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Tumor Promotion by Citrus Oils: Tumors of the Skin and Urethral Orifice in Mice [1, 2]

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Tumor Promotion by Citrus Oils: Tumors of the Skin and Urethral Orifice in Mice [1, 2]

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SUMMARY

Oils derived from the peel of four citrus fruits, sweet orange, lemon, grapefruit, and lime, were found to cause epidermal hyperplasia in mice of the inbred strain 101. These oils were tested for tumor-promoting activity by weekly application to the mouse skin after a single application of 300 μg. of 9,10-dimethyl-1,2-benzanthracene (DMBA): all gave rise to skin papillomas after 5 to 12 weeks. Several malignant skin tumors have also arisen, and more are expected as observation continues. Dilution of orange oil with acetone reduced the tumor-promoting effect. Undiluted orange oil promoted tumors in mice previously treated topically or parenterally with urethan. Orange oil, undiluted or diluted with acetone, was not carcinogenic for mouse skin. Normal mice and urethan-pretreated mice were given 7 intradermal injections of undiluted orange oil; no tumors arose during a 30-week observation period. Preliminary experiments indicate that the promoting activity of orange oil is probably due to its content of *d*-limonene, which constitutes more than 90 percent of the oil, and not to the terpene alcohols and other oxygenated compounds present. Papillomatous tumors of the urethral orifice were seen in 7 mice: 4 treated with DMBA and diluted orange oil, 2 with diluted orange oil only, and 1 with DMBA and the nonterpene fraction of orange oil. The diluent, acetone, probably facilitated the absorption of the active component of orange oil since no urethral tumors arose in mice painted with the undiluted oil. The experimental production of these tumors has not, to our knowledge, been reported previously.—J. Nat. Cancer Inst. 24: 1389–1403, 1960.

A PRELIMINARY communication (*1*) reported that the oil obtained by expression from the peel of the sweet orange, *Citrus sinensis*, promoted skin-tumors in strain 101 mice previously treated with a single tumor-initiating dose of 300 μg. of 9,10-dimethyl-1,2-benzanthracene.[4] The

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[2] This investigation was aided by a block grant from the British Empire Cancer Campaign.
[3] The authors wish to express their gratitude to Dr. J. S. Fawcett, Department of Experimental Biochemistry, London Hospital, and to Mr. D. F. Salaman, for the purification of 2 fractions of orange oil; and to Dr. M. H. Salaman, for his advice and encouragement during the course of this work. They are also indebted to Mr. A. L. Stiff, Mrs. J. Cohen, Mrs. G. Moger, Mr. W. J. Milton, and Miss M. Orford for technical help.
[4] *Chemical Abstracts'* nomenclature: 7,12-dimethylbenz[*a*]anthracene.

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result of that experiment has now been confirmed and the studies have been extended.

MATERIALS AND METHODS

Mice.—Mice of the inbred strain 101, bred and maintained in this laboratory, were used for all the experiments. They were housed in metal cages (zinc or galvanized iron), 10 mice per cage. At 6 to 8 weeks of age they were vaccinated on the tail with sheep lymph, as a precaution against ectromelia. They were fed cubes of the Rowett Institute formula and water *ad libitum*. Mice were 8 to 9 weeks old at the beginning of the experiments.

Technique of application to the skin.—Dorsal hair was removed from all mice, both test and control groups, by electric clippers before the beginning of the experiments and thereafter at 2- to 3-week intervals. The area from the neck to the root of the tail was clipped.

Test substances were delivered from calibrated pipettes, care being taken to ensure even spread over the whole of the clipped area.

Recording of tumors.—The mice were examined, at fortnightly intervals, for tumors of the skin and other tissues. Sick mice were killed and, together with mice found dead, examined post mortem for tumors of internal organs and for other lesions. Otherwise mice were kept under observation during their natural lifespan.

Histological examination.—Specimens of skin, skin tumors, regional lymph nodes, and other organs showing pathological changes were taken for histological examination. They were fixed in Zenker's fluid, embedded in paraffin wax, and stained with hematoxylin and eosin.

