agonal units some 650 to 1000 Å in diameter. The cytoplasm contains scanty, granular, endoplasmic reticulum, a feature correlated with the presence of a low concentration of cytochemically detectable RNA (*6*). Mitochondria are sparsely scattered throughout the cytoplasm, and multivesicular bodies are common. The latter are composed of spheres some 300 to 650 Å in diameter, are sometimes limited by a membrane, and are frequently closely associated with the basal zone of the microvilli (Fig. 3*B*). They were never seen within the nerve which leaves the median frontal organ. The size (about 1 to 2 μ), location, and abundance of the multivesicular bodies suggest that they are the small fuchsinophil inclusions visible with the light microscope. Membranous configurations reminiscent of the Golgi complex are present, and are occasionally associated with microvesicles similar to those constituting the multivesicular bodies.

Because the microvillar elaborations of the median frontal organ clearly resemble retinal photoreceptor elements found in many other animals (*11*), and because the location and innervation pattern of this organ suggested homology to the median ocellus of insects, a comparison with the fine structure of a thysanuran median ocellus seemed in order. Fortunately, several living specimens of *Tricholepidion gertschi* Wygodzinsky (*12*) were made available to us (*13*). The rhabdomere subunits of the median ocellus of this species and the microvillar elaborations of the median frontal organ of *Thermobia* are strikingly similar in form and size (Fig. 3*A*, *B*). In addition, both types of cells possess multivesicular bodies, and lack a well-developed granular endoplasmic reticulum. Among the more salient differences between the two is the formation of the *Tricholepidion* rhabdom by the apposition of three cells rather than the usual two seen in the median frontal organ. It also seems that the ocellar receptor cells of *Tricholepidion* possess a greater concentration of mitochondria.

Although the question of whether or not a cell is functioning as an endocrine gland cannot be answered by cytological examination per se, previous comparative studies have consistently revealed the presence of characteristic elementary neurosecretory granules in cells known or suspected of serving a neuroendocrine function (*9*). Such granules were not found in the median frontal organ of *Thermobia*. The only formed elements which might represent secretory products are the multivesicular bodies, and this interpretation seems doubtful in view of the widespread occurrence of such bodies in nonendocrine organs. On the other hand, the morphological evidence that the median frontal organ may be a photoreceptor is convincing. The location of this organ in *Thermobia*, its innervation, and its fine structure all indicate that it is homologous to the median ocellus of other Thysanura. The absence of a median frontal organ in those Thysanura which possess a median ocellus (*4*) also favors this interpretation.

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13. We thank Howell V. Daly, Department of Entomology and Parasitology, University of California, Berkeley, for supplying us with these insects.
14. Aided by research grants NSF G-8805 and USPHS NB-03845. We thank Mrs. Emily Reid for assistance in preparing Fig. 1.

4 May 1964

Tumor-Promoting Activity of Extracts of Unburned Tobacco

Abstract. *Both an aqueous $Ba(OH)_2$ extract and an acetone-benzene extract of unburned cigarette tobacco produced skin tumors when painted on mice previously treated with 125 μg of 7,12-dimethylbenz[a]anthracene. The extract from as little as 0.5 cigarette per day was effective. The data are of interest in connection with epidemiological evidence that tobacco chewing is a cause of oral cancer. The data also suggest that tumor-promoting agents in cigarette smoke may have their source in the unburned tobacco.*

Clinical observations that tobacco chewing is associated with oral cancer have been reported for many years (*1*). This relation is most striking in the Far East, where tobacco is often chewed in conjunction with betel nut, flavorings, and lime (*2*, *3*). In the United States, where tobacco chewing is less extensive, similar observations have been reported, but the association of tobacco chewing with oral cancer is much less striking (*4*). Peacock *et al.* suggest that tobacco chewing alone is not carcinogenic but acts as a tumor-promoting stimulus to men exposed to other carcinogenic agents (*5*). Regardless of mechanism, it is noteworthy that most tobacco chewers or snuff dippers place the quids at the same site for many years, and it is at this site that the cancers develop.

