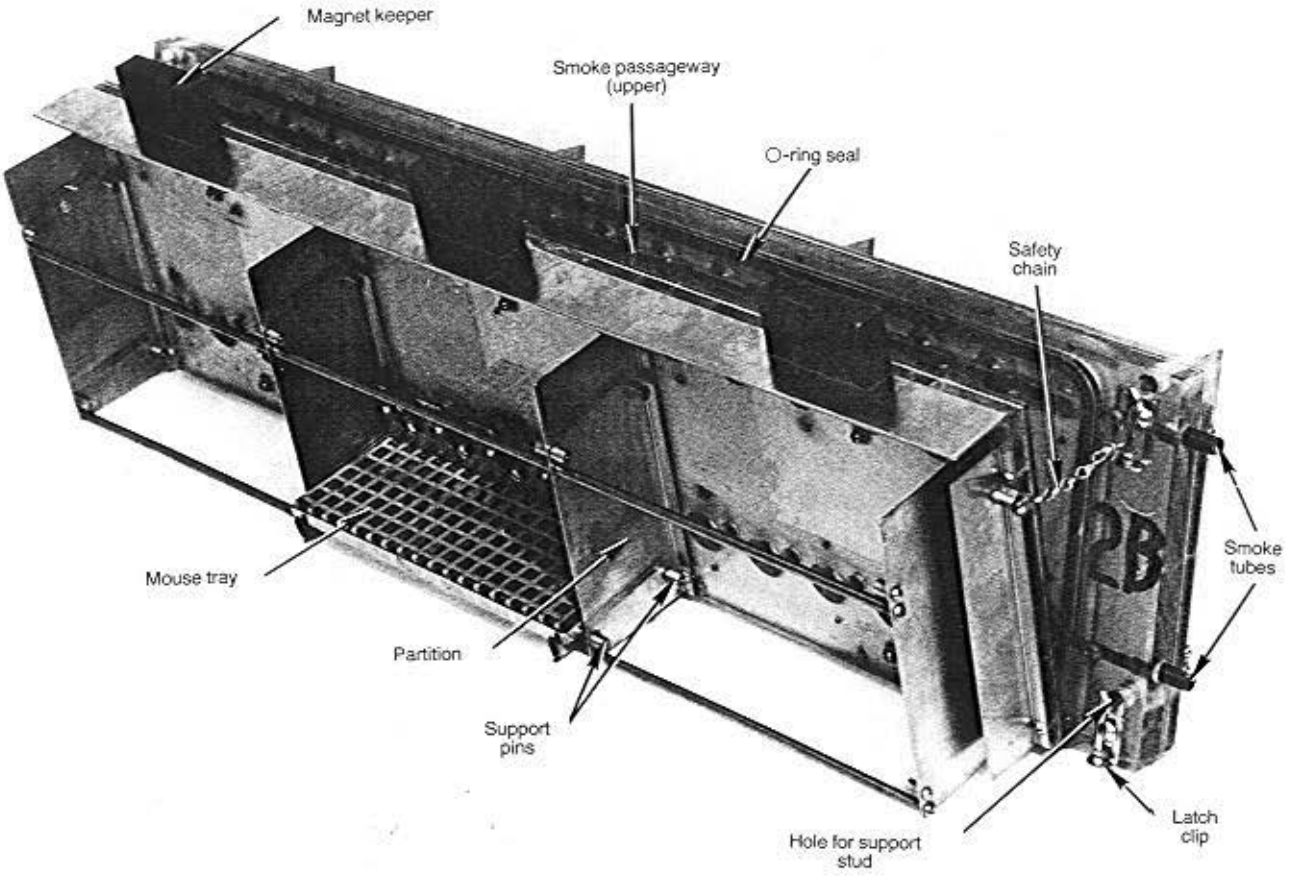


Figure 17. Cross section of a module showing internal construction.