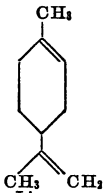
Chemicals

Citrus oils.—The citrus oils, sweet orange, lemon, grapefruit, and lime, were supplied by a well-known British firm which specializes in the importation, processing, and distribution of essential oils. The oils were obtained by simple expression from the peel of the fruit. Prior to use the oils were stored in well-stoppered amber-glass bottles at room temperature.

These oils may have contained a variety of contaminants, such as pesticides, colorants, and atmospheric soot. No attempt was made at this stage to identify or remove them. Every effort was made to test the materials in the form and condition in which they are used as food additives.

The terpene and nonterpene fractions of orange oil.—Orange oil is commercially separated into 2 fractions by vacuum distillation: Fraction *A*, about 95 percent of the oil, consists mainly of terpenoid hydrocarbons of which *d*-limonene (I) is the major component. Fraction *B*, about 5 percent of the oil, consists of terpenoid alcohols and esters, together with a substantial amount of terpenoid hydrocarbons.

(I)



Limonene

Further purification of both commercial fractions was undertaken to obtain a *terpene* (hydrocarbon) fraction free of terpenoid alcohols and a *nonterpene* (hydrocarbon-free) fraction. Commercial fraction *A* was purified chromatographically: 80 gm. in 150 ml. of petroleum ether (b.p. 60–80° C.) was passed through an aluminium oxide column (300 gm.). The column was then washed with 500 ml. of petroleum ether and the solvent from the combined eluates was removed by distillation, yielding 75 gm. of terpene fraction. Fraction *B* was purified in a similar way: 40 gm. in 100 ml. of petroleum ether was passed through an aluminium oxide column (300 gm.). The column was washed with about 500 ml. of petroleum ether until the washings were hydrocarbon-free. The required *nonterpene* fraction was then eluted from the column with 400 ml. of a mixture of equal parts of ethanol and diethyl ether. After evaporation of the solvent, a yield of 8.5 gm. of nonterpene material was obtained.

Other chemicals.—Urethan and acetone, Analar grade, were obtained from British Drug Houses, Poole, Dorset, England, and 9,10-dimethyl-1,2-benzanthracene from L. Light and Company, Colnebrook, Bucks, England.

EXPERIMENTAL

Experiment I: Tests for Tumor Promotion in Mouse Skin by Certain Citrus Oils

Four citrus oils, namely, sweet orange, lemon, grapefruit, and lime, as received from the supplier, were tested at different times for tumor-promoting action. Preliminary tests showed that all of them gave rise to considerable epithelial hyperplasia when applied to the skin of strain 101 mice. The results with orange oil have already been reported (*1*) but are given again here for comparison.

For each test 10 male and 10 female mice were used. All received a single application of DMBA—300 μg. in 0.2 ml. of acetone for the first 4 groups, and 225 μg. in 0.15 ml. of acetone for Group 5. Group 1 was a control group and received no further treatment except for periodic hair clipping; it was contemporaneous with Groups 3 and 4. Weekly applications, 0.25 ml. each, of the test substances were begun 3 weeks after the application of DMBA in Groups 2 to 5.

Results

Benign Tumors of the Skin

Papillomas began to arise during the 5th week of secondary treatment in Group 3 (lemon oil), Group 4 (grapefruit oil), and Group 5 (lime oil), and during the 12th week in Group 2 (orange oil). Table 1 shows the incidence of papillomas in survivors after 33 weeks of treatment with the test substance (only 12 weeks with lime oil). The incidence was similar in the 4 test groups and obviously differed significantly from that in the control Group 1 treated with DMBA only, in which only 1 papilloma arose, not on the treated skin but in the submandibular region.

In addition to the papillomas shown in table 1, a female mouse in Group 3 developed 1 sebaceous-gland tumor of the nipple. Tumors of the nipples have not to our knowledge been recorded before in this kind of experiment, although mammary carcinomas have resulted from the application of DMBA to the skin of female rats [(*2*), *see also* results of expt. II, p. 1395].

Malignant Tumors of the Skin

In Group 2 treatment was stopped after 42 weeks, at which time there were 9 survivors. No malignant tumors have arisen in this group: 2 mice are still under observation at the 65th week. Groups 3 and 4 stopped treatment after 40 weeks and Group 5 is at present in the 34th week of treatment. Two mice of Group 3 and 2 of Group 4 developed malignant tumors between the 36th and 55th weeks after the beginning of secondary treatment. Three of these tumors were examined microscopically and showed active infiltration of the dermal tissues down to the panniculus muscle: all were squamous-cell carcinomas. No malignant tumors have arisen in the control group treated with DMBA only, which is contemporaneous with Groups 3 and 4.