Extracts of unburned tobacco have not been tested extensively for carcinogenic activity. Wynder and Wright

Table 1. Tumor promotion by extracts of unburned tobacco.

Treatment	Tobacco equivalent (cigarettes per day)	Ratio of No. of tumors to No. of mice with tumors*	
		30 weeks	36 weeks
DMBA and acetone extract	2.5	8/5	16/7
DMBA and conc. $Ba(OH)_2$ extract	0.5	19/7	18/8
DMBA and dilute $Ba(OH)_2$ extract	.5	11/2	6/2
DMBA alone	0	0	0
Acetone extract alone	2.5	0	0
Conc. $Ba(OH)_2$ extract alone	0.5	0	0
Dilute $Ba(OH)_2$ extract alone	.5	0	0
Untreated controls	0	0	0

* Thirty mice per group, 36 weeks of promoting stimulus.

reported papillomas in 18 percent of 80 mice painted for 22 months with a methanol–methylene chloride extract of cigarette tobacco (*6*). Ranadive *et al.* observed that acetone extracts of Indian tobaccos possessed both tumor-promoting activity and complete carcinogenic activity (*7*). Muir and Kirk were the only workers to test a tobacco extract obtained in a manner related to tobacco chewing (*3*). They found that an aqueous extract of whole betel-tobacco preparation (betel leaf, betel nut, "gambir," tobacco, and lime) was weakly carcinogenic to the skin of mice, and they suggested that tobacco acted in combination with other stimuli.

These observations are important not only because they may lead to a more complete understanding of oral cancer, but also because transfer of carcinogens or tumor-promoting agents from the cigarette to the smoke may account for a part of the carcinogenic activity of cigarette tar, which contains both "complete" carcinogens and tumor-promoting agents. We felt it desirable to extend the observations of Muir and Kirk to determine whether or not the carcinogenic activity was due to the tobacco alone. In addition, we decided to test aqueous alkaline extracts of unburned tobacco for tumor-promoting activity. The results showed that unburned tobacco is, indeed, an excellent source of tumor-promoting agents, and that these promoters can be extracted readily by alkaline aqueous solutions. The unexpected activity of extracts of small amounts of tobacco has prompted this report.

Three extracts of commercial cigarette tobacco were used in the study. The first was prepared by extracting 400 g of tobacco twice with 2 liters of acetone at −10°C for 16 hours. The residue was then extracted with 2 liters of benzene for 16 hours at room temperature. The combined acetone and benzene extracts were concentrated at reduced pressure to yield 15 to 20 g of product, which was dissolved in acetone to a final volume of 40 ml. Thus the daily dose of this acetone solution contained the extract from 2½ cigarettes.

The second extract was prepared by treating 400 g of tobacco with 1 liter of saturated aqueous $Ba(OH)_2$ at room temperature for 4 hours. The *p*H was initially high, but gradually fell during this period. In all cases, however, the *p*H remained above 9 for at least 15 minutes. The mixture was filtered, and the residue was extracted two additional times with 500 ml of distilled water. The combined extracts were poured slowly into a 2-liter cylinder containing 100 to 200 g of dry ice. A small amount of Dow antifoam A was used to suppress foaming. After the evolution of CO_2 had ceased, the $BaCO_3$ was removed by filtration; the filtrate had a *p*H of 5 to 6 and was free of detectable Ba^{++}. The filtrate was condensed to 200 ml, which contained 130 to 150 g of product. This was labeled "concentrated $Ba(OH)_2$ extract." Each mouse received a daily dose of material recoverable from ½ cigarette.

Table 2. Characteristics of extracts.

