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Some newly discovered tumour-promoting substances

by

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Before I begin my main contribution (and before Prof. Graffi takes out his stop-watch) I think it would be advisable for me to define the terms I shall be using.

A *complete carcinogen* is capable of converting normal cells to tumour cells.

There are two types of *incomplete carcinogens* the actions of which are complementary to each other. Firstly there are the *tumour-initiating* agents, and secondly the *tumour-promoting* agents. When the second type is administered repeatedly after the first, tumours result. When the first type is administered after the second, no tumours result. Nor do tumours result from the action of either agent alone.

Tumour initiators may cause little or no histologically detectable change in the tissues. But all tumour-promoting agents cause hyperplasia of the cells in the tissue concerned. Tumour initiators may act at sites remote from the site of application. Promoting agents always act where they are applied. Most tumour initiators are either weakly carcinogenic for the tissue concerned (i. e. they may produce tumours when applied in higher dosage than necessary for tumour-initiation), or for other tissues, or for other species.

Prolonged administration of tumour-promoting agents sometimes elicits a few tumours.

Despite the occasional production of tumours by both types of incomplete carcinogen, we feel that these distinctions may have practical importance, and that this concept of the mechanism of carcinogenesis should not be discarded because the distinction between the two of incomplete carcinogen is not absolutely clear-cut.

Until recent years carcinogenic activity was generally regarded as a highly *specific* property. Very small amounts of certain polycyclic hydrocarbons had been shown to produce tumours. In the past 15 years, however, many unrelated classes of compounds have been shown to possess this property, and today it is possible to induce histologically identical tumours by a variety of different stimuli, chemical, physical, and viral. It is, then, with caution that we should refer to carcinogenic activity as a *specific* property. But I feel that the term „specific" still has some meaning in this connection, for, despite the strenuous efforts of many of us attending this Symposium, there is still a vast preponderance of noncarcinogenic substances, — or should I say, of substances which have not yet been shown to be carcinogenic?

Most of the substances which hitherto have been found to possess *tumour-initiating* activity, are known to be carcinogenic on their own. Hence the tumour-initiating process is probably of about the same degree of specificity as carcinogenic activity.

The position of the *tumour-promoting* process is less clear. For many years *croton oil* has been in a class by itself — many times more potent than any other substance. From time to

time workers have reported that irritant substances which cause hyperplasia of the skin of mice do not promote tumour-formation. These two facts pointed to the conclusion that tumour-promotion is an even more specific process than carcinogenesis or tumour-initiation. We feel however, that information concerning the tumour-promoting process is at present too scanty to come to any such conclusion.

The experiments briefly described here were designed to study the relationship between the production of epidermal hyperplasia and tumour-promotion.

A number of substances mostly of plant origin, were tested to see if they would give rise to epidermal hyperplasia in the skin of mice of the '101' inbred strain. Those which did so were then tested for tumour-promoting activity. A single application of 300 μg 9,10-dimethyl-1,2-benzanthracene (DMBA) in 0,2 ml acetone was followed after an interval of 3 weeks by once-weekly applications of the test substance. Control mice received the single application of DMBA without further treatment.

Results

The experiments are incomplete, but the results so far point to there being a close relationship between the induction of epidermal hyperplasia and tumour-promotion. All substances which have given rise to epidermal hyperplasia, and maintained this state for a period of 30 weeks or more, promoted tumour-formation.

One interesting observation was that mice were able to adapt themselves to the irritating properties of some of the substances. An example of this phenomenon was seen in the case of linseed oil. A group of mice were painted once with linseed oil (undiluted); three days later marked hyperplasia on the epidermis was seen. A second group of mice were painted once-weekly with linseed oil; three days after the 20th application the epidermis appeared normal (Figures 1 and 2). This phenomenon of adaptation may account for some of the previously reported examples of substances which cause epidermal hyperplasia but do not promote tumour-formation.

The citrus oils

Table 1 summarizes very briefly some of our results in tests with 4 different citrus oils: orange, lemon, grapefruit, and lime. All promoted the development of many papillomas. In the case of lime oil the experiment is still at a very early stage, but the rapid tumour-development suggests that this oil may be more potent than the others. Several malignant tumours have also arisen in these tests.