In Group 5, which is only in the 34th week of treatment, so far only 1 malignant tumor has been seen. The mouse that bore this tumor was subsequently found dead and satisfactory histological examination of the tumor was not possible. It is expected that more malignant tumors will arise in this group in which 14 mice are still alive.

It is unfortunate that survival of strain 101 mice beyond 11 to 12 months is poor, since in this type of experiment malignant tumors of the skin only appear after a long latent interval, about 46 weeks (*3*). The main reason for the poor longevity of this strain is the very high incidence of the renal disease, papillonephritis (*vide infra*). Thus only 7 mice of Group 2, 8 of Group 3, and 7 of Group 4 were alive 46 weeks after the beginning of the experiment and it is not surprising that so few malignant tumors were seen. It is feared that survival will be no better in Group 5.

Tumors of Other Sites

One mouse of Group 2 developed a hemangioma of the subcutaneous tissues. This tumor, which first appeared after 7 weeks and grew to a

TABLE 1.—Incidence of cutaneous papillomas after 33 weeks of treatment with test substance

Number	Survivors	Mice with papillomas	Total papillomas

TABLE 1.—Incidence of cutaneous papillomas after 33 weeks of treatment with test substance

Group	Tumor-initiating treatment	Substance tested for tumor promotion	Number of mice	Survivors	Mice with papillomas	Total papillomas
		Experiment I: Tumor promotion by undiluted citrus oils applied to the skin				
1	300 μg. DMBA	None	20	16	1	1*
2	"	Orange oil	20	18	13	39
3	"	Lemon oil	20	15	10	38
4	"	Grapefruit oil	20	15	13	37
5	225 μg. DMBA	Lime oil	20	19†	10	22
		Experiment II: Effect of dilution with acetone on tumor promotion by orange oil applied to the skin				
6	300 μg. DMBA	None	30	22	4	5*
7	"	40% Orange oil	20	10	5	13
8	"	80% Orange oil	20	15	10	31
2	"	Undiluted orange oil	20	18	13	39
		Experiment III: Tests for carcinogenic effect of orange oil on mouse skin applied to the skin				
9	None	40% Orange oil	20	17	0	0
10	"	80% Orange oil	20	15	1	1*
11	"	Undiluted orange oil	20	16	0	0
		Experiment IV: Tests comparing the tumor-promoting activity of orange oil after initiation by DMBA and urethan				
12	240 mg. Urethan to skin	Orange oil	20	13‡	3	3
13	64 mg. Urethan intraperitoneally	" "	20	13‡	2	3
2	300 μg. DMBA	" "	20	20‡	9	9
		Experiment V: Tests for carcinogenicity and tumor promotion by orange oil in dermal tissues				
14	None	Intradermal injections of 0.1 ml. undiluted orange oil	20	11‡	0	0
15	64 mg. Urethan intraperitoneally	" " " "	20	12‡	0	0
		Experiment VI: Tests of two fractions of orange oil for tumor promotion on mouse skin				
16	300 μg. DMBA	80% Terpene fraction or orange oil	20	15	8	29
17	"	20% Nonterpene fraction of orange oil	20	13	1	1
1	"	None	20	16	1	1

*Outside the treated area.
†After only 12 weeks of treatment.
‡After only 20 weeks of treatment with test substance.

diameter of 10 mm. during the following 9 weeks, was removed by biopsy and has not recurred. It was regarded as probably malignant. A mouse of Group 4 developed a spindle-cell sarcoma of the subcutaneous tissues beneath the treated epidermis. This tumor grew rapidly and was invasive. No tumors of internal organs have so far been observed.

Incidence of Papillonephritis

A high incidence of this disease in untreated strain 101 mice has already been recorded, with references to the relevant literature (*4*). Because of the disease, it is unusual for more than 50 percent of mice, whether treated or not, to survive 12 months. The usefulness of the strain for experiments involving long-term treatment and observation is therefore limited. Unfortunately this disadvantage was not apparent until the experiments were far advanced. There was no evidence that treatment with DMBA and/or the citrus oils affected the incidence of the disease.

