Further cytological and cytochemical studies on the biological significance of the gas phase of fresh cigarette smoke[1] [2]

Inhibition of RNA synthesis and of normal growth of cultured mammalian cells (mouse kidney) and syncytium of myxomycete physarum polycephalum after exposure to the gas phase, and acrolein, a gas phase constituent of cigarette smoke

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Zusammenfassung

Primärkulturen von Mäusenieren und des Myxomyceten Physarum polycephalum, die ein einziges Mal zu Puffen der Gasphase von frischem ungefiltertem Zigarettenrauch ausgesetzt wurden, zeigen eine Sequenz von Veränderungen wie Hemmung der Synthese von Ribonukleinsäure, Verlust von Ribonukleinsäure, Pyknose und Zelltod in einer Zeitspanne von 1–24 Stunden nach der Berauchung.

Passiert die gleiche Gasphase vorher ein aus aktivierter Holzkohle bestehendes Filter, so bleiben beide Arten von Kulturen unversehrt, d.h. zeigen keine Veränderungen.

Akrolein, ein Bestandteil der Gasphase von frischem Zigarettenrauch, bewirkt im wesentlichen den gleichen schädigenden Effekt an Mäusenieren- und Schleimpilzkulturen wie die Gasphase von ungefilterten Zigaretten.

Summary

Cultures of primary mouse kidney tissue and of the myxomycete physarum polycephalum, exposed once to puffs of the gas phase from fresh unfiltered cigarette smoke, display a sequence of inhibition of RNA synthesis, loss of RNA, pycnosis and cell destruction from 1–24 hours after exposure. The same gas phase after passing through an activated charcoal filter does not produce any alterations in both types of cultures. Acrolein, a gas phase constituent of fresh cigarette smoke, has essentially the same cell damaging effects on mouse kidneys and slime mold as the gas phase from unfiltered cigarettes.

In contrast to the extensive experimental work on the biological activity of cigarette smoke condensates or extracts, there are few experimental investigations concerned with the biological effect of fresh native cigarette smoke on cells, and its interaction with cell metabolism. Since results obtained in experiments with fresh cigarette smoke would appear to be more directly related to the important problem of the role of cigarette smoking for etiology and patho-

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genesis of human lung cancer than those obtained with cigarette smoke condensates or extracts, the *Leuchtenberger's* attempted to develop model systems in which the biological effect of fresh cigarette smoke can be studied *in situ* directly on cells from tissues of living animals and in cultures. Exposing mice to chronic inhalation of intermittent puffs from fresh cigarette smoke, imitating as closely as possible the human habit of cigarette smoking, the *Leuchtenberger's* et al. observed an increase of frequency and of spectrum of tumors over those of controls. Tumors were observed in various tissues not only after chronic inhalation of fresh whole smoke, but also after inhalation of the gas phase of cigarettes (8, manuscript in preparation). In order to elucidate the mechanism by which fresh cigarette smoke contributes to cell transformation, these *in vivo* studies were complemented by *in vitro* investigations on tissue and organ cultures. A model system was developed in which cell-, tissue-, and organ cultures can be exposed directly to puffs of fresh cigarette smoke, controlling important factors of smoke, such as volume, duration of and intervals between puffs. This *in vitro* bioassay permits rapid detection of very early cell alterations and assessment of sequence of events evoked by cigarette smokea t the cell, tissue, andorgan level *in situ* [9, 11]. Using primary mouse kidney tissue, and embryonic mouse lung organ cultures, and assessing the sequence of events simultaneously from a cytological and cytochemical point of view, striking cell damage, characterised by a succession of loss of RNA, pycnosis and cell destruction was observed as early as from 6–24 hours after one exposure to puffs of unfiltered fresh cigarette smoke. While whole fresh smoke and the gas phase of unfiltered cigarettes evoked essentially the same cell damage, activated charcoal filtered smoke from the same cigarettes did not produce any cell damage [9, 11].