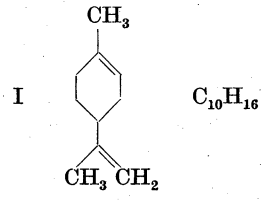
All 4 oils are separated commercially into a terpene (hydrocarbon) and non-terpene (hydrocarbon-free) fractions. The latter, which constitutes only 5 to 15% of the oil, contains terpene alcohols and esters.

Table 1

Tumour-promotion by Citrus Oils

Initiating treatment	Promoting treatment	Papillomas/survivors at 33 weeks
DMBA (3000 μg)	orange oil	83/43
,, ,,	lemon oil	38/15
,, ,,	grapefruit oil	37/15
,, ,,	lime oil	22/19 (12 weeks)
DMBA (300 μg)	none	0/38
none	orange oil	1/48

Using chromatographic methods we purified the two commercial fractions of orange oil, and tested them separately. The purified terpene fraction which consists mainly of d-limonene (I) gave rise to considerable



I $C_{10}H_{16}$

epidermal hyperplasia and promoted tumour-formation. The purified terpene-free fraction, on the other hand, was inactive in both respects, even when applied at 4 times the concentration present in the original oil (see Table 2).

Tumours of other sites were seen in some of these experiments. The most noteworthy of these were the papillomatous tumours of the urethral orifice (caruncles) which arose in 7 female mice treated with orange oil (Table 3 and Figures 3 and 4).

These remarkable tumours arose in mice treated with orange oil diluted with acetone (or the terpene-free fraction of orange oil diluted with acetone). Two arose in mice which had not been pretreated with DMBA. None of the mice treated with DMBA only, or DMBA followed by undiluted citrus oils, developed these tumours. We think it probable that acetone

Table 2

Tumour-promotion by fractions of Orange Oil

Initiating treatment	Promoting treatment	Papillomas/survivors at 33 weeks
DMBA (300 μg)	Terpene fraction of orange oil	29/15
,, ,,	Non-terpene fraction of orange oil	1/13

facilitated the absorption through the skin of the active substance or its precursor. The site of origin of these tumours suggests that the causative agent was secreted in the urine. Further work in this field could lead to the discovery of a new mechanism by which carcinogens are produced endogenously from inactive precursors.

Four malignant haemangiomas, a large uterine tumour, and two subcutaneous sarcomas were among the other tumours seen in these experiments.

Table 3

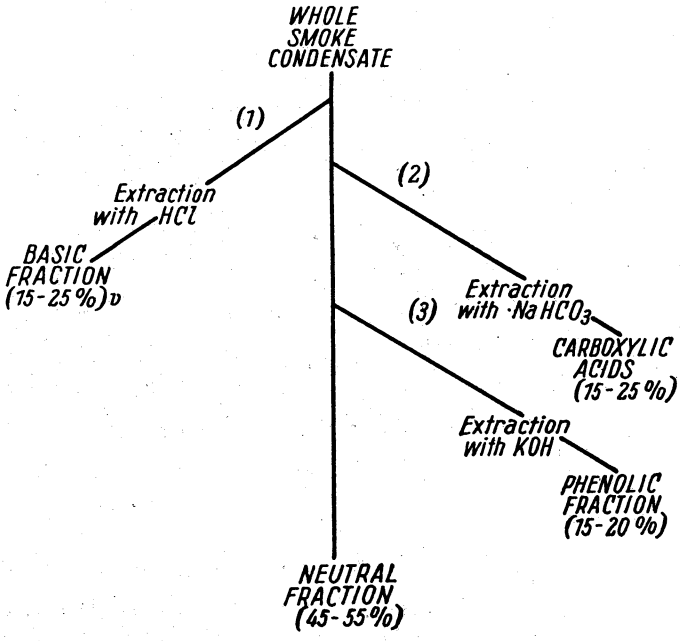
Tumours of the Urethral Orifice in Female Mice

Treatment	Urethral tumours/Mice
DMBA only	0/35
DMBA + diluted* 00	4/20
Diluted 00 only	2/20
DMBA + undiluted 00	0/10
Undiluted 00 only	0/10
DMBA + Terpene fraction of 00	0/10
DMBA + Non-terpene fraction of 00	1/10

* Diluted in Acetone.