Conclusion

Despite poor survival of mice due to papillonephritis, the results clearly indicate that the 4 citrus oils tested possess pronounced tumor-promoting activity for mouse skin.

Experiment II: Effect of Dilution with Acetone on Tumor-Promoting Effect of Sweet Orange Oil

The addition of acetone to orange oil enhanced its irritating effect on mouse skin. Biopsy specimens of skin taken 3 days after application of either 80 or 60 percent concentrations of orange oil in acetone showed epidermal necrosis, ulceration, crusting, and inflammatory infiltration of the dermal tissues. When the concentration was reduced to 40 percent in acetone, these more severe signs of irritation were far less marked but the degree of hyperplasia of the epidermis was greater. Thus the histological effect of 40 percent orange oil in acetone was similar to that of the undiluted oil. After an application of 20 percent orange oil in acetone, epidermal hyperplasia was only moderate.

During a course of repeated once weekly applications of 80 percent orange oil in acetone, mice became progressively less susceptible to the irritating effects of the treatment, necrosis and crusting became less severe, and the degree of hyperplasia of the epidermis increased. After 20 weekly applications, it was difficult to see any difference between mouse skin treated with 80 percent orange oil in acetone and that treated with a 40 percent concentration.

Thirty-five male and 35 female mice were divided into 3 groups; Group 6 had 15 mice of each sex, and Groups 7 and 8, 10 of each. Mice of all 3 groups received a single application of DMBA (300 μg./0.15 ml. acetone). After 3 weeks, Group 7 received the first application of 0.25 ml. of 40 percent orange oil in acetone, and Group 8 a similar application but with

80 percent orange oil These applications were thereafter given weekly for 37 weeks. Group 6 received no further treatment.

Results

Papillomas began to arise on the treated skin of mice in Groups 7 and 8 during the 12th week of secondary treatment; that is, at the same time as they first appeared on mice painted with undiluted orange oil after DMBA (expt. I, Group 2).

Table 1 shows the incidence of papillomas on mice after 33 weeks of secondary treatment, and, for comparison, that in Group 2 of experiment I.

It will be seen that there were 5 papillomas in 4 of 22 survivors of the control group which received DMBA only. All these tumors arose outside the treated area of the skin, on the face and head. The first was seen 28 weeks after the application of DMBA, *i.e.*, equivalent to the 25th week of secondary treatment in Groups 7, 8, and 2. Of the 13 papillomas in Group 7 only 1 was outside the treated area; similarly only 1 of the 31 papillomas in Group 8 was outside it. In both cases the ectopic tumors were on the head. All the 39 papillomas of Group 2 arose from treated skin.

Sebaceous-cell tumors arose in the nipples of 4 mice: 1 mouse in Group 7 (2 tumors), 1 in Group 8 (1 tumor), and 2 in Group 6 (2 and 3 tumors respectively). Since these tumors appeared in both test and control groups, there is no reason to think that orange oil played any specific role in their production.

Two malignant tumors of the skin were seen. Both of them arose in a pre-existing papilloma, 1 on a female mouse of Group 7, during the 34th week of secondary treatment, and the other on a female of Group 8, 19 weeks after the end of treatment. On histological examination both tumors were found to be squamous-cell carcinomas infiltrating the panniculus muscle.

As in the previous experiment, papillonephritis has caused the death of many mice before malignant skin tumors are expected in this type of test.

Tumors of the Urethral Orifice in Female Mice

Tumors of the urethral orifice arose in 4 female mice of Group 7. A full description and discussion of these tumors is given later. They all arose between the 10th and 21st weeks of treatment. None were seen in Groups 2, 6, or 8.

Conclusion

Both 80 and 40 percent concentrations of orange oil in acetone were effective in promoting tumor development after a single application of DMBA. There was little difference between the effect of 80 percent orange oil in acetone and the undiluted oil, but 40 percent orange oil was

definitely, though proportionately, less effective than the higher concentrations.

In this experiment we had the first indication that the addition of acetone to orange oil led to the induction of urethral tumors in female mice (*vide infra*).