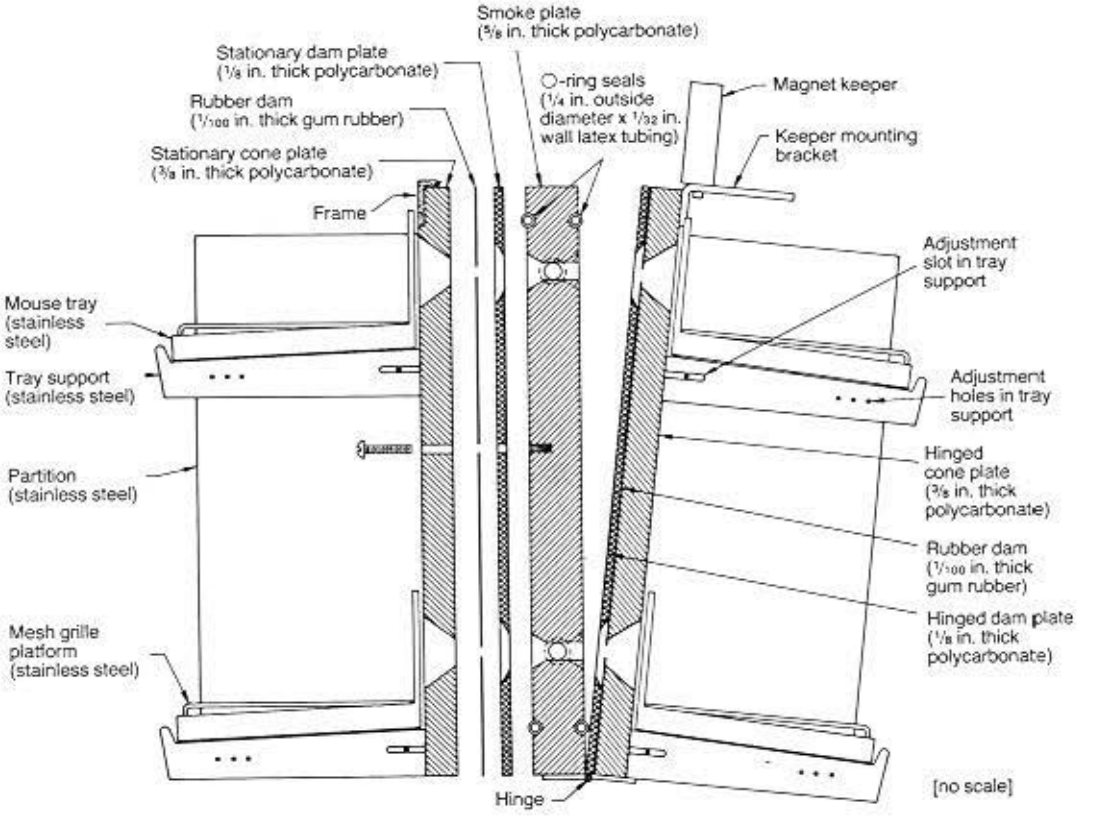
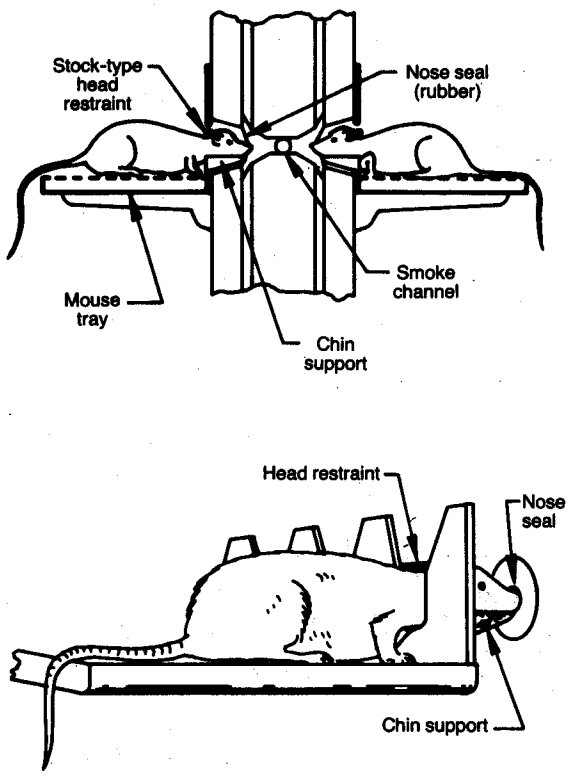


