PRO EXPERIMENTIS

The Assessment of Tobacco Smoke Toxicity in Tissue and Organ Culture: an in vitro Arrangement for Exposure under Defined Conditions

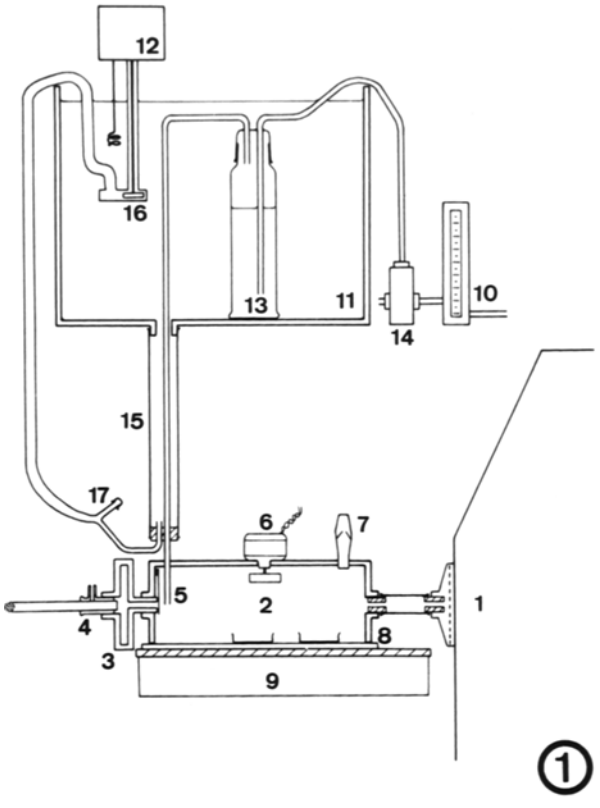
Although the toxicity of tobacco smoke for the living cell is generally accepted, its quantitative measurement in tissue or organ culture has been attempted by only a very few workers (see RYLANDER[1] for review). One of the reasons for such a lack of investigation in this field is, undoubtedly, the difficulty in establishing a technique which will overcome the variations inherent in the smoking of a cigarette, and which is valid over a long series of experiments.

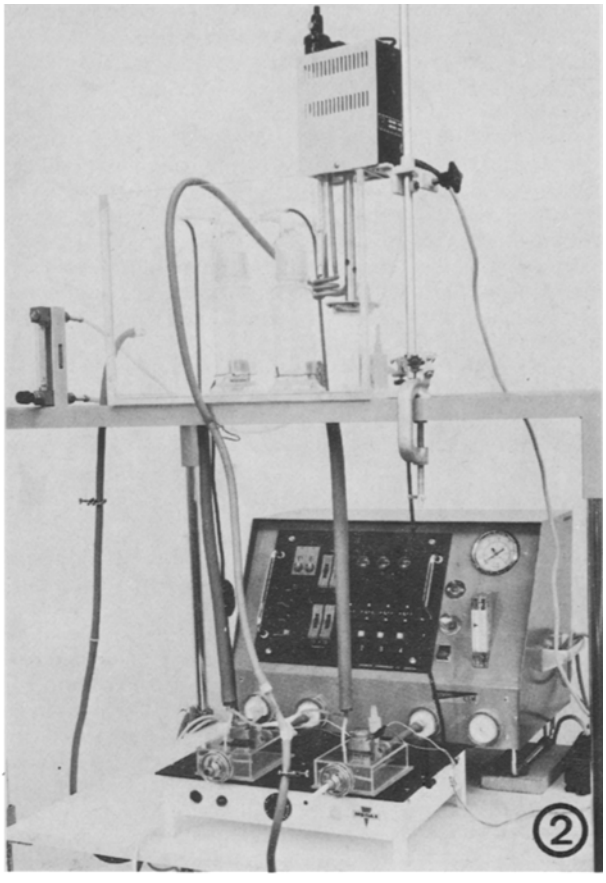
A variety of machines have been developed for exposing cell or organ cultures to fresh smoke. KENSLER and BATTISTA[2] constructed an apparatus for their studies on ciliary transport in tracheae excised from rabbits, and later in chicken tracheae[3]. A noteable feature of their machine is its careful maintenance of physiological temperature and humidity, and also the fact that the smoke can be retained within the exposure chamber for as long as desired before being flushed out by a stream of moist air. However, the ciliated epithelium is exposed to smoke and to moist air alternately, so that a thorough mixing of both together, as occurs in the human smoker, is not acheived. The LEUCHTENBERGERS have worked extensively with an apparatus which has enabled them to study the effects of tobacco smoke, and the smoke from marijuana cigarettes, on human and mouse lung explants. At first, they used a smoking machine of their own manufacture[4], but later incorporated a commercially produced automatic smoking machine of a similar type to the one used in the apparatus forming the subject of this paper[5-8]. Their machine, however, has no control of temperature or humidity, nor is there any mixing of mainstream smoke with air.

