Tumor Promotion in the Forestomach Epithelium of Mice by Oral Administration of Citrus Oils [1, 2, 3]

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SUMMARY—The tumor-promoting and carcinogenic effects of lime oil, orange oil, and *d*-limonene on the forestomach epithelium of mice were studied. Mice, given a single dose of either 100 μg 7,12-dimethylbenz[*a*]-anthracene (DMBA) or 200 μg benzo[*a*]pyrene (BP) in polyethylene glycol (PEG) by stomach tube, after food had been withheld overnight, were examined 10 weeks later and onward, and it was found that they had developed a moderate number of benign stomach tumors. Smaller single doses of DMBA (50 μg) or BP (50 or 12.5 μg) in PEG evoked fewer tumors or none. When these single doses of DMBA or BP were followed by 40 once weekly treatments by stomach tube of 0.05 ml undiluted lime oil, the tumor incidence in the forestomach was always markedly increased and in some experiments malignant tumors were induced. Treatment with lime oil, after administration of PEG only, regularly evoked a few tumors. When two intragastric doses of urethan (16 mg each) in water were given instead of DMBA or BP, a few forestomach tumors were elicited in the groups treated with lime oil. The tumor-promoting effect of lime oil was not destroyed by being heated under reflux condenser for 3 hours. Orange oil and highly purified *d*-limonene irritated the forestomach epithelium far more than lime oil. Both induced a few tumors when given once weekly by stomach tube after a single dose of 50 μg BP in PEG. However, essentially the same result was obtained when orange oil or *d*-limonene was given in the same way after administration of PEG. The effects on the gastrointestinal tract of mice of lime oil administered in the diet and of orange oil added to the drinking water were also investigated. It was found that the addition of lime oil to the diet

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considerably increased tumor incidence following administration of a single dose of 50 μg BP and that the effect was related to the concentration of oil in the diet. Lime oil mixed with the diet appeared to be as effective in the promotion of tumors as it was when given by stomach tube after food was withheld overnight. The results with orange squash were doubtful and puzzling. Orange squash appeared to be a weak tumor promoter for the mouse forestomach, regardless of its orange oil content. Anatomical differences and considerations of dosage render it unlikely that the citrus oils are a serious tumor-promoting hazard for man.—J Nat Cancer Inst 35: 771–787, 1965.

ORANGE OIL was found to be a tumor-promoting agent for mouse skin when applied repeatedly after a single dose of 300 μg 7,12-dimethylbenz[*a*]anthracene (DMBA) (*1*). It was later demonstrated that grapefruit, lemon, and lime oils possessed similar activity and that the tumor-promoting activity was associated with the terpene fraction of the oils. It was thought that in all probability the major constituent, *d*-limonene, was the active principle in orange oil. The terpeneless fraction showed neither hyperplastic nor tumor-promoting activity (*2*).

These results warranted further investigation, as citrus oils are present in many foodstuffs. It was decided to test the oils for carcinogenicity and tumor-promoting activity in the gastrointestinal tract of the mouse. In the first three experiments, lime oil was given by stomach tube once weekly for 40 weeks after initiating treatment with either DMBA, benzo[*a*]pyrene (BP), or urethan. In the second experiment, orange oil, pure *d*-limonene, and heated lime oil were also tested for carcinogenicity and tumor-promoting activity. As the results of the first experiment were encouraging, we decided to test lime oil in a way that more closely resembled normal exposure in man. In experiment 4, lime oil was added to the basic powdered diet, and, in experiment 5, orange squash was substituted for the drinking water.

Two polycyclic hydrocarbons, DMBA and BP, were used as initiating agents, the former because of its exceptional potency as a tumor initiator for mouse skin, and the latter because it is ubiquitous in the human environment. Berenblum and Haran (*3*) administered these and other hydrocarbons followed by repeated doses of croton oil by stomach tube to mice, in an attempt to induce tumors of the gastrointestinal tract by a two-stage process. However, the dose given (1500 μg) produced so many tumors itself that any effect croton oil may have had was obscured. Later, Bock and King (*4*) concluded from their own results that in mice the forestomach is more sensitive than the skin to chemical carcinogenesis. We used a range of doses of BP, the highest (200 μg) being about one eighth of that used by Berenblum and Haran. The two lower doses were 50 and 12.5 μg. We hoped that one of the doses within this range would be subcarcinogenic but sufficient to initiate tumors. It was not possible to test a similar range of doses of DMBA owing to lack of animal accommodation. A dose of 100 μg was selected. In experiment 2 this was reduced to 50 μg because 100 μg was above the subcarcinogenic range. The highest dose of BP was omitted from the experiment for the same reason.

Polyethylene glycol 400 was used as the solvent for DMBA and BP since it had been shown to be a suitable solvent for carcinogenic hydrocarbons administered *per os* (*5*). It is a lipophylic, hydrophilic substance of low toxicity that allows the hydrocarbon (*6*, *7*) to penetrate both the forestomach and glandular epithelium.

Urethan was tried as an initiator because it is chemically dissimilar to the polycyclic hydrocarbons. It was interesting to ascertain that the two-stage production of tumors occurring in the earlier experiments was not restricted to combinations of citrus oils with polycyclic hydrocarbons.