The finding that the gas phase of fresh unfiltered cigarette smoke produced essentially the same effect on cells as did whole fresh cigarette smoke containing particulate matter, such as tar, made a further *in vitro* exploration of early interaction between gas phase of cigarette smoke or its components with constituents of the cell itself, particularly of the nucleic acids, of interest. Such studies should help in clarifying the mechanism of action, and in attempts of characterisation and elimination of cell damaging factors contained in whole fresh smoke and in the gas phase from cigarettes [10]. These investigations may also contribute towards the elucidation of the significance of early cell damage observed *in vitro* after one exposure to cigarette smoke [9, 11] for cell transformation observed later *in vivo* after chronic inhalation [8, 10].

The present report deals with an experimental study in which the three following questions pertinent to the problems were explored:

1. Does the gas phase of fresh unfiltered cigarette smoke affect the nucleic acids, especially the RNA metabolism of the cell, and if so, is the cell damage a consequence of this interference?

2. Is the response of cultured mammalian cells to fresh whole smoke or the

gas phase from unfiltered and charcoal filtered cigarettes similar or different from those of cultured non-mammalian cells, particularly those containing mucus, such as the myxomycete physarum polycephalum?

3. Does a single gas phase constituent of cigarette smoke, such as acrolein, evoke the same sequential alterations in cultured mammalian tissues and slime molds as does the gas phase of fresh cigarette smoke?

Material and Methods

For the cytological and cytochemical studies on the effect of fresh cigarette smoke, and of acrolein, primary cultures of kidneys from Snell's mice and cultures of the myxomycete physarum polycephalum were prepared as previously described [1, 2, 3, 7, 9, 11, 12]. However, there were some slight modifications in that inoculums of myxomycete physarum polycephalum were smaller (0.05 ml instead of 0.3 ml). Furthermore, the cultures were placed on filterpaper with 2 ml of media in small plastic petri dishes. Medium was not changed after exposure to cigarette smoke or to acrolein with the exception of the cultures which were kept for 24 hours. In this case the cultures were transferred to large glass petri dishes containing fresh media.

The cultures were exposed to puffs of whole fresh smoke or the gas phase from unfiltered cigarettes and to puffs of the same cigarettes, but after letting

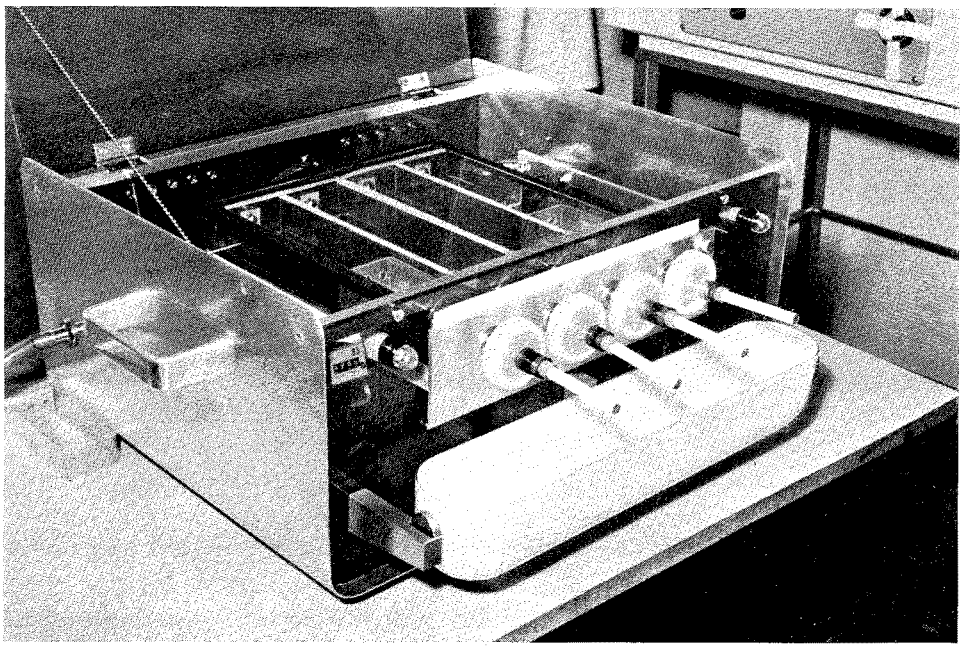

Fig. 1 Photograph of smoking machine for exposure of cultures to puffs of fresh cigarette smoke.

the smoke pass through activated charcoal. Duration of each puff was 5 seconds with a 15 seconds interval and exposure was carried out in a smoking machine shown in Fig. 1. A detailed description of the smoking machine and of the exact procedure of exposure of cultures to fresh cigarette smoke has been described recently by *C.* and *R. Leuchtenberger* [9, 11].