We describe here an apparatus which attempts to parallel more closely the conditions prevailing in the bronchial tree of the human smoker by providing for: 1. the maintenance of temperature and humidity; 2. the mixing of fresh cigarette smoke and air in controlled proportions; 3. facility and speed in introducing material into the machine and also in its removal after exposure.

Apparatus and operation (see Figures 1, 2 and 3). As a basis, the system utilises the CSM Mark II smoking machine (Cigarette Components Ltd., London) which has inputs for 4 channels. In present experimental practice, however, only the 2 central channels are used, thus allowing for test and control material. The machine is set for the standard *Coresta* cycle, viz. a 35 ml. intake of cigarette smoke over 2 sec, operating once every min. Connected with the machine inputs by vacuum-tight rubber tubing are 2 plexiglass chambers, each constructed to give an internal volume of 350 ml. The opening directed away from the smoking machine receives a plastic Cambridge filter holder which itself takes the cigarette holder (both are standard issue with the CSM machine). Separating the cigarette from the exposure chamber is a mechanical valve which operates at the appropriate time to let the smoke pass into the chamber, whilst for the rest of the time it remains closed. On the top of each chamber is a small opening allowing for the passage of a plastic tube which, in the intervals between puffs, distributes an air/5% CO_2 mixture at 37 °C and 100% relative humidity flowing at the rate of 350 ml per min. Also inserted into the top of each chamber is a small fan whose function is to ensure perfect mixing of the air/CO_2 and smoke during inhalation. Both fans can be regulated by a variable transformer. The dimensions of each chamber and the volume of each puff give an immediate 10 to 1 mixing of air/CO_2 and smoke respectively. This represents the ratio deduced from studies on the dilution of smoke by air in the trachea and bronchi of humans[9]. A one-way plastic valve is also fitted into the top of each chamber. It permits the air/CO_2-smoke mixture to pass out, but closes tight when the vacuum pump within the machine operates on that particular channel during the intake of a standard puff. Each chamber rests on a detachable glass plate a few millimetres thick, and a rubber seal ensures a vacuum-tight joint. The arrangement allows three or four 35 mm diameter Falcon® petri dishes to be placed inside. To match physiological conditions the chambers stand on a hot-plate, the temperature of which is set at 37 °C, and they are held in place by rubber rings which pass tightly over them and are secured by screw hooks set into the hot-plate itself. In addition, the incoming air/CO_2 mixture is also at a temperature of 37 °C and a relative humidity of 100%. This is achieved by passing the air/CO_2 through 2 glass wash bottles containing autoclaved distilled water, which are themselves placed in a tank of water kept hot by a thermostatically controlled heater. In practice, it was found convenient to place the tank directly above the exposure chambers. However, this produced a certain loss of heat in the air-flow, together with a condensation of its water content as it passed from the wash bottles to the chambers. Accordingly, the tubes containing the humid air/CO_2 mixture are surrounded by jackets through which hot water from the heating tank can be pumped. Little flexibility is lost by this arrangement and the tubes can be connected to the exposure chambers within a matter of sec. Two wash-bottles are necessary because otherwise the vacuum system of the machine would work against itself. In order to prevent loss of smoke volume due to gas being sucked from the main supply tank and associated tubing, a pair of electromagnetic valves are inserted into the system before the air/CO_2 is heated and humidified. Each valve is connected with the appropriate electrical channel in the smoking machine so that when either channel operates, the valve closes off the communication to the gas supply tank, and a full 35 ml puff of cigarette smoke (or air in the case of the control channel) is taken into the chamber. A certain heat loss still prevails along the tubing to the chambers so that water in the heating tank has to be kept at several degrees above 40 °C to ensure that the air/CO_2 reaching each chamber is at 37 °C. There is no difficulty in achieving this, however, and once the temperature has been reached it remains stable. To provide for a correct air/CO_2 flow of 350 ml/min through