Experiment III: Tests for the Carcinogenic Effect of Sweet Orange Oil on Mouse Skin

Three groups, 9 to 11, of 20 mice, 10 of each sex, were used in this experiment. They were treated once weekly with orange oil, either 40 percent in acetone (Group 9), 80 percent in acetone (Group 10), or undiluted (Group 11). Each application consisted of 0.25 ml. Treatment was continued for 38 weeks in Groups 9 and 10, and for 46 weeks in Group 11.

Skin Tumors

One papilloma arose on the head of a mouse in Group 10 during the 33d week of treatment. No other papillomas and no malignant skin tumors were seen in any of the mice.

Tumors of the Urethral Orifice

One female mouse of Group 9 and 1 of Group 10 developed tumors of the urethral orifice; the former after 40 weeks and the latter after 10 weeks (*vide infra*).

Tumors of Other Organs

No tumors of other sites have so far been seen either in the mice which have died or in those still under observation.

Conclusion

The occurrence of 2 urethral tumors and 1 papilloma outside the treated area suggests that orange oil by itself may not be completely devoid of tumorigenic power. However the fact that none of these tumors arose on the treated area of skin indicates an indirect rather than a direct tumorigenic action (*see* p. 1399, "Tumors of Urethral Orifice in experiments I to VI").

Experiment IV: Test for Promotion of Epidermal Tumors by Orange Oil After Initiation by Urethan

Forty female mice were divided into 2 equal groups. Group 12 was given 4 applications each of 60 mg. urethan in 0.3 ml. acetone, to the clipped dorsal skin, at 3-day intervals [total dose of urethan = 240 mg.]. Group 13 was given 4 intraperitoneal injections each of 16 mg. urethan in 0.1 ml. distilled water, also at 3-day intervals [total dose of urethan = 64 mg.]. After 3 weeks, both groups began a course of once weekly applications of undiluted orange oil, 0.25 ml. per application, to the dorsal skin.

Results

Papillomas appeared on 3 mice of Group 12, after 10, 12, and 14 weeks of treatment with orange oil respectively, and in 2 mice of Group 13, after 10 and 12 weeks respectively. This is a much smaller yield than that in Group 2 in which DMBA was used as the initiator. Table 1 shows the incidence of tumors in Groups 12, 13, and 2, after 20 weeks of secondary treatment. Survival of mice was poor in the 2 urethan-treated groups: by the 30th week when treatment was stopped there were only 19 survivors of the original 40 mice in the 2 groups. No more papillomas arose after the 20th week, and no urethral tumors were seen at any time.

Pulmonary adenomas were present in most of the mice of Groups 12 and 13 that died more than 18 weeks after the administration of urethan.

Although there were no contemporary control groups treated with urethan only, previous experience with the strain 101 and many other strains leaves us with little doubt that the tumors seen in Groups 12 and 13 would not have arisen if orange oil treatment had been withheld. The report by Lindsay (*5*) that skin tumors had appeared 10 to 18 months after treatment with urethan only has not, to our knowledge, been confirmed; but in any case the 6 papillomas reported here arose after only 2 to 3 months.

Conclusion

Orange oil has the power to promote tumors in mouse skin after initiation with urethan, but the combined effect of 240 mg. urethan on the skin, or 64 mg. urethan intraperitoneally, followed by weekly applications of orange oil, is very weak compared with the effect of 300 μg. DMBA on the skin followed by similar treatment with orange oil.

Experiment V: Tests for the Carcinogenic and Tumor-Promoting Effects of Orange Oil on the Subcutaneous Tissues of the Mouse

This experiment was designed (1) to see whether orange oil is carcinogenic for the dermal tissues, and (2) to see whether tumors could be promoted, either in the dermis or overlying skin, by injecting orange oil intradermally into urethan-initiated mice. A similar technique was used by Salaman and Glendenning (*6*) to demonstrate the tumor-promoting action of proflavine.

Two groups of 20 female mice were used. The first, Group 14, was given intradermal injections at different sites on the back, each injection consisting of 0.1 ml. of undiluted orange oil. The first 4 injections were given at weekly intervals, and the subsequent 3 at 2- to 3-week intervals. Abscesses and ulcers developed at most of the injection sites within a few days. These lesions sometimes persisted for several weeks but eventually disappeared. Because of the severity of this inflammatory response, it was not possible to continue the course of injections as originally planned.