Figure 16.
A schematic of the nose-only exposure technique illustrating the module and mouse tray.



the opposing sides of the module in two tiers of 15 mice per tier per side for a total of 60 mice. The tiers align with the passageways and as the mice in trays are loaded onto the module, the noses of the animals protrude through a dental rubber dam diaphragm forming a seal that prevents exposure of the body to the smoke aerosol (see Figure 16 for a schematic of the holding system). Figure 17 is a transverse, cross-sectional drawing of a module and Figure 18 shows an end-on view of a module mounted on an exposure rack. One entire side of the module is hinged at the bottom along its length so that it can be swung open to expose the passageways or channels to room air. In this manner, the mice breathe room air whenever the module is open. The O-ring seal prevents leakage of air or smoke from the module when it is closed. Electromagnets, composed of a magnet and a magnet keeper, hold the modules closed (Figure 18). The magnets are powered by a direct current source and release automatically causing the modules to open if the auxiliary air line pressure drops below 5 pounds per square inch or if one of the two flow sensors in a channel detects a pre-set low-flow rate of either smoke or air.

Exposure Rack: The exposure rack holds all of the components that make up the animal containment system. It consists of a base with 3 vertical supports to which are attached 4 horizontal cross supports of different levels. Each level or channel holds 2 modules for a total of 8. Figure 19 is a view of a rack with modules mounted and components labeled. Figure 20 shows a rack assembled and loaded with animals during an actual exposure.

DISCUSSION

The facilities and equipment described here were designed for long-term, standardized nose-only inhalation exposure of large numbers of mice. The system has been used on a daily basis for over 4 years with minimal maintenance and very good day-to-day reproducibility. Hard copies of data from the smoke monitoring and safety systems are available from every exposure session.

The animal exposure modules provide for smoke exposure only via the nose. Contamination of the animals with urine and feces during exposure and temperature buildup that occur in whole body tubes are avoided with this system. These modules are compatible for use with other aerosol generation equipment and thus can be used to effect nose-only exposure of a variety of material other than cigarette smoke. Recent studies using aerosolized catechol have been presented elsewhere (11) as well as studies using biological materials (12).

Previous studies from this laboratory have shown that the deposition of smoke particulates was primarily in the respiratory tract of mice exposed to whole smoke from the SEM II machine (8, 13). Smoke conditions can be varied with this system so that deposition between 30 μg TPM / lung to 200 μg TPM / lung can be obtained. Greater than 80% of the TPM deposition was found in the total respiratory tract and the mouse-to-mouse variation along the length of the exposure module was less than 20%. Moreover, the mice did not learn to avoid the smoke, because deposition was similar after 3 weeks, 3 months or 6 months of smoke exposure. These data are in direct contrast to those from exposures utilizing inhalation exposure chambers where only 20—40% of particulate material was found in the respiratory tract and 60—80% was found in the gastrointestinal tract after exposure (14). The cost of performing an inhalation study with this nose-only system is comparable to a chamber exposure study. However, if the test material is available in small amounts, is relatively expensive, or is to be tested under conditions where most of the aerosol is deposited in the lungs (i.e. not gastrointestinal tract) of the test animals, then the nose-only system described here has obvious advantages. Our laboratories have recently developed a stock-like holder for rats to provide nose-only exposure. Thus, inhalation studies using both mice and rats may now be performed using the equipment described in this paper.

Figure 18.
End view of a rack with an opened module in place.