A preliminary report (*8*) of the first experiment was published before the full results were available.

MATERIALS AND METHODS

Mice.—Stock albino mice obtained from S. Schofield and Company, Intake Head, Delph, Nr. Oldham, Lancashire, England, or mice bred in this laboratory from parents obtained from this source, were used for all experiments. The animals were housed in groups of 10 in zinc or galvanized iron cages and bedded on white wood shavings. The mice in experiments 1, 2, 3, and 5 were fed cubed diet 41B,[7] obtained from E. Dixon and Son Limited, Ware, Hertfordshire, England, and given water *ad libitum*. The powdered basic diet used in experiment 4 was also prepared according to the formula for diet 41B, but it was obtained from Messrs. J. Rank Ltd., London E.C.3.

All the mice were vaccinated on the tail with sheep lymph at 6 to 8 weeks of age as a precaution against ectromelia. Only positive reactors were used. Female mice were used in experiment 1 and mice of both sexes in all others.

Chemicals.—7,12-Dimethylbenz[*a*]anthracene (DMBA) and benzo[*a*]pyrene (BP) were obtained from L. Light and Company, Colnebrook, Buckinghamshire, England, and polyethylene gylcol (PEG) of average molecular weight 400 and urethan (ethyl carbamate) from British Drug Houses Limited, Poole, Dorset, England. The arachis oil used in the fourth experiment was of British pharmacopoeia grade.

A well-known British firm specializing in the importation and processing of essential oils kindly supplied the oils of lime and sweet orange. The lime oil was from the West Indies and the orange oil came from Florida and contained no artificial colorants.

A specially purified sample of *d*-limonene was kindly sent to us by Dr. W. L. Stanley of the Agricultural Research Service, U.S. Department of Agriculture, South Chester Avenue, Pasadena, California. It was prepared as follows: Terpene and terpeneless fractions were obtained from orange oil by fractional distillation. The terpene fraction was purified chromatographically with the use of powdered silicic acid, free from aluminum oxide that causes polymerization of terpenes. The purified terpene fraction was then separated into the component terpenes by gas chromatography. The *d*-limonene produced by this procedure was shown by gas chromatographic analysis to be contaminated with less than 0.1 percent *p*-cymene, and it was thought unlikely that it contained any high boiling-point polymeric contaminants.

Lime oil of the same origin as that used in the other experiments was heated in a boiling bath under a reflux condenser for 3 hours and used in the second experiment.

For experiment 4, the special diets were made as follows: The powdered form of diet 41B (obtained from Messrs. Rank Ltd.) was the basic ingredient of all the diets. For more even dispersion, the amounts of lime oil were added to 20 ml of arachis oil. This solution was added to the 41B meal and mixed for at least 10 minutes in an electric mixer. Sufficient water was added to form a dough. This was rolled out, divided into squares, and dried at room temperature. The control diet was made in the same way by the addition of 20 ml arachis oil to each kg of 41B meal. The diets were made up once or twice weekly and never stored over 10 days.

Samples of the diets were analyzed for their content of lime oil immediately after preparation and after storage for 10 days. Irrespective of the initial dose level, approximately 75 percent of the lime oil was lost during preparation but little, if any, was lost during storage. Thus the diet to which 8 ml per kg of lime oil had been added gave the same total dose over 40 weeks as that given in experiments 1 and 2.

In experiment 5, the two orange squashes, control and standard, were specially prepared by the same British firm that supplied the orange and lime oils. The composition of the two squashes was:

Control orange squash
- Six times concentrated orange juice—9 fluid oz.
- Unconcentrated orange juice—55 fluid oz.
- Citric acid monohydrate—Sufficient to produce a final concentration of 1 percent w/v
- Syrup, British Pharmacopoeia—73 fluid oz.

[7] Diet 41B consists of wheatmeal, 47%; Sussex ground oats, 40%; white fish meal, 8%; dried skim milk, 3%; dried yeast, 1%; sodium chloride, 1%. To each ton of diet is added a stabilized vitamin supplement which supplies: Vitamin A, 4 million units; vitamin D3, 1 million units; vitamin B2, 1.5 g; vitamin B12, 3.25 mg; vitamin B1, 0.5 g; pantothenic acid, 0.5 g; nicotinic acid, 2.5 g; vitamin E, 1.25 g; vitamin K, 0.5 g; choline chloride, 25.0 g.

Sucrose—4 lb.
Sodium bisulfite—Sufficient to produce a final concentration of 350 ppm of SO_2
Water—Sufficient to make 1 imperial gallon
Standard squash
Oil of sweet orange—5.75 fluid oz.
Powder gum acacia, British Pharmacopoeia—2.875 oz.
Water—5.75 fluid oz.
Made into an emulsion and added to 6 imperial gallons of the control squash.
Both of these squashes were diluted 1 in 3 with tap water immediately before use.

Treatment.—In all experiments, the initial dose in 0.2 ml of solvent was given by stomach tube, introduced into the esophagus. In experiments 1 to 3, the secondary treatment was 40 weekly doses of 0.05 ml of the appropriate citrus oil by stomach tube. Berenblum and Haran (*5*) found that the tumor yield was increased if food was withheld from the mice for 18 hours before each treatment. Accordingly, all mice were denied food overnight before each treatment. Food was also withheld overnight from control groups given no secondary treatment. Details of treatment in each experiment are shown in the tables.