As shown in table 1, survival has been poor: only 11 mice were alive

20 weeks after the beginning of treatment. The experiment is now in its 30th week and there are 8 survivors. No tumors of the dermis or overlying skin have so far been seen.

The second, Group 15, was first given 4 intraperitoneal injections of urethan, 16 mg. in 0.1 ml. distilled water per injection, at 3-day intervals. After a further 2-week interval they were given a course of intradermal injections of undiluted orange oil in the same way as Group 14.

The experiment is in its 30th week and there are 11 survivors. No tumors have arisen so far.

As in Groups 12 and 13, pulmonary adenomas were present in mice of Group 15 that died more than 18 weeks after administration of urethan.

Conclusion

There is at present no evidence that orange oil is carcinogenic for the dermal tissues of the mouse nor that it will promote tumors in this site after urethan has been administered systemically.

Experiment VI: Tests of a Terpene (Hydrocarbon) and a Nonterpene Fraction of Orange Oil for Tumor Promotion

The terpene and nonterpene fractions of orange oil were prepared as outlined in "Materials and Methods." Both the fractions were first tested for irritant action on mouse skin at approximately ⅓ and ⅔ their concentrations in orange oil. It was found that 80 and 40 percent concentrations of the terpene fraction in acetone produced a histological effect 3 days after application, similar to that produced by 80 and 40 percent concentrations of orange oil itself. Mice treated with 4 and 2 percent concentrations of the nonterpene fraction in acetone showed little or no hyperplasia and no other evidence of irritation of the skin.

It was therefore decided to test the terpene fraction for tumor promotion at a concentration of 80 percent, but the nonterpene fraction at 4 times its concentration in the original oil, namely, 20 percent in acetone.

For each test 10 mice of each sex were used. Both groups were first given a single application of 300 μg. DMBA. Then, after a 3-week interval, weekly applications of 0.25 ml. each of the fractions were begun.

Tumors of the Skin

Group 16 which received the terpene fraction began to develop papillomas after 11 weeks, and by the 33d week of secondary treatment there were 29 of these tumors on 8 of the 15 survivors. But in Group 17 treated with the nonterpene fraction, 13 survived for 33 weeks, and only 1 papilloma arose after 3 weeks.

Table 1 shows the incidence of papillomas in the 2 groups and, for comparison, that in a control group treated with DMBA only (Group 1).

A malignant tumor arose during the 36th week in a mouse of Group

16. On histological examination it was found to be a squamous-cell carcinoma penetrating the panniculus muscle.

Tumors of the Urethral Orifice and Perineal Region

One urethral tumor appeared in a female mouse of Group 17 during the 12th week of secondary treatment. It was removed 8 weeks later when it had reached a diameter of 1.0 cm. In addition a rapidly growing tumor arose in the perineal region of a female mouse during the 32d week of secondary treatment. This mouse was killed 3 weeks later, and on microscopic examination the tumor was found to be a fibrosarcoma of high malignancy.

Conclusion

The results of this experiment indicate that the tumor-promoting properties of the terpene (hydrocarbon) fraction of orange oil are approximately equal to that of orange oil itself (*cf.* 29 papillomas on 8 of 15 survivors in Group 16, and 31 papillomas on 10 of 15 survivors in Group 8 of expt. II).

With the nonterpene fraction the only papilloma seen was possibly due to treatment with DMBA only, since it arose so early in the experiment. However it did arise on the treated area of the back, whereas no tumors were seen on the backs of mice in either of the control groups (Groups 1 and 6) that received DMBA only.

The fact that a tumor of the urethral orifice and a sarcoma of the perineal region arose in mice of Group 17 suggests that the nonterpene fraction of orange oil may have some carcinogenic or cocarcinogenic activity.

TUMORS OF THE URETHRAL ORIFICE IN EXPERIMENTS I TO VI

Table 2 summarizes the results of the 6 experiments on the production of urethral tumors in female mice—7 of these tumors were seen. Although this incidence is not high, there are several interesting points to be made: (1) In addition to the mice of the present experiments, we have had considerable experience with strain 101 and also with mice of many other strains, but have never before seen tumors of this kind. (2) Table 2 shows that 6 of 7 of these tumors arose in mice treated with orange oil diluted with acetone, whereas none arose in groups treated with the undiluted citrus oils. (3) Two of these tumors arose in mice that received no DMBA, and none were seen in the control groups that received DMBA only. (4) It is noteworthy that 1 of the tumors arose in a mouse treated with DMBA plus the nonterpene fraction of orange oil.