For the acrolein experiments each culture received repeatedly for 5 seconds acrolein, with an interval of 15 seconds, imitating the procedure utilised for exposure of cultures to the gas phase of fresh cigarette smoke. The amount of acrolein, during a 5 seconds exposure was 5 μg, corresponding to that contained in a 5 seconds puff of the gas phase from fresh cigarette smoke.

For the studies of RNA synthesis, cultures were pulse-labeled with 10 μg tritiated uridine per ml. The uridine was left for 10 minutes on cultures of slime mold and for 30 minutes on those of mouse kidneys. Techniques of extraction and counting were done according to the instructions of *R. Braun* [2].

For the cytological studies of the slime mold cultures, smears were prepared, and cultures were imbedded in paraffin for cutting sections and stained with H.E.

For the cytochemical studies of RNA acridine orange staining, for DNA the Feulgen reaction [6], and for the polysaccharides the PAS reaction [4, 13] were used.

Results

a) *Comparison of early effects of fresh smoke from unfiltered and charcoal filtered cigarettes on metabolism of cultured cells from mouse kidney tissue and myxomycete physarum polycephalum.*

The salient features of the sequential cytochemical and cytological alterations observed in primary mouse kidney and myxomycete cultures after one exposure to fresh cigarette smoke are summarised in Table I and described below.

No significant differences were observed in cell alterations between mouse kidney and slime mold cultures, after exposure to whole fresh smoke and the gas phase from unfiltered cigarettes, both displayed a sequence of inhibition of RNA synthesis, loss of RNA, pycnosis and cell destruction. Slime mold syncytium which contains appreciable amounts of mucus also revealed considerable breaking up and destruction of mucus after exposure to fresh smoke from unfiltered cigarettes.

Fig. 2 and Fig. 3 show examples of inhibition of RNA synthesis, as illustrated by decrease of uptake of tritiated uridine, observed as early as from 1–2 hours after exposure of cultures of mouse kidney and slime molds to fresh puffs of whole smoke, or its gas phase from unfiltered cigarettes.

No inhibition of RNA synthesis or any cell damage were seen in mouse

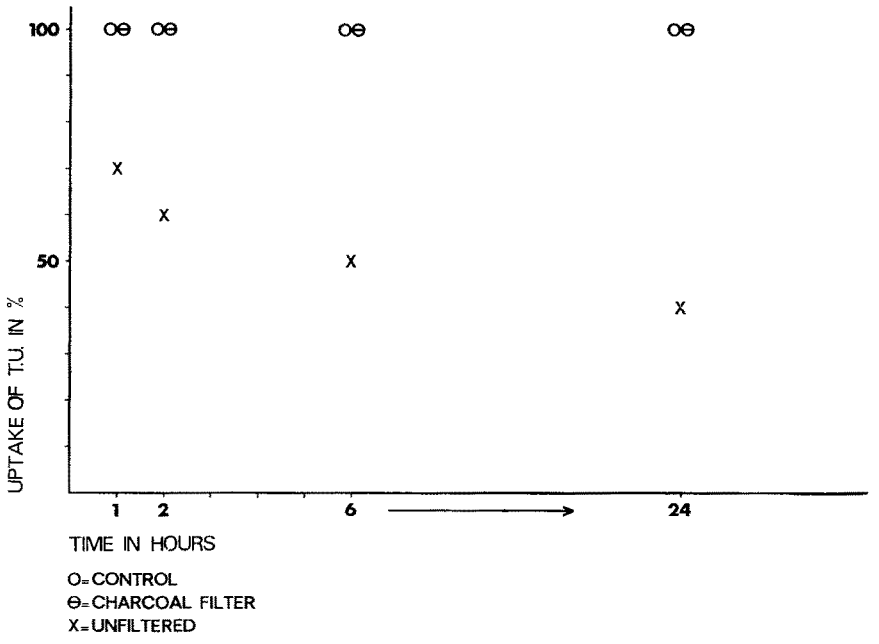

Fig. 2 Uptake of tritiated uridine (T. U.) by primary kidney tissue cultures after exposure to 20 puffs from unfiltered and charcoal filtered cigarettes.*