[1] R. RYLANDER, Rev. Envir. Hlth. *1*, 55 (1972).
[2] C. J. KENSLER and S. P. BATTISTA, New Engl. J. Med. *269*, 1161 (1963).
[3] S. P. BATTISTA and C. J. KENSLER, Arch. Envir. Hlth. *20*, 318 (1970).
[4] C. LEUCHTENBERGER and R. LEUCHTENBERGER, Cancer Res. *29*, 862 (1969).
[5] C. LEUCHTENBERGER and R. LEUCHTENBERGER, Expl. Cell Res. *62*, 161 (1970).
[6] C. LEUCHTENBERGER and R. LEUCHTENBERGER, Nature, Lond. *234*, 227 (1971).
[7] C. LEUCHTENBERGER, R. LEUCHTENBERGER and A. SCHNEIDER, Nature, Lond. *241*, 137 (1973).
[8] C. LEUCHTENBERGER, R. LEUCHTENBERGER, U. RITTER and N. INUI, Nature, Lond. *242*, 403 (1973).
[9] T. DALHAMN and R. RYLANDER, Scand. J. resp. Dis. *50*, 273 (1969).





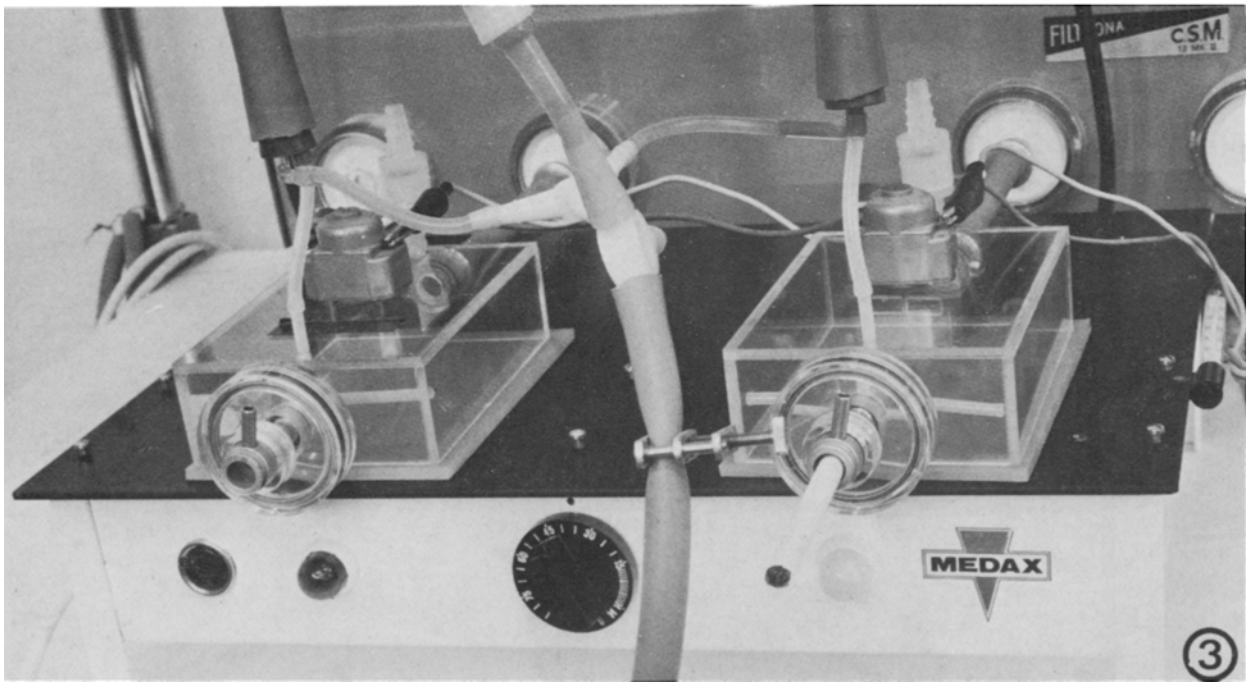


Fig. 1. Diagrammatic representation of the apparatus. 1. CSM 12 Mark II Smoking Machine. 2. Exposure chamber. 3. Cambridge filter holder. 4. Cigarette holder. 5. Humidified warm air/CO_2 inlet. 6. Electric fan. 7. Air/CO_2-smoke outlet valve. 8. Glass plate. 9. Hot plate. 10. Flow meter. 11. Warm water tank. 12. Thermostatically controlled heating element and pump. 13. Humidifying bottle. 14. Solenoid valve. 15. Heating jacket. 16. Warm water inlet to heating jacket. 17. Draining pipe.

Fig. 2. General view of the complete apparatus.

Fig. 3. Close-up view of the exposure chambers and hot-plate.

each chamber, a steel-ball-type flow meter is inserted at a point before the air is heated and humidified.