Tumour-promotion by a phenolic fraction of cigarette smoke

Turning to another field I would like to mention briefly a recent finding in the field of tobacco-smoke carcinogenesis. We have found that the phenolic fraction of cigarette smoke condensate, which constitutes only 15 to 20% of the smoke, possesses marked tumour promoting activity. The phenolic fraction is derived from the whole smoke condensate by extraction with potassium hydroxide after the majority of the carboxylic acids have been removed by previous extraction with sodium bicarbonate (Text-Fig. 1). Our results in this experiment are given in Table 4. Several malignant tumours arose after treatment was stopped at 40 weeks (Fig. 5).



Text-Fig. 1

Hitherto it has been difficult to understand why mice painted with large amounts of cigarette tar develop any tumours at all. A mouse painted thrice weekly with 40 mg of tar for a year receives altogether only 1—3 μg of 3,4-benzpyrene. In addition it receives small doses of other carcinogens. But the sum of the carcinogenic effects of all these substances is far from sufficient to account for the tumours seen in such experiments. On the other hand the sum of the effects of these substances is sufficient to *initiate* the tumours seen. Hence the demonstration of potent tumour-promoting substances in cigarette smoke is important because it enables us to regard tobacco-carcinogenesis as a two-stage process. At the same time it relieves us of the obligation of finding more potent carcinogens than 3,4-benzpyrene in tobacco smoke.

Table 4

Tumour-promotion by the Phenolic Fraction of Cigarette Smoke

Initiating treatment	Promoting treatment	Papillomas/Survivors at 40 weeks
DMBA (300 μg)	6 to 12 mg Phenolic Fraction × 3 weekly	65/15
"	none	0/37
None	6 to 12 mg Phenolic Fraction × 3 weekly	0/16

Tumour-promotion by a dilute extract of Euphorbia ingens

Croton oil is obtained from the seeds of *Croton tiglium*, a member of the *Euphorbia* family. Many members of this family produce a latex in their stems which is irritant to the skin of

man. We tested an extract of the latex derived from *Euphorbia ingens*, a cactus-like plant found in South Africa.

We found that a 1% acetone extract of the latex produced marked hyperplasia of mouse epidermis, and strongly promoted tumour development (Table 5).

Table 5

Tumour-promotion by an acetone extract of the Latex of Euphorbia ingens

Initiating treatment	Promoting treatment	Papillomas/Survivors at 30 weeks
DMBA (300 μg)	1% Euphorbia extract	80/19
None	1% Euphorbia extract	0/12
DMBA (300 μg)	none	0/42

This finding may be important firstly because of the low concentration necessary to produce the effect, and secondly because the chemical analysis of this material may prove easier than that of croton oil itself. Work in all these new fields is being continued.

Zusammenfassung

Einige neu entdeckte tumorauslösende Substanzen

Anfang 1958 stellten wir uns die Frage, ob die Realisierungswirkung der Geschwulstbildung ein spezifischer Vorgang ist. In Voruntersuchungen prüften wir verschiedene von Pflanzen stammende Stoffe, um zu sehen, ob sie eine Epithelvermehrung in der Mäusehaut hervorrufen Wir fanden, daß einige dieser Stoffe Zellvermehrung verursachten, und zwar auf unbeschränkte Zeit, wenn man sie oft anwandte. Diese Substanzen und einige andere, die weniger Zellvermehrung hervorriefen, wurden dann auf ihre Realisierungswirkung (tumorauslösende Wirksamkeit) hin geprüft. Mäuse wurden einmal mit 9,10-Dimethyl-1,2-Benzanthrazen und dann einmal wöchentlich mit der Testsubstanz behandelt.

Bei diesen Untersuchungen ergaben sich vier wichtige Befunde:

1. Wir entdeckten, daß das Öl, welches man von 4 verschiedenen Zitrusfrüchten (Apfelsinen, Zitronen, Grapefruit und Limonen) auspressen kann, eine ziemlich starke geschwulstauslösende Wirkung hat. Die wirksame Substanz ist wahrscheinlich ein einfacher Kohlenwasserstoff, d-Limonin ($C_{10}H_{16}$).

2. Apfelsinenöl führte zur Bildung von Tumoren in der Harnröhrenöffnung von weiblichen Mäusen, die vorher nicht behandelt worden waren.