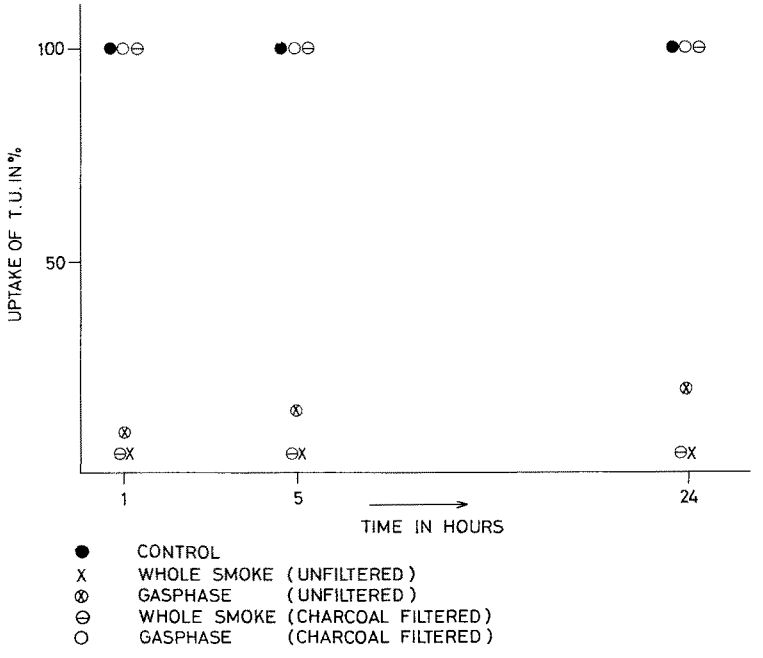

Fig. 3 Uptake of tritiated uridine (T.U.) by the slime mold physarum polycephalum after exposure to 80 puffs of fresh whole smoke and gas phase from unfiltered and charcoal filtered cigarettes. (Note especially the negative and positive results after exposure to charcoal filtered whole smoke.)

Table 1 Comparison of sequential cytochemical and cytological effects on growth of cultured mouse kidney cells (K) and slime mold physarum polycephalum (S) 1–24 hours after exposure to puffs of fresh whole smoke or its gas phase from unfiltered, activated charcoal filtered cigarettes, and after acrolein.

Treatment				Cytochemical and cytological sequence											
Type of cigarette		Dose (number of puffs)		Breaking up of mucus	Inhibition of RNA synthesis		Loss of RNA		Pycnosis		Destruction		Inhibition of growth		Stimulation of growth	
		S	K	S	S	K	S	K	S	K	S	K	S	K	S	K
Whole smoke	unfiltered	80	20	++	++	++	++	++	++	++	++	++	++	++	0	0
	activated charcoal filtered	80	20	0–+	0–+	0	0–+	0	0–+	0	0–+	0	0–+	0	0–(+)	(+)
Gas phase	unfiltered	80	20	+	+	+	+	++	+	++	+	++	+	++	(+)	0
	activated charcoal filtered	80	20	0	0	0	0	0	0	0	0	0	0	0	(+)	0
Acrolein		400μg	60μg	++	++	++	++	++	++	++	++	++	++	++	0	0