Results and discussion. The design and arrangement of the chambers is such that they can be cleaned and sterilized by UV treatment prior to exposure, and tissue or organ cultures installed in a quite convenient manner. In addition, the set-up is easy to dismantle so that cultures can be viewed or treated almost immediately after exposure. Although the system has been in operation for only a short time, it has already proved reliable. Work is now progressing, and in a future publication we hope to present quantitative data on the reaction of cells in monolayer and organ culture to mainstream cigarette smoke under such conditions[10].

Zusammenfassung. Eine Apparatur zur Berauchung von Zell- und Organkulturen unter kontrollierten Bedingungen (Anzahl und Dauer der Puffs, Temperatur, Luftfeuchtigkeit) wird beschrieben. Das System basiert auf der Verwendung der Filtrona-Berauchungsmaschine (Cigarette Components Ltd.); die zusätzlichen Elemente können leicht aus laboreigenem Material zusammengestellt werden. Frischrauch und wasserdampfgesättigte Gasmischungen (z.B. Luft/CO_2) können in der Berauchungskammer in variablen Proportionen gemischt werden.

P. DAVIES and G. S. KISTLER

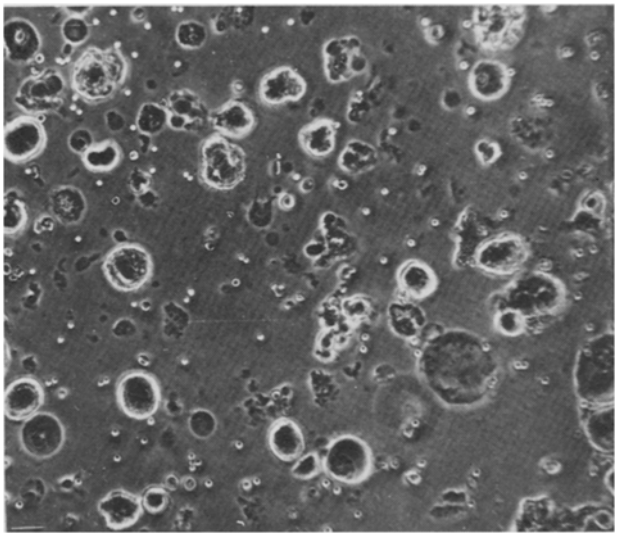
Department of Anatomy, Division of Electron Microscopy, University of Zürich, Gloriastrasse 19, CH-8006 Zürich (Switzerland), 29 October 1973.