Extract	Yield of ext. (% solids of fresh tobacco)	Concn. (g/ml)	Equiv. wt. tobacco (g/ml)
Acetone	4–5	0.2–0.3	10
Conc. $Ba(OH)_2$	30–40	0.6–0.7	2

The third extract was prepared by treating the tobacco a single time with aqueous $Ba(OH)_2$ and then neutralizing. The neutral extract was used directly. The extraction procedure was not very efficient and yielded only about 32 g of product from 400 g of tobacco. The solution was thus about one-fourth as concentrated as the concentrated aqueous $Ba(OH)_2$ solution.

Female ICR Swiss mice at 57 days of age were painted a single time with 125 μg of 7,12-dimethylbenz[*a*]anthracene (DMBA) in 0.25 ml of acetone. After 21 days, 0.25 ml of the various test solutions (Table 1) were applied once a day, five times a week, for the duration of the experiment.

Each of the extracts showed tumor-promoting activity (Table 1). In 36 weeks, the acetone-benzene extract of 2½ cigarettes per day produced 16 tumors in 7 of 30 mice; the $Ba(OH)_2$ extract of only ½ cigarette per day produced 20 papillomas in 8 of 30 mice; and the dilute aqueous extract produced 11 tumors in 2 of 30 mice. No tumors were found in untreated mice, in mice treated with DMBA alone, or in mice treated with one of the three extracts but not with DMBA.

The first tumor appeared after 9 weeks of treatment. Up to the present time, all of the tumors are rather small papillomas, ranging in maximum diameter from 1 to 9 mm. A few have regressed, as expected, in spite of continued treatment. There appeared to be no significant difference in the gross appearance of the tumors among the experimental groups.

The concentrated aqueous extract and the acetone-benzene extract are compared in Table 2. Much more material was extracted from the tobacco by the aqueous $Ba(OH)_2$, and hence this extract was dissolved in a larger volume. The solutions applied to the animals were of comparable activity. Nevertheless, the daily dose of acetone-benzene extract represented 2½ cigarettes, whereas the daily dose of aqueous $Ba(OH)_2$ extract represented only ½ cigarette. It is conceivable that different active materials were present in the two extracts, or that the agent was more effective when applied to the skin in the aqueous solution. On the other hand, acetone is a volatile solvent permitting rapid penetration of many solutes into the skin, and it would seem unlikely that acetone was less effective as a solvent than water (*8*). It seems probable then that extraction with aqueous $Ba(OH)_2$ produced the best recovery of active material in this experiment. Hydrolysis of the cigarette tobacco by the alkaline solution may have played a role in liberating "bound" tumor-promoting agents.

The results of the present study are of interest in connection with the epidemiological and clinical evidence that chewing mixtures of tobacco and lime is a cause of many oral cancers. The data may also have a bearing on the problems of cigarette smoking and lung cancer. It is probable that the carcinogenic hydrocarbons of cigarette smoke (*9*) result from destructive distillation of the cigarette components. Nevertheless, other carcinogens or tumor promoters in unburned cigarette tobacco might be volatilized by pyrolysis and pass into the smoke, which is known to contain a number of carcinogens and tumor-promoting agents (*10*). If such a process does take place, removal of tumor "promoters" from the unburned product should aid in the development of less hazardous cigarettes. The feasibility of this approach can be ascertained when the tumor promoters in unburned tobacco are identified and compared with promoting agents found in the smoke.

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28 May 1964

Virogenic Hamster Tumor Cells: Induction of Virus Synthesis

Abstract. *Virogenic cell cultures of a hamster ependymoma originally produced by Simian Virus 40 were treated with proflavine, hydrogen peroxide, or mitomycin C. Induction of virus synthesis was observed. Similar experiments with hamster cells transformed by adenovirus type 12 or polyoma virus gave negative results.*

The properties of cells of hamster ependymomas produced by intracerebral inoculation of newborn animals with Simian Virus 40 (SV_{40}) have been described (*1*). No infectious virus was detectable in cell-free extracts of both primary and tissue-cultured tumor cells. However, when intact, viable tumor cells were seeded onto sensitive indicator cells (cultures from kidneys of African green monkeys), characteristic viral lesions appeared in the indicator cells adjacent to the tumor cells. It was postulated that the tumor cells harbored a persistent subviral infection which is transmissible to indicator cells by cell-to-cell transfer of subviral units. The term "virogenic state" was suggested to describe this virus-cell relationship.