All mice were weighed every 2 weeks and their average weight per group was recorded graphically.

Examination.—Mice were examined weekly during the first 25 weeks of experiments and daily thereafter. Abnormalities were noted. Sick mice were killed and, like those found dead, were autopsied.

The entire gastrointestinal tract was examined from the pharynx to the anus. The stomach and intestines were distended with formol saline. The intestines were unraveled and examined for tumors and other lesions by transmitted light and palpation. The stomach was fixed in formol saline for 24 hours, slit open, and examined with a hand lens. Representative neoplastic lesions, if any, and/or at least one, usually several, segments were taken for histological examination from all stomachs.

Histology.—Segments from the stomach and any organ showing gross pathological change were fixed in formol saline, embedded in paraffin wax, and stained with hematoxylin and eosin. Other stains were used as indicated.

EXPERIMENT 1

Tumor Promotion by Lime Oil in the Forestomach After a Single Dose of 7,12-Dimethylbenz[a]-anthracene or Benzo[a]pyrene

Details of treatment are given in table 1. Half the mice surviving at the 41st week were killed and autopsied. Results at this stage were published elsewhere (*8*). The remaining mice were allowed to complete their natural lifespan and the few still alive were killed at 569 days.

Results

Hyperplasia of the forestomach.—Sections from the forestomach of all mice given lime oil showed hyperplasia of the epithelium, due mainly to an increased number of prickle cells (acanthosis). Hyperkeratosis accompanied the hyperplasia in most mice. At 40 weeks, the hyperplasia was

TABLE 1.—Incidence of tumors of forestomach in mice of experiment 1

Group	Tumor-initiating treatment	Tumor-promoting treatment	Number of mice*	Number of mice with tumors	Total tumors	Total carcinomas	Average No. of tumors per survivor
1	100 μg DMBA†	Lime oil	14	14	78	2	5.5
2	100 μg DMBA	None	17	15	45	0	2.6
3	200 μg BP‡	Lime oil	17	15	58	2	3.2
4	200 μg BP	None	17	8	27	0	1.5
5	50 μg BP	Lime oil	12	10	32	2	2.7
6	50 μg BP	None	19	0	0	0	0
7	12.5 μg BP	Lime oil	17	10	20	2	1.1
8	12.5 μg BP	None	17	3	3	0	0.2
9	Solvent (PEG)§	Lime oil	13	5	5	0	0.4
10	None	Lime oil	15	1	1	0	0.1
11	None	None	17	0	0	0	0

*Mice surviving more than 60 days and autopsied within 24 hours of death.
†7,12-Dimethylbenz[a]anthracene.
‡Benzo[a]pyrene.
§Polyethylene glycol.

slight or moderate, but it did not further decrease after the cessation of treatment. Hyperplasia was rarely seen in mice not treated with lime oil and, if present, was never more than slight.

Tumors of the forestomach.—The first tumor was seen in a mouse of group 1 dying in the 10th week of the experiment. The 12 mice that died before this and the 33 with autolysis which was too far advanced for an adequate autopsy were excluded from the results.

Table 1 gives the incidence of benign and malignant tumors of the forestomach in all groups. The incidence was significantly higher in the groups receiving lime oil after a single dose of DMBA or BP than it was in groups receiving comparable doses of DMBA or BP only, or lime oil only.

Figure 1 shows the stomach of a mouse in each of groups 1 and 2 viewed by transmitted light. The tumors seen in the mouse of group 1 are larger and more numerous than those in the group 2 mouse. The size and number of lesions in both mice were typical of those seen in their respective groups. Figure 2 shows the microscopic appearance of a forestomach papilloma in a mouse of group 1.

Microscopic examination showed that most of the tumors were benign papillomas. Eight malignant tumors were seen, all in mice given polycyclic hydrocarbon plus lime oil. The first arose in a mouse of group 3 at 41 weeks and the others at intervals thereafter. All were squamous cell carcinomas. Invasion of the muscle coat was taken as the essential criterion of malignancy. One carcinoma in a mouse of group 1 had penetrated the stomach wall and invaded adjacent parts of the liver, spleen, diaphragm, and body wall (figs. 3 and 4). No metastases from any of these tumors were seen.

Tumors of the glandular stomach.—In most instances, the glandular mucosa of the stomach was normal. Slight atrophy and cyst formation such as those described by Stewart *et al.* (*9*) were seen occasionally. Neoplasms were observed in 7 mice, 2 before and 5 after the 70th week of the experiment. Most of these lesions were benign, but one that was larger than the others was possibly malignant. Microscopically, it was composed of cuboidal cells and had a well-differentiated, adenomatous structure. The cells had penetrated the muscularis mucosa but had not reached the deeper muscle coats. Stewart *et al.* (*9*) described similar lesions which they thought were premalignant in rats fed a diet containing *N*,*N*′-2,7-fluorenylenebisacetamide. Adenocarcinomas of the glandular stomach have also been reported in rats fed *N*-nitroso-*N*-alkylurethans (*10*). The tumors in the present experiment were seen in both test and control groups and therefore cannot be attributed to treatment.

Tumors of other tissues.—The incidence of tumors of all sites is summarized in table 2.