[10] Acknowledgements. We wish to thank Mr. W. F. SCHERLE for skilful technical assistance. This work is supported by a research grant from the ASFC (Association Suisse des Fabricants de Cigarettes).

Free Cell Suspensions from Rat Placental Tissue: Metabolism of Pregnenolone-4-^{14}C and Progesterone-4-^{14}C

In this laboratory we have shown the disappearance of 17α-hydroxylase and 17–20 lyase activity after homogenization of the placental tissue. In vitro conversion of labelled C_{21} precursors to androgens occurs only in undamaged placental tissue[1]. These studies were carried out with tissue slices or small cubes of cut tissue. In such experiments, several methodological problems are encountered: broken cells, difficulties in getting homogenous tissue replicates, and gradients of penetration into the tissue of precursors. In work with adrenal[2] and ovarian[3] tissue, these problems are partially overcome by preparing free cell suspensions. We believe that a method of preparing free cells from placenta will make it possible to isolate the hormonal active cells from this morphologically and functionally complicated organ, and will facilitate studies on the regulation of steroid secretion.

We shall describe here a technique of preparing free cells from rat placental tissue. Our results indicate that suspensions of dissociated placental cells can be used to study the metabolism of labelled steroids.



Cell suspension prepared from rat placenta by treatment with trypsin (1:200, Biomed, Warsaw), collagenase (W.S.S., Warsaw) and DNAse (2,000 U/mg, B.Z.S.S., Warsaw). The cells are seen in phase contrast. ×250.

Preparation of placental cell suspensions. Wistar rats were used in these studies. Proestrous females were placed with males; the day that sperm were found in the vaginal smear was designated as day 1 of pregnancy. The animals were decapitated on day 15. The placentas, quartered and washed with Krebs-Ringer bicarbonate buffer, and then chopped and transferred (1.0 g of tissue) into a 25 ml Erlenmeyer flask containing 10.0 ml of 0.25% solution of trypsin, 0.25% collagenase and 0.013% DNAse in medium of pH 7.2–7.3 containing per 100 ml NaCl 0.8 g, KCl 0.02 g, Na_2HPO_4 0.115 g, KH_2PO_4 0.02 g and $MgCl_26H_2O$ 0.01 g. DNAse was added to digest DNA released from damaged cells[4] which otherwise would form a slimy material entrapping the liberated cells. The flask was gassed with 95% O_2–5% CO_2 and incubated at 37 °C for 15 min with shaking. At the end of the incubation period, the fluid containing freed cells was transferred with a siliconized pasteur pipette to a cold siliconized 50 ml centrifuge tube. To the residual 10 ml of fresh digestion medium was added and the process was repeated 3 times. The cell suspensions were combined and centrifuged at $100 \times g$ for 10 min at 4 °C. After centrifugation, the supernatant was removed and the pellet was washed twice with 10 ml portions of Krebs-Ringer bicarbonate buffer. Cell viability was tested by mixing the cell suspension with an equal volume of 0.2% methylene blue. Only the nulcei of living cells take up this stain. The number of cells was estimated by using a Bürker's counting chamber. A total yield of $5.0–6.0 \times 10^6$ cells was obtained from 1.0 g of placental tissue.

Incubation. The pellet was resuspended in Krebs-Ringer bicarbonate medium (pH 7.4) containing 0.2% glucose and 0.2 μCi of pregnenolone-4-^{14}C (S.A. 24 mCi/m*M*) or progesterone-4-^{14}C (S.A. 60 mCi/m*M*, Radiochemical Centre Amersham, England). The suspension

[1] R. REMBIESA, M. MARCHUT and A. WARCHOŁ, Steroids *19*, 65 (1972).

[2] G. SAYERS, R. PORTANOVA, R. J. BEALL, S. SEELIG and S. MALAMED, Acta endocr. Copenh. suppl. *153*, 11 (1971).

[3] D. GOSPODAROWICZ and F. GOSPODAROWICZ, Endocrinology *90*, 1427 (1972).

[4] M. S. STEINBERG, Expl. Cell Res. *30*, 257 (1963).