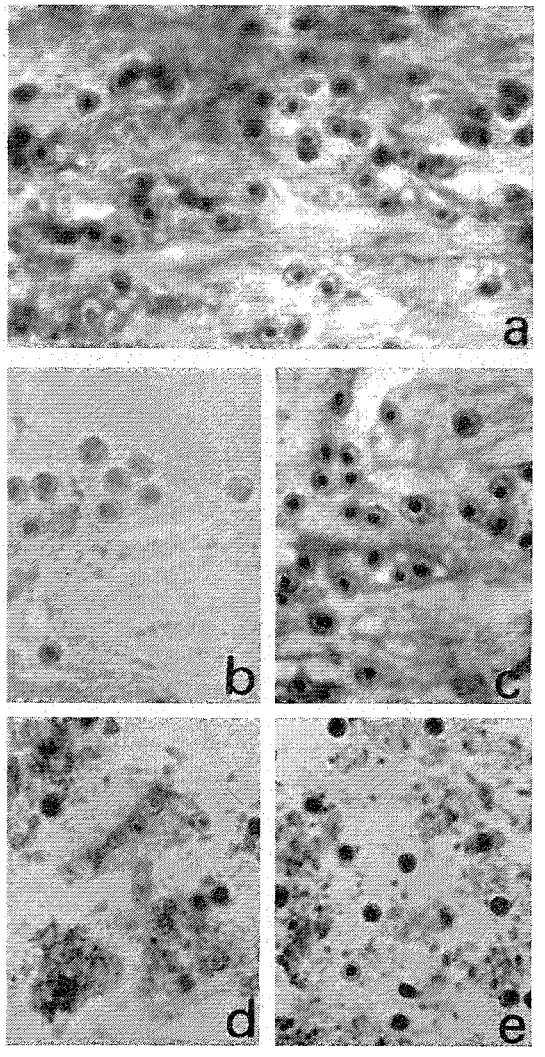

Fig. 4 Slime mold cultures exposed to puffs of fresh smoke from 4 cigarettes.

a) Untreated control.
Note strong staining of nucleolar RNA.

b) 5 hours after exposure to the gas phase of unfiltered cigarettes.
Note weak staining of nucleolar RNA.

c) 24 hours after exposure to the gas phase of charcoal filtered cigarettes.
Note similarity with control.

d) 24 hours after exposure to whole fresh smoke of unfiltered cigarettes.
Note pycnosis and weak staining of nucleolar RNA.

e) 24 hours after exposure to whole fresh smoke of charcoal filtered cigarettes.
Note partial pycnosis and partial strong staining of nucleolar RNA.

Smears, H.E. 625×

kidney cultures, exposed either to whole smoke or the gas phase of the same cigarettes, after the smoke passed first through activated charcoal (Table 1, Fig. 2). However, slime molds responded differently to whole smoke and to the gas phase from charcoal filtered cigarettes. While whole smoke of charcoal filtered cigarettes caused in some cultures inhibition of RNA synthesis and a sequence of cell damage, the gas phase of charcoal filtered cigarettes did not affect the normal slime mold cell metabolism (Table 1, Fig. 3). The loss of RNA from nucleoli and occurrence of pycnosis in slime molds after exposure to the gas phase of unfiltered cigarettes, as contrasted with the normal appearance of such cultures after exposure to the gas phase of charcoal filtered cigarettes, is illustrated in Fig. 4.

b) *Comparison of early effects of acrolein, a gas phase constituent of cigarette smoke, on metabolism of cultured cells from mouse kidney tissue and the myxomycete physarum polycephalum.*

As seen in Table 1, acrolein, a single gas phase constituent of cigarette smoke, evoked essentially the same alterations as those observed after whole fresh smoke or the gas phase from unfiltered cigarettes in both, mammalian and slime mold cells. Fig. 5 and Fig. 6 illustrate the decrease of uptake of tritiated uridine

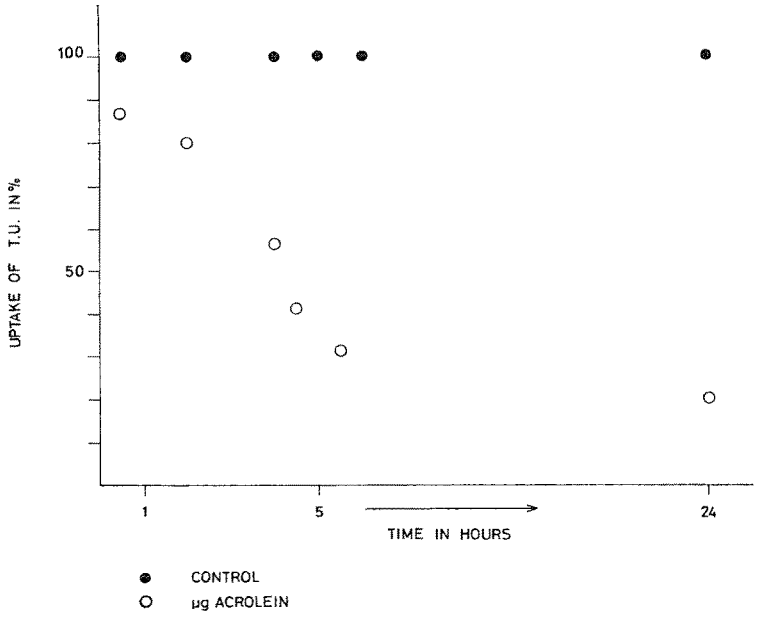

Fig. 5 Uptake of tritiated uridine (T. U.) by primary mouse kidney tissue cultures after exposure to 70 μg acrolein.