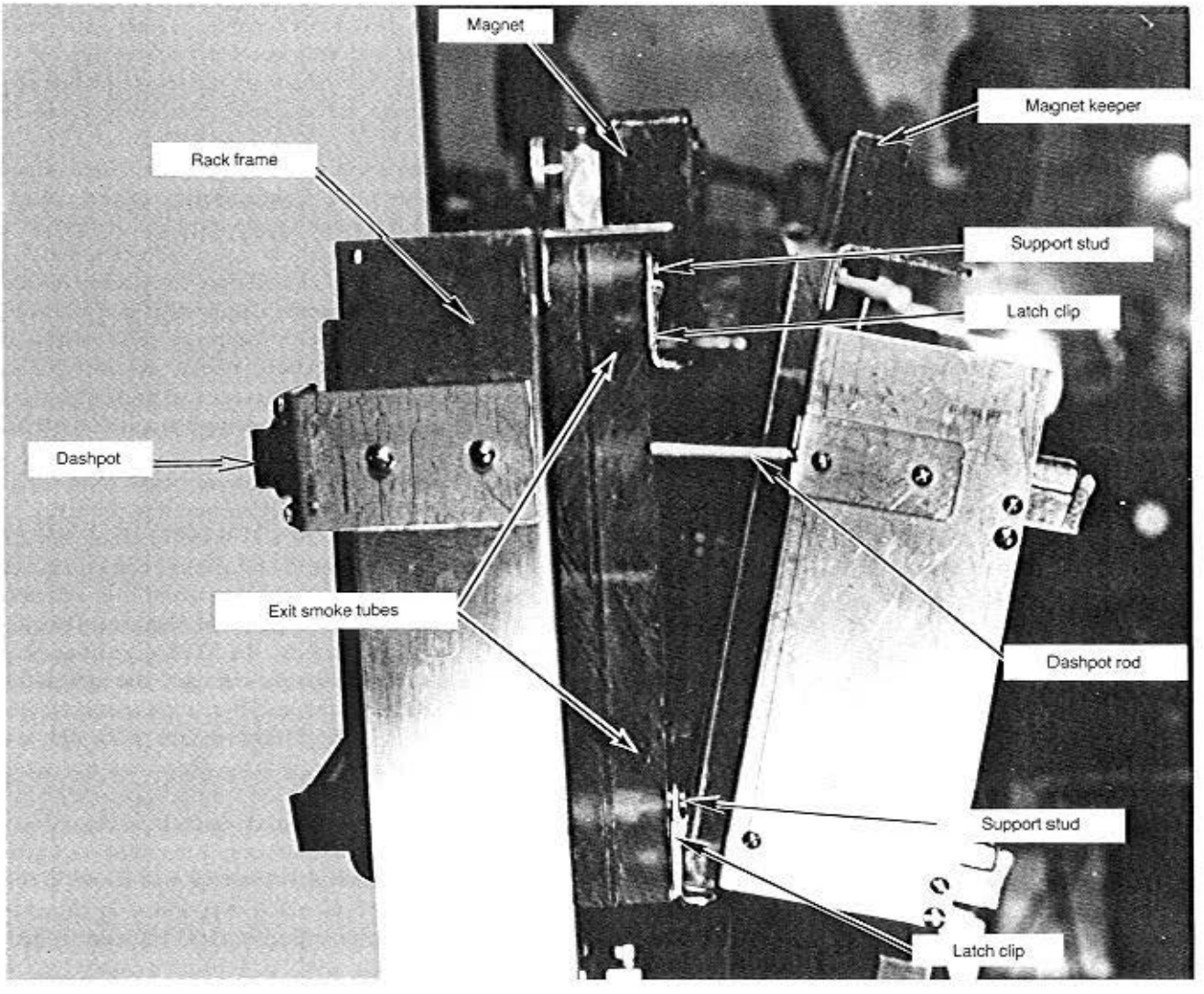


Figure 19.
A rack assembly with modules in place.