Five of the 7 tumors arose between the 9th and 12th weeks of secondary treatment, 1 during the 22d week, and 1 during the 40th week, 3 weeks after secondary treatment was stopped.

TABLE 2.—Incidence of tumors of the urethral orifice

Group	Tumor-initiating treatment	Tumor-promoting treatment	Number of female mice	Number of mice with urethral tumors	Week of appearance in relation to beginning of secondary treatment
1	300 μg. DMBA	None	10		
2	"	Undiluted orange oil	10		
3	"	" lemon oil	10		
4	"	" grapefruit oil	10		
5	225 μg. DMBA	" lime oil	10		
6	300 μg. DMBA	None	10		
7	"	40% Orange oil	15		
8	"	80% " "	10	4	10, 10, 11, 21
9	None	40% " "	10		
10	"	80% " "	10		
11	"	Undiluted orange oil	10	1	40*
12	240 mg. Urethan	" "	10	1	10
13	64 mg. Urethan, intraperitoneal	" "	10		
14	None	" "	10		
15	64 mg. Urethan, intraperitoneal	" " intradermal	10		
		" " intradermal	10		
16	300 μg. DMBA	80% Terpene fraction of orange oil	10	1	12
17	300 μg. DMBA	20% Noniterpene fraction of orange oil	10		

*This tumor appeared 4 weeks after the end of treatment with 40 percent orange oil.

Four of the 7 tumors have been examined histologically after removal by biopsy. All were heavily infected, papillomatous tumors and all were actively growing and showed many mitotic figures (figs. 1, 2, and 3). Three of them recurred after removal. Unfortunately none of the mice which bore these urethral tumors could be examined satisfactorily post mortem: in all mice decay was too far advanced when death was discovered. Thus it is not known whether any of the tumors had metastasized, or whether there were primary tumors elsewhere in the urinary tract, or in other sites. In one mouse a large swelling, 20 ×10 ×10 mm., was observed in the left uterine horn, but advanced decay precluded histological examination. No metastases have been observed in living mice.

The apparent enhancing effect of acetone on tumor production at the urethral orifice may be due to facilitation either of surface contamination or of absorption through the skin. In the latter case, which seems more probable, it may be that an active constituent or metabolite of the oil is excreted in the urine.

DISCUSSION

The results of the experiments reported here indicate that there is a component in each of 4 citrus oils, which is capable of promoting skin tumor development in previously initiated strain 101 mice. Furthermore it is probable that the active component is a hydrocarbon, and possible that it is the terpene, *d*-limonene, which is a major constituent of all 4 oils. For reasons given, it is important that the nonterpene fractions of these oils be examined further.

Clearly, additional work is required to identify the active component or components. The possibility that it or they are contaminants and not a part of the natural fruit has been considered. It would be difficult to obtain a natural product of this kind which had not been exposed to pesticide sprays or atmospheric dust. However, we may claim that the material applied to the mice in these experiments was of the same degree of purity as that ingested daily by man. Moreover it is most unlikely that the tumor-promoting activity observed is due to a contaminating carcinogenic hydrocarbon such as benzo[*a*]pyrene. For it is well established that such substances in doses too small to produce tumors by themselves may act as tumor initiators, but not as promoters, in mouse-skin experiments. For this reason too, and because controls in these and other similar experiments were negative, it is very unlikely that the tumor-promoting action observed was due to accidental contamination in the laboratory.

The results are of possible interest in three ways:

(1) They suggest that a new class of tumor-promoting substance has come to light, and this may be of importance in basic research on the 2-stage mechanism of carcinogenesis.

(2) They raise the question whether these oils, to which man is frequently exposed, constitute carcinogenic hazards.

(3) It appears that orange oil contains a substance that can give rise to tumors of the urethral orifice which have not previously been induced experimentally. It is not at present clear whether these tumors were due to surface contamination or excretion in the urine of a component or metabolite.

Work now in progress may help to solve some of the new problems arising out of these findings.