Skin tumors were seen only in mice given DMBA or BP. It is interesting that 5 of the 7 skin tumors arose on the head. This agrees with the observation that tumors arising after a single relatively small application of DMBA to the dorsal skin tend to be situated on the head and neck (*11*).

From table 2 it appears that treatment with lime oil reduces the incidence of mammary adenocarcinomas. It is difficult to find a logical reason for this, but the observation may warrant further investigation.

EXPERIMENT 2

Confirmation of the Tumor-Promoting Effect of Lime Oil and the Testing of Orange Oil, *d*-Limonene, and Heated Lime Oil for Similar Activity

Three hundred and thirty mice were distributed at random among the nine experimental groups. Between 20 and 30 mice were allotted to each group, the two sexes being represented almost equally. Details of treatment are shown in table 3.

At the end of secondary treatment the mice were allowed to complete their lifespan. An outbreak of ectromelia seriously depleted the number of mice, despite revaccination at the start of the epizootic. Losses in the different groups and in the two sexes were similar.

All mice were autopsied as previously described.

Results

Hyperplastic and inflammatory changes in the forestomach.—The findings in groups treated with lime oil and groups receiving no secondary treatment were similar to those described for experiment 1. The epithelium of the forestomach was much more

TABLE 2.—Incidence of tumors of all sites in mice of experiment 1

Group	Treatment	Number of mice*	Forestomach tumors	Tumors of glandular stomach	Leukemia	Mammary adenocarcinomas	Skin tumors	Lung adenomas	Other neoplasms
1	100 μg DMBA† and lime oil	14	78‡	0	1	0	0	5	1 uterine sarcoma
2	100 μg DMBA only	17	45	1	3§	1	2	3	1 hepatoma, 1 sarcoma of subcutaneous tissue
3	200 μg BP‖ and lime oil	17	58‡	1	2	0	2	1	None
4	200 μg BP only	17	27	2	1	2	2¶	5	None
5	50 μg BP and lime oil	12	32‡	0	1	0	0	1	None
6	50 μg BP only	19	0	0	2	2	0	0	1 sarcoma of abdominal wall, 1 sebaceous cell adenoma of nipple
7	12.5 μg BP and lime oil	17	18‡	0	1	0	1	4	1 lipoma
8	12.5 μg BP only	17	3	0	1	2	0	1	None
9	Solvent and lime oil	13	5	2	1	0	0	2	None
10	Lime oil only	15	1	0	0	0	0	2	1 uterine adenocarcinoma
11	None	17	0	1	4**	2	0	2	1 uterine sarcoma

*Mice surviving more than 60 days and autopsied within 24 hours of death.
†7,12-Dimethylbenz[a]anthracene.
‡Two squamous carcinomas.
§One with thymic involvement.
‖Benzo[a]pyrene.
¶One squamous carcinoma.
**Two with thymic involvement.

TABLE 3.—Incidence of tumors of the forestomach in mice in experiment 2

Group	Tumor-initiating treatment	Tumor-promoting treatment	Number of mice*	Number of tumor-bearing mice	Total tumors	Carcinomas	Average No. of tumors per survivor
1	50 μg DMBA†	Lime oil	25	25	108	4	4. 3
2	50 μg DMBA	None	19	12	26	0	1. 4
3	50 μg BP‡	Lime oil	22	10	25	0	1. 1
4	50 μg BP	Orange oil	23	8	22	1	0. 95
5	50 μg BP	*d*-Limonene	23	5	8	0	0. 3
6	50 μg BP	Heated lime oil	23	14	29	0	1. 3
7	50 μg BP	None	17	2	2	0	0. 1
8	12.5 μg BP	Lime oil	17	9	12	1	0. 7
9	12.5 μg BP	None	18	2	3	0	0. 1
10	Solvent	Lime oil	21	2	3	0	0. 1
11	Solvent	Orange oil	18	8	17	0	0. 95
12	Solvent	*d*-Limonene	15	2	3	0	0. 2
13	None	Lime oil	22	2	2	0	0. 1
14	None	None	18	0	0	0	0

*Surviving more than 60 days and autopsied within 24 hours of death. *Note:* Survival time after the 60th day was similar in all groups and no obvious sex difference in response was observed. The results for the sexes have therefore been combined.
†7,12-Dimethylbenz[*a*]anthracene.
‡Benzo[*a*]pyrene.

severely damaged by orange oil and *d*-limonene than by lime oil. The forestomach of mice in groups receiving either of these two substances was frequently grossly shrunken and scarred and occasionally there were small ulcers in the squamous epithelium. Microscopic examination showed marked hyperplasia of the squamous epithelium, with ulceration and much chronic inflammation, including a marked increase in the collagen in the submucosa. These changes were especially severe in mice dying early in the experiment. The incidence of this severe damage was 15 of 38 in mice treated with *d*-limonene, 15 of 41 in those treated with orange oil, and 2 of 107 in mice treated with lime oil.

Tumors of the forestomach.—The first papilloma of the forestomach was seen in a mouse of group 1 dying during the 9th week of secondary treatment. This latent period was very similar to that seen in the previous experiment. Twenty mice dying before this time and another 28 with autolysis too advanced for adequate autopsy were excluded from the results.

The results are summarized in table 3. There was no significant sex difference in the incidence of forestomach tumors. Six mice receiving treatment with both initiator and promoter developed squamous carcinomas of the forestomach. Three of these tumors, 2 in mice of group 1 and 1 in a mouse of group 4, had penetrated all the layers of the stomach wall and invaded adjacent organs. In one mouse of group 1 there was a single metastasis in the local lymph gland, and in another, both direct invasion of the liver (fig. 5) and multiple separate metastases in it, as well as a metastatic deposit in the pancreas, separate from the main tumor mass (fig. 6).

Pathology of the glandular stomach.—As in the previous experiment, the glandular mucosa was usually normal. Several very wasted mice had small ulcers that appeared to be similar to those described by Hulse (*12*). Four neoplasms of the glandular mucosa were seen (table 4); one, in a mouse of group 14 that received no treatment whatsoever, was an anaplastic carcinoma penetrating the entire stomach wall.

Tumors of other sites.—Tumors of other organs are listed in table 4. The range of tumors was similar to that in the previous experiment, though the total number was less. In this experiment there was no tendency for skin tumors to localize on the head and neck.

Growth and general pathology.—The average weight of adult mice in this experiment was less than that in experiment 1, but the form of the weight curves was similar.

Routine autopsy revealed that the most frequent cause of death was ectromelia, which killed more than a third of the mice at risk. Otherwise, the

TABLE 4.—Tumors of all sites found in mice of experiment 2

Group	Treatment	Number of mice*	Fore-stomach tumors	Tumors of glandular stomach	Leukemia	Mammary adeno-carcinomas	Skin tumors	Lung adenomas	Other neoplasms
1	50 μg DMBA† and lime oil	25	108‡	1	1	1	1	1	None
2	50 μg DMBA only	19	26	0	0	1	1	1	1 sarcoma
3	50 μg BP§ and lime oil	22	25	0	0	0	0	2	None
4	50 μg BP and orange oil	23	22‖	0	0	0	0	0	None
5	50 μg BP and *d*-limonene	23	8	1	0	0	0	0	1 hepatoma
6	50 μg BP and heated lime oil	23	29	0	1	0	0	0	None
7	50 μg BP only	17	2	1	0	0	0	4	None
8	12.5 μg BP and lime oil	17	12‖	0	0	0	0	2	None
9	12.5 μg BP only	18	3	0	2	0	0	2	None
10	Solvent and lime oil	21	3	0	0	0	0	1	None
11	Solvent and orange oil	18	17	0	0	0	1	3	None
12	Solvent and *d*-limonene	15	3	0	0	0	1	4	Multiple sarcomatous nodules in subcutaneous tissue
13	Lime oil only	22	2	0	1	1	1	3	1 hepatoma, 1 carcinomatosis, site of primary unknown
14	None	18	0	1	1	0	0	2	None

*Mice surviving more than 60 days and autopsied within 24 hours of death.
†7,12-Dimethylbenz[*a*]anthracene.
‡Four squamous carcinomas.
§Benzo[*a*]pyrene.
‖One squamous carcinoma.

TABLE 5.—Incidence of tumors of forestomach in mice of experiment 3

Group	Tumor-initiating treatment*	Tumor-promoting treatment*	Number of mice†	Number of tumor-bearing mice	Total tumors‡
1	32 mg urethan	0.05 ml lime oil weekly for 40 weeks	31	7	8
2	32 mg urethan	None	37	1	2
3	Distilled water	0.05 ml lime oil weekly for 40 weeks	32	2	3
4	None	None	34	1	1

*All treatments given by stomach tube.

†Mice surviving more than 100 days and autopsied within 24 hours of death. *Note:* Survival was similar in the 4 groups and there was no obvious sex difference in response.

‡Benign papillomas.

autopsy findings were similar to those described in the preceding experiment in type and incidence.

EXPERIMENT 3

Tumor Promotion by Lime Oil After Initiation by Urethan

One hundred and sixty mice were divided at random into 4 groups of 30 males and 10 females each. Details of treatment are shown in table 5.

One week after the 40th dose of lime oil was administered, the surviving mice were killed and autopsied, like those found dead earlier in the experiment.

Results

Seven mice that died before the 100th day of secondary treatment and another 19 with autolysis too far advanced for adequate autopsy were excluded from the results.

The alimentary canal.—The incidence of tumors of the forestomach is summarized in table 5. The tumor yield was slightly greater in the group receiving both urethan and lime oil (8 tumors in 7 of 31 mice) than in the group receiving lime oil only (3 tumors in 2 of 32 mice). All the tumors were benign. The significance of this difference is doubtful.

In groups 1 and 3 severe damage to the squamous epithelium of the forestomach, similar to that described in experiment 2, occurred in 6 of 63 mice. As in experiments 1 and 2, the epithelium of the forestomach was hyperplastic in all mice treated with lime oil and normal in those receiving no secondary treatment.

In most mice the glandular epithelium of the stomach was normal. An adenoma was seen in a mouse of group 2, and in one of group 4 there was marked hyperplasia of the glandular mucosa.

The only abnormalities seen in the intestines (all in urethan-treated mice) were blood cysts in the Peyer's patches in 4 mice.

General pathology.—Lung adenomas were observed, particularly in animals of the 2 urethan-treated groups. In group 1 there were 277 adenomas in 29 of 30 mice, in group 2, 287 in 34 of 37 mice, in group 3, 6 in 6 of 32 mice, and in group 4, 1 in 1 of 34 mice. Urethan, therefore, markedly increased the incidence of lung adenomas, as was expected. Lymphocytic leukemia was observed in 4 mice: 2 in group 1 and 1 each in groups 2 and 3. The only other neoplasms seen were 4 skin papillomas, all in urethan-treated mice.

Other pathological changes were similar to those in previous experiments.

EXPERIMENT 4

Effect on Mouse Forestomach of the Addition of Lime Oil to the Standard Diet

Three hundred and twenty mice were divided at random into 8 groups of 20 males and 20 females each.

After food had been withheld overnight, a single dose of 50 μg BP in 0.2 ml PEG was given by stomach tube to mice of groups 1 through 4. Groups 5 through 8 received 0.2 ml PEG only. Three weeks later the mice were put on special diets. They were fed solely on these diets until the experiment was ended at 42 weeks. Mice in groups 1 and 5 received a diet without lime oil, groups 2 and 6 a diet containing 2 ml of lime oil

per kg of dry meal, groups 3 and 7 a diet containing 8 ml per kg, and groups 4 and 8 a diet containing 32 ml per kg. Details of the preparation of the diet have been given previously.

During the 42d week all the surviving mice were killed and necropsied, like those dying earlier in the experiment.

Results

Twenty-one mice died before the 100th day of the experiment, and autolysis was too far advanced in another 19 for a satisfactory necropsy. These mice were excluded from the results.

Findings in the forestomach.—Hyperplasia of the forestomach epithelium was not as marked as that observed after the administration of lime oil by stomach tube and it was always slight. As the concentration of lime oil increased, so did the number of mice in the group showing hyperplasia. In no instance was scarring of the forestomach seen. Ulceration was observed in one mouse in each of groups 4 and 8; both had received the diet with the highest lime oil content.

The incidence of tumors of the forestomach is summarized in table 6. Most tumors were benign papillomas, but a squamous cell carcinoma was seen in each of groups 1, 2, 3, and 4. These tumors had penetrated the muscularis mucosa but had not reached the deeper muscle coats. The number of tumor-bearing mice was higher in groups 2, 3, and 4 than in group 1, but the difference was not statistically significant by the χ^2 test. A comparison of the average number of tumors per survivor showed a more convincing difference between groups 2, 3, and 4 combined and group 1, and analysis by the t test gave $P<0.01$. The incidence of tumors in groups 3 and 4 was higher than in group 2, but the difference between groups 3 and 4 was slightly in the opposite direction. The mice receiving the control diet after a single dose of BP bore many more tumors, including one squamous cell carcinoma, than were expected from the results of previous experiments. The difference in tumor incidence between mice of group 1 of this experiment and those in the groups of experiments 1 through 3 that had received 50 μg BP could be explained either by a difference in the basic diet or by a tumor-promoting effect attributable to arachis oil.

Survival and response were essentially similar in the two sexes.

Incidence of tumors of other sites.—Table 7 summarizes these findings.

Growth and general pathology.—Examination of the weight charts showed that neither the growth rate nor the adult weight was altered by the addition of lime oil to the diet.

The general health of the mice was good and very few died early in the experiment. An epizootic of ectromelia killed 15 mice and an outbreak of Tyzzer's disease another 8. With the exception of bronchopneumonia, the incidence of other pathological changes was similar to that seen in previous experiments. Bronchopneumonia was observed in only 2 mice. This low incidence suggests it was the method of administration, *i.e.*, by stomach tube, rather than the lime oil itself, that caused the excessive incidence of this disease in the previous experiments.

TABLE 6.—Incidence of tumors of forestomach in mice of experiment 4

Group	Tumor-initiating treatment*	Tumor-promoting treatment	Number of mice†	Number of tumor-bearing mice	Total tumors	Total carcinomas	Average No. of tumors per survivor
1	50 μg BP‡	Control diet	37	13	18	1	0.5
2	50 μg BP	2 ml lime oil per kg diet	35	16	28	1	0.8
3	50 μg BP	8 ml lime oil per kg diet	30	17	43	1	1.4
4	50 μg BP	32 ml lime oil per kg diet	38	18	41	1	1.1
5	Solvent	Control diet	36	0	0	0	0
6	Solvent	2 ml lime oil per kg diet	36	1	1	0	0.03
7	Solvent	8 ml lime oil per kg diet	29	2	2	0	0.07
8	Solvent	32 ml lime oil per kg diet	39	3	3	0	0.08

*Given in 0.2 ml polyethylene glycol by stomach tube.

†Surviving more than 100 days and autopsied within 24 hours of death. As no obvious sex difference in response was observed, the results for both sexes are shown together.

‡Benzo[a]pyrene.

TABLE 7.—Incidence of tumors of all sites in mice of experiment 4

Group	Treatment	Number of mice	Forestomach tumors	Tumors of glandular stomach	Leukemia	Lung adenomas	Skin tumors	Tumors of other organs
1	50 μg BP* followed by control diet	37	18	0	0	3	0	None
2	50 μg BP followed by 2 ml lime oil per kg diet	35	28	0	2	4	0	None
3	50 μg BP followed by 8 ml lime oil per kg diet	30	43	0	1	0	0	None
4	50 μg BP followed by 32 ml lime oil per kg diet	38	41	2†	1	4	1	1 granulosa cell tumor of the ovary
5	Solvent followed by control diet	36	0	1	2	4	1	1 mammary adenocarcinoma
6	Solvent followed by 2 ml lime oil per kg diet	36	1	2	0	2	0	1 mammary adenocarcinoma
7	Solvent followed by 8 ml lime oil per kg diet	29	2	0	1	3	1	None
8	Solvent followed by 32 ml lime oil per kg diet	39	3	0	0	4	0	1 hepatoma

*Benzo[*a*]pyrene.
†One adenocarcinoma.

TABLE 8.—Incidence of tumors of forestomach in mice of experiment 5

Group	Tumor-initiating treatment (given by stomach tube)	Tumor-promoting treatment	Number of mice*	Number of tumor-bearing mice	Total tumors	Average No. of tumors per survivor
1	50 μg BP†	Standard orange squash	38	12	22	0. 6
2	Solvent	Standard orange squash	37	2	4	0. 1
3	50 μg BP	Control squash	30	9	16	0. 5
4‡	50 μg BP	None	36	2	2	0. 06

*Mice surviving more than 100 days of tumor-promoting treatment and autopsied within 24 hours.
†Benzo[*a*]pyrene.
‡This group consisted of mice treated with 50 μg BP only in experiments 1 and 2.

EXPERIMENT 5

Possible Carcinogenicity and Tumor-Promoting Activity of Orange Squash in the Forestomach

One hundred and twenty mice were divided at random into 3 groups of 20 males and 20 females

After food was withheld overnight, a single tumor-initiating dose of 50 μg BP in 0.2 ml PEG was given by stomach tube to groups 1 and 3 and 0.2 ml PEG to group 2.

The orange squashes, at a dilution of 1 in 3, were substituted for drinking water after 3 weeks and thereafter were the only source of liquid available to the mice. Groups 1 and 2 received the "standard squash" and group 3 the "control squash" (*see* Materials and Methods). Group 4, as indicated in table 8, was not contemporary with the other 3 groups and therefore not a strictly valid control. It consisted of 2 groups of mice from experiment 1 (group 6) and experiment 2 (group 7) that had received 50 μg BP and no further treatment. In view of the result of the experiment, it is a pity that a strictly valid control group was not set up. Shortage of animals and space led to this omission.

The standard squash differed from that available to the public in that it had a constant concentration of 0.6 percent orange oil instead of the usual 0.2 to 0.3 percent. Fractional distillation of the control squash showed that the concentration of orange oil was less than 0.01 percent.

Surviving mice were killed after 42 weeks on the experimental squashes and were then autopsied, as were those that had died earlier in the experiment.

Results

Five mice died before the 100th day of the experiment and autolysis was too far advanced in another

10 for an adequate autopsy. These mice were excluded from the results.

Changes in the forestomach.—Hyperplasia was seen in groups 1, 2, and 3. It was slight in most cases but more marked in a few. No ulceration or scarring of the forestomach epithelium was observed. This contrasts with the findings after administration of orange oil by stomach tube.

All the tumors seen in this experiment were papillomas, though some had changes suggestive of incipient malignancy, such as frequent mitotic figures, loss of the basement membrane, and invasion of the subepithelial connective tissue.

As table 8 shows, the incidence of tumors in groups 1 and 3 was similar and the addition of orange oil to the water-soluble extract of the fruit did not appear to enhance the tumor-promoting effect of the squash. A comparison of the number of tumors in group 4 with those in these groups indicates that the squash appeared to have promoting activity. However, such a comparison is not strictly valid since group 4 was not contemporary with groups 1 through 3. The occurrence of a few tumors in group 2 mice, which did not receive an initial dose of BP, suggests that the standard orange squash was not only a tumor promoter but also a weak carcinogen.

Changes in the glandular stomach.—The administration of orange squash did not affect the glandular mucosa in any demonstrable way. As in other experiments, a few cases of ulceration and slight atrophy were seen. The glandular mucosa was cystic in a mouse of group 2. Adenomas were found in 2 mice; one in each of groups 1 and 2.

Growth and general pathology.—From the weight charts it was deduced that orange oil given in the form of a squash had no effect either on growth rate or adult weight.

Routine autopsy revealed changes similar in type and incidence to those found in the previous experiments except that only 1 mouse had bronchopneumonia. This observation supports the previously expounded theory that it was the method of administration of oils and not the oils themselves which caused the high incidence of this disease in other experiments.

Tumors of sites other than the forestomach were seen in 12 mice. Lung adenomas were in 3 mice of group 1, 5 of group 2, and 1 of group 3. Lymphocytic leukemia occurred in 2 mice of group 1 and 1 of group 2. The incidence of tumors of other sites in group 4 is shown in tables 2 (group 6) and 4 (group 7).

DISCUSSION

Action of Citrus Oils in Carcinogenesis

The foregoing experiments have shown that lime oil is a tumor-promoting agent for the forestomach of the mouse. Treatment with either polycyclic hydrocarbon (DMBA or BP) alone, or with lime oil alone, induced occasional papillomas of the forestomach. However, repeated treatments with lime oil after a single dose of either of the polycyclic hydrocarbons increased the incidence of both papillomas and carcinomas of the forestomach. This increase was significant and could not be explained by simple summation of the separate effects of lime oil and the polycyclic hydrocarbons. This conclusion applies equally to male and female mice.

Orange oil and *d*-limonene gave more puzzling results. Both were weakly carcinogenic for the forestomach epithelium, but pretreatment with 50 μg BP did not increase the tumor yield. Thus it appears that neither orange oil nor its main constituent, *d*-limonene, promotes tumor development when given after a single dose of BP known to be sufficient for initiation before promotion with lime oil. This finding is the more unexpected because both these materials are good promoting agents for mouse skin, for which they are not carcinogenic. The explanation of this curious finding must await the results of further experiments. With the orange oil, it cannot be due to differences in the composition of various batches of oil, since positive results were obtained in skin-painting experiments when the same batches of oil were used. During this work it became increasingly apparent that the skin and epithelium of the forestomach differ in their reaction to various chemical agents. The explanation of the results with orange oil and *d*-limonene could lie in this inherent difference. Alternatively, the scarring and extremely marked hyperplasia seen frequently in the forestomachs of mice treated with orange oil or *d*-limonene, but only rarely in mice treated with lime oil, may indicate that the concentration of

oil reaching the cells of the epithelium was too high. In the authors' opinion this is the most likely explanation. In the light of the results obtained, it is unfortunate that the experiments with *d*-limonene in the skin and forestomach were conducted with samples of different origin and purity. There was not enough of the purer material to carry out a skin-painting test. The cruder sample was not tested in the forestomach because it was assumed that *d*-limonene was the active principle. Obviously the next step is to obtain the missing information.

It is, of course, possible that the active principle is present in the crude sample of *d*-limonene as an impurity which is lost on purification by gas chromatography. This and other possible explanations need only be considered if the pure sample of *d*-limonene is found to be inactive and the crude material active under identical experimental conditions.

The tumor-promoting activity of lime oil was not destroyed by being heated to 100° C at atmospheric pressure for 3 hours. Thus it is unlikely that the tumor-promoting activity of citrus oils would be destroyed during cooking.

The tumor yield was slightly greater in the group receiving both urethan and lime oil (8 tumors in 7 of 31 mice) than in the group receiving lime oil only (3 tumors in 2 of 32 mice). However, the significance of this difference is doubtful, not only because it is small, but also because the incidence is similar to that seen in the groups of mice in experiments 1 and 2 treated with PEG and lime oil (8 tumors in 7 of 34 mice). Either urethan is not an initiator for the forestomach or, more probably, the dose given was insufficient for initiation before promotion with lime oil. A similar conclusion was reached in an experiment on mouse skin (*2*) in which urethan was used as the initiator and orange oil as the promoter.

The addition of lime oil to the diet in concentrations insufficient to affect the general health of the mice significantly increased the incidence of tumors of the forestomach after a single dose of 50 μg BP. The tumor-promoting effect in response to lime oil administered in the diet was very similar to that of the same total dose of lime oil administered weekly by stomach tube. An average of 1.4 tumors per survivor occurred in mice receiving lime oil in the diet and of 1.7 tumors per survivor in those receiving lime oil by stomach tube. This indicates that the total dose of lime oil, rather than the concentration of lime oil reaching the stomach, determines the final tumor incidence. It would be interesting to know whether mice in the group receiving the lowest concentration of lime oil in the diet would eventually have had the same tumor incidence as those in groups receiving the two higher concentrations of lime oil in the diet.

These results are important in the assessment of the possible significance for man of the tumor-promoting activity of the citrus oils in mouse tissues. They show clearly that it is not essential for the cells to be exposed to a high concentration of oil, as in experiments 1 through 3. Exposure to a low concentration over a longer period, by mixture of the material with the diet, seems to be equally effective.

Orange squashes in a form very similar to that prepared for human consumption appeared to possess tumor-promoting activity for the forestomach of the mouse. However, the experiment that indicated this included no strictly valid control group treated with BP only and, from a quantitative point of view, the results were somewhat puzzling because the tumor-promoting activity of the squashes was similar, whereas their content of orange oil differed 60-fold.

The experiments described constitute the first clear demonstration of the two-stage mechanism of carcinogenesis in the forestomach. The fact that both the skin and the lining of the forestomach are squamous epithelium gives them a similarity more apparent than real. The absence of hair follicles and sebaceous glands makes the forestomach a much simpler structure than the skin. This being so, there were no *a priori* grounds for the assumption that the two-stage mechanism, so easily demonstrated in the former, would be demonstrated in the latter. The present demonstration makes it appear more likely that the two-stage process is a feature of carcinogenesis in general.

Possible Implications for Man

In any assessment of the relevance of experimental results for man it is essential to consider at least 3 points: 1) species variation; 2) wheth

the experimental conditions are applicable to those normally encountered by man; 3) any relevant epidemiological evidence.

The tumors induced in the experiments described all originated in the epithelium of the forestomach. The forestomach in the rodent has no analogous counterpart in man; hence any relevance of these results in