3. Es zeigte sich, daß eine Phenolfraktion des Zigarettenrauches eine bedeutende tumorauslösende Wirksamkeit hat. Die Tumoren, die sich bei Pinselung von Mäusen mit Tabakteer bilden, könnten somit das Resultat eines zweiphasigen Vorganges sein. Die kleine Menge von 3,4-Benzpyren, die allein zur Tumorbildung unzureichend ist, genügt hingegen für den Initialvorgang, während die Phenolfraktion eine tumorauslösende Wirkung hat. In diesem Falle brauchten wir nicht weiter nach einem stärkeren Cancerogen im Tabakteer zu suchen, um seine cancerogene Wirkung in der Mäusehaut zu erklären.

4. Crotonöl ist in den Samen von Croton tiglium enthalten. Wir fanden, daß ein Acetonextrakt aus der Flüssigkeit des Stengels einer verwandten Pflanze, Euphorbia ingens, bedeutende Realisierungswirkung besitzt. Wir hoffen, daß weitere Untersuchungen Klärung über die Eigenschaften des aktiven Prinzips im Crotonöl selbst bringen werden.

Die Arbeit wird auf diesen neuen Gebieten weitergeführt.

Fig. 1: Mouse-skin 3 days after an application of undiluted linseed oil

Fig. 2: Mouse-skin 3 days after the last of 20 weekly applications of undiluted linseed oil

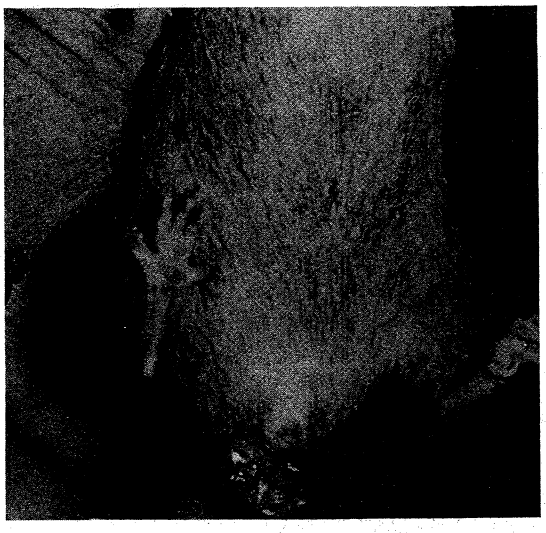


Fig. 3: Papillomatous tumour of urethral orifice in a female mouse treated with 14 once-weekly applications of 40% orange oil in acetone

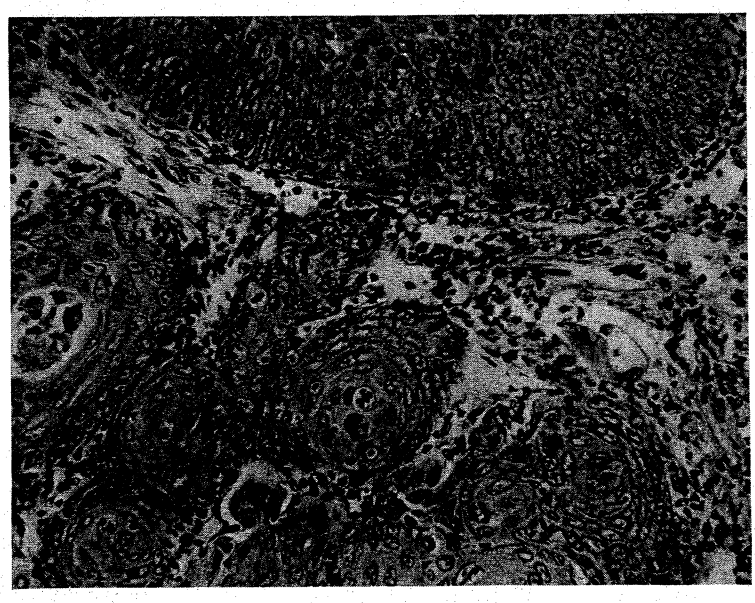


Fig. 4: Photomicrograph of tumour shown in Fig. 3. Stained with haematoxylin and eosin. (×50)

Fig. 5: Malignant tumour of skin in mouse treated with DMBA followed by thrice-weekly applications of phenolic fraction of cigarette smoke for 40 weeks