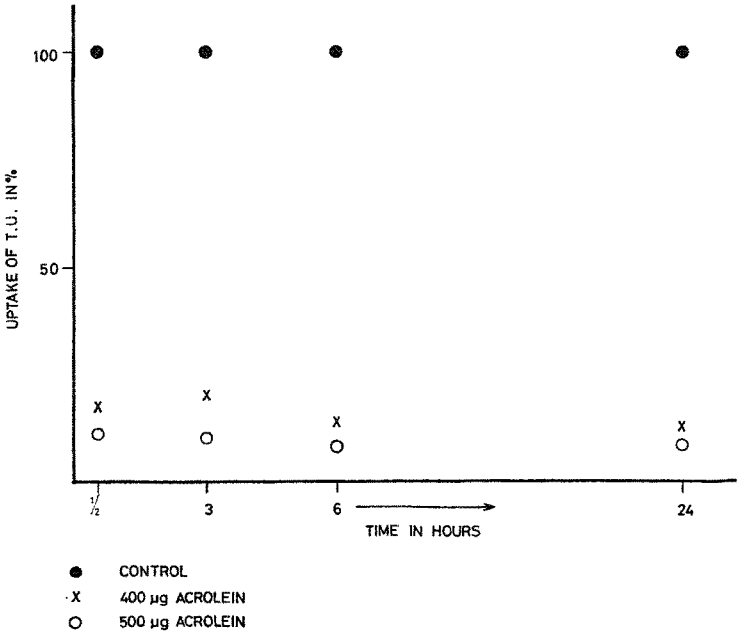

Fig. 6 Uptake of tritiated uridine (T.U.) by the slime mold physarum polycephalum after exposure to 400 and 500 μg acrolein.

after exposure of cultures of mouse kidney and slime molds to acrolein. The similarity between the cell damage in mouse kidney cultures after exposure to the gas phase of fresh cigarette smoke, and after that to acrolein only, is illustrated in Fig. 7.

Discussion of Results

To facilitate the discussion, the essential findings are briefly summarised.

a) Puffs from whole fresh smoke and from the gas phase of unfiltered cigarettes evoke essentially the same cytochemical and cytological alterations in mouse kidney and slime mold cultures.

b) The gas phase from the same cigarettes, after passing through activated charcoal, does not elicit damage in cultured mouse kidney and slime mold syncytium. However, while the same holds true for kidney cultures exposed tow hole fresh smoke from charcoal filtered cigarettes, slime mold syncytium exhibits sometimes damage after exposure to charcoal filtered whole smoke, similar to that after unfiltered smoke (Fig. 4e).

c) Acrolein, a gas phase constituent of cigarette smoke, evokes essentially

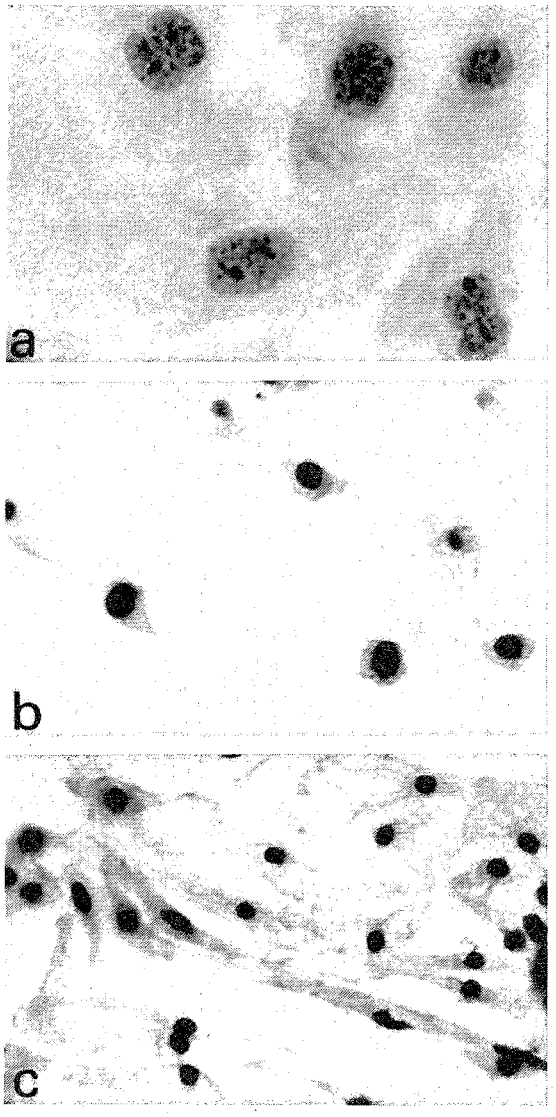

Fig. 7 Primary mouse kidney cultures exposed to 80 μg of acrolein, and to 10 puffs of the gas phase of fresh smoke from an unfiltered cigarette.

a) 5 days old control culture.

b) 24 hours after exposure to acrolein.
Note striking pycnosis and destruction of culture.

c) 24 hours after exposure to the gas phase of cigarette smoke.
Note marked pycnosis and destruction of culture.

H.E., 500×

the same cell alterations in both, mouse kidney and slime mold cultures, as those observed after whole smoke and its gas phase from unfiltered cigarettes.

The finding that whole fresh smoke or the gas phase from unfiltered cigarettes inhibit not only RNA metabolism and growth of cultured *mammalian cells* but also of slime mold is noteworthy if one takes into consideration the striking differences existing between these two types of cultures. In addition to the basic differences between mammalian and non-mammalian cells, monolayers prepared from trypsinized mouse kidneys show contact inhibition, that is hardly any DNA synthesis or cell replication, while slime molds undergo more or less continuous RNA synthesis, DNA synthesis, and rapid synchronised cell division in a relatively short time (3 cycles within 24 hours) [1, 3]. Furthermore, slime mold cultures display large quantities of extra cellular mucus, containing polysaccharides [4, 13]. One would have expected that this mucus material prevents penetration of cigarette smoke into the nuclei. That the mucus may inhibit penetration of the smoke into the cells at least in part, is suggested by the observation that much higher doses of cigarette smoke are needed to evoke inhibition of RNA synthesis in slime molds (80 puffs) than in kidney cells (20 puffs). The necessity of giving such large doses in slime molds may also explain why whole smoke from charcoal filtered cigarettes elicited some cell damage. It is not unreasonable to suggest that in this particular instance the cell damage is caused by the deposition of large quantities of particulate matter on the surface of the slime mold cultures, in other words obstruction of cell metabolism may be simply due to an "unspecific mechanical effect".

The observation that the *gas phase* of fresh cigarette smoke will evoke inhibition of RNA synthesis in mammalian and non-mammalian cells points to the biological importance of gas phase constituents for nucleic acid metabolism and cell growth. This concept, which has been stressed repeatedly by the *Leuchtenberger's* [8, 9, 10, 11] is further supported by the results obtained in this study with acrolein. This single gas phase constituent of cigarette smoke is able to produce alone all the essential cell alterations seen after exposure to the complete gas phase of fresh unfiltered cigarette smoke. While it would be unwise to ascribe the interference in cell metabolism observed after the gas phase of cigarette smoke, to acrolein only, there are two findings which at least suggest the important role of acrolein[1] for these changes. 1. Acrolein acts in doses comparable or even less to those contained in the gas phase of cigarette smoke. 2. Acrolein is known to be absorbed by activated charcoal, and this property explains our previous and present findings, that the gas phase of fresh smoke from activated charcoal filtered cigarettes does not produce any cell damage.

[1] During the preparation of this manuscript a paper by *Izard* et al. (1967) was brought to our attention which describes an inhibitory and mutagenic effect of acrolein in the algae Dunaliella bioculata [5].

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