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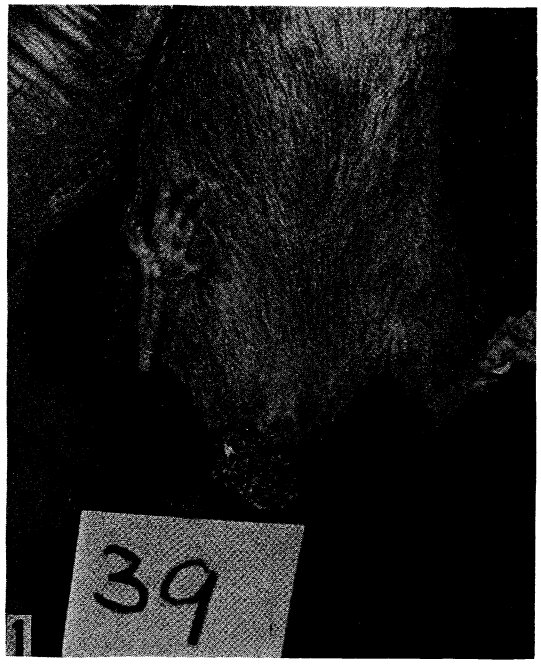
PLATES

PLATE 131

FIGURE 1.—Papillomatous tumor of urethral orifice in a female mouse of strain 101 treated with a single application of 300 μg. DMBA followed by 15 once weekly applications of 40 percent orange oil in acetone.

FIGURE 2.—Section of tumor shown in figure 1. × 56

FIGURE 3.—High-power view of same section. *Note* inflammatory infiltration by polymorphonuclear cells. × 250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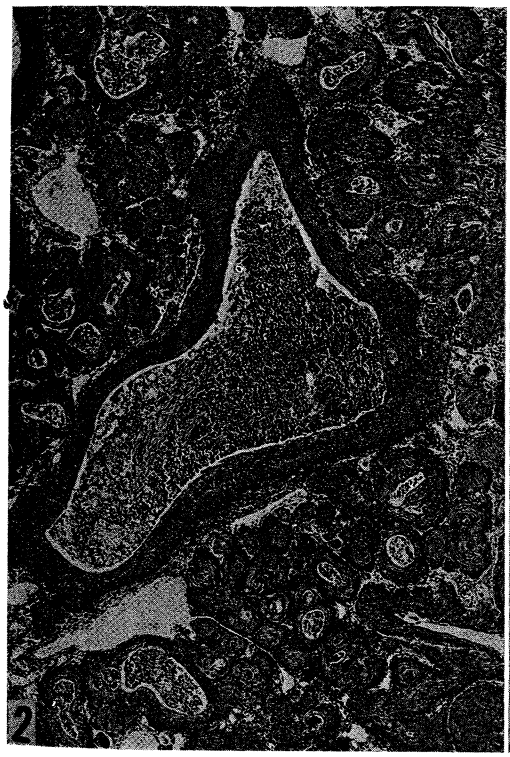
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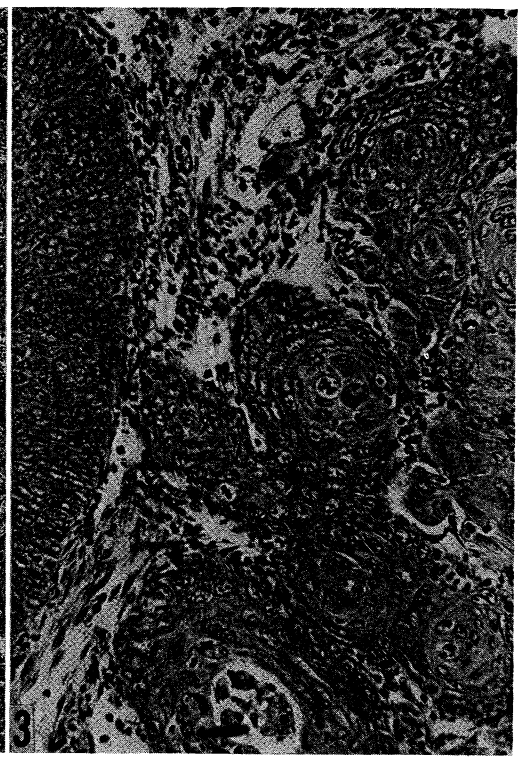
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