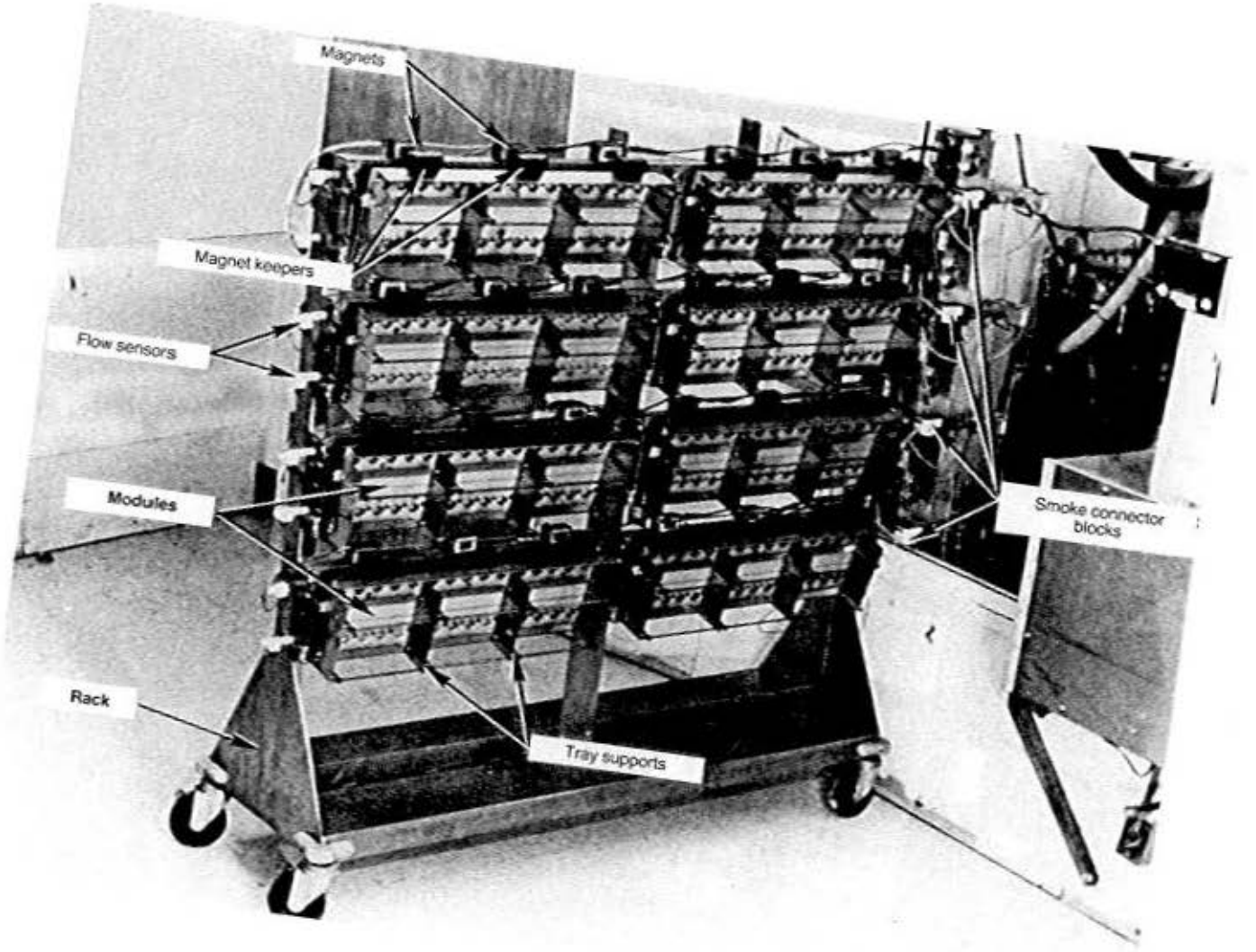
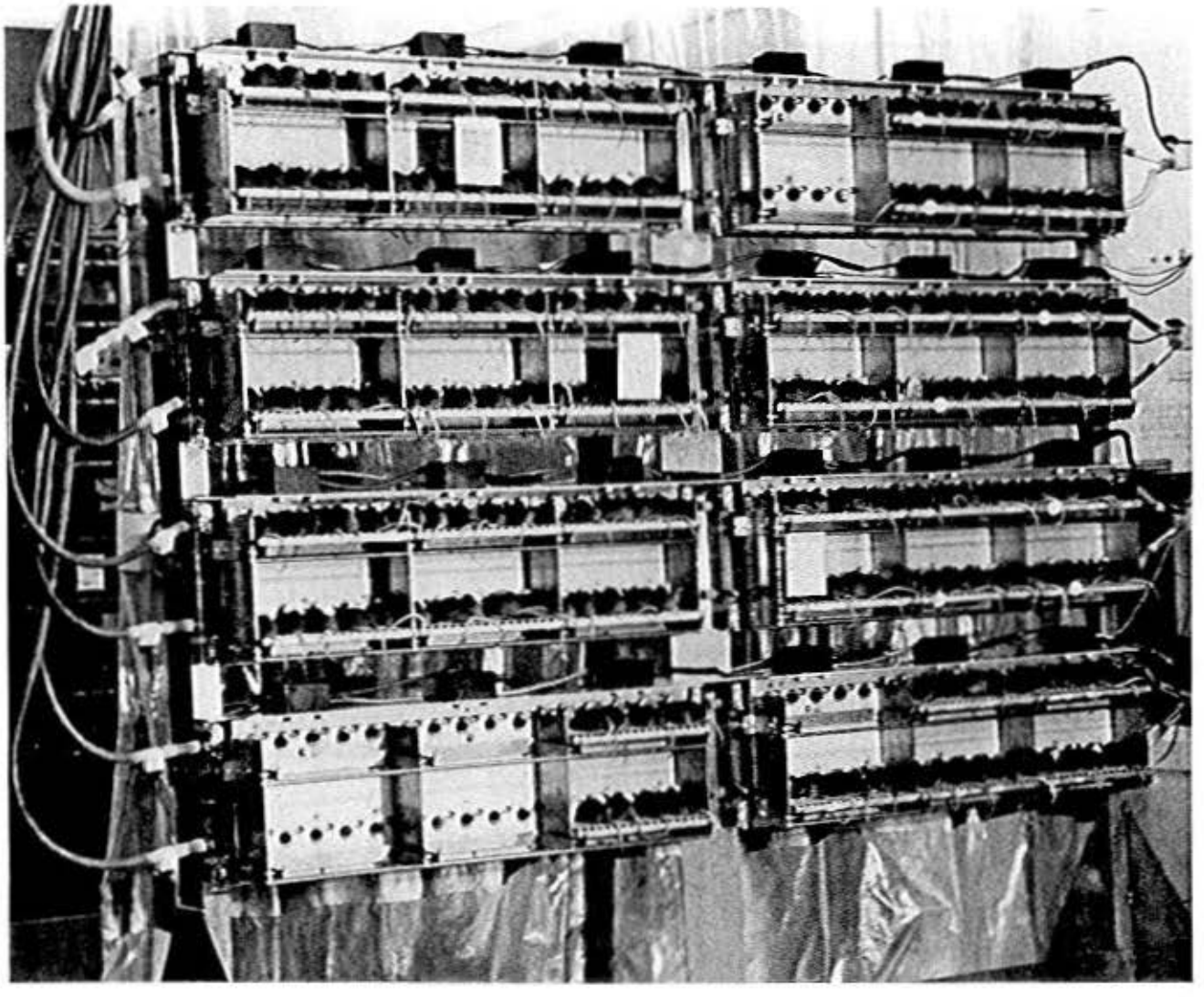


Figure 20.
The assembled system during an actual exposure.



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Acknowledgement

We thank Mrs. Patricia Harbin for assistance in preparation of the manuscript.

Authors' address:

Division of Toxicology and Molecular Biology,
Microbiological Associates,
5221 River Road,
Bethesda, Maryland, 20816, U.S.A.