When our attempts to induce virus synthesis in virogenic cells by exposure to ionizing radiation gave irregular results, we explored other means of virus induction. The results of such studies with proflavine, hydrogen peroxide, and mitomycin C are the subject of this report.

Three transformed hamster cell cultures free of detectable virus were employed in this study. They were the EP_A cell line derived from an ependymoma produced in hamsters by SV_{40}, a tissue culture preparation of a primary hamster sarcoma produced by adenovirus type 12 (*2*), and the TC-1 line of Stoker, a hamster kidney cell line transformed in vitro by polyoma virus (*2*).

Cell-free preparations of these malignant cells showed no evidence of infectious virus when tested in cultures of appropriate indicator cells. Tests for subviral infectivity were performed by seeding intact malignant cells directly on indicator cell cultures. After 8 and 14 days of incubation, the mixed cultures were disrupted by freezing and thawing and ultrasonic treatment. The cell-free preparations were assayed for presence of virus. As seen in Table 1, only cells transformed by SV_{40} gave evidence of persistent subviral infectivity.

In attempts to induce viral synthesis in these transformed cells we evaluated the effects of proflavine, hydrogen peroxide, and mitomycin C. Monolayer tube cultures of transformed cells were treated as shown in Table 2. The medium used in all experiments consisted of Eagle's minimum essential medium (*3*) containing 2 percent calf serum. Proflavine treatment was carried out in subdued light, and the treated cultures were shielded from light during the entire period of incubation. At 6 to 24 hours after treatment, slight cytotoxic effects were noticed in cultures treated with proflavine or mitomycin C. These cultures were thoroughly washed and incubated with drug-free maintenance medium. The cultures treated with hydrogen peroxide showed no significant toxic changes and were not refed. All cultures were placed on roller drums and were maintained with 2 ml of medium per tube at 36°C. Eight to ten days after treatment, the cells were frozen, thawed, and treated with high-frequency sound. The cell-free fluids were tested for the presence of virus. The results (Table 2) show the successful induction of virus synthesis in tumors produced by SV_{40}. The relatively small amount of virus recovered from induced cultures suggests that only a fraction of the virogenic cells were inducible under the present experimental conditions. By contrast, none of the treatments resulted in virus induction in the cells transformed by adeno- or polyoma virus. Failure to induce virus production in cells transformed by polyoma virus confirms previously published findings (*4*). Other investigators (*5*) have shown that "virus-free" tumors produced by SV_{40}, polyoma, or adenovirus type 12 release complement-fixing antigens specific for the virus or tumor.

Table 1. Detection of subviral infectivity in transformed hamster cells.

Hamster cells transformed by	Cell extracts	Intact cells seeded onto	Infectivity
SV_{40}	Negative	GMK*	Positive
Adenovirus type 12	Negative	HEK*	Negative
Polyoma virus	Negative	ME*	Negative

* GMK, green monkey kidney; HEK, human embryonic kidney; ME, mouse embryo.

Table 2. Attempts to induce virus production in transformed hamster cells.

Dose	Period of treatment	$TCID_{50}$* per culture of cells transformed by:		
		SV_{40}	Adeno-12	Polyoma
	No treatment			
None		0	0	0
	Proflavine			
1–5 μg/ml	6–8 hr	2	0	0
	H_2O_2			
$9 \times 10^{-5} M$	6–8 days	15	0	0
	Mitomycin C			
0.2–0.4 μg/ml	7–24 hr	10	0	0

* Fifty percent tissue culture infective dose.

The observations recorded in this study together with the evidence on complement-fixing antigens suggest a basic difference among cells transformed by these three oncogenic viruses with respect to the nature of the viral genetic material and its relationship to cellular DNA.

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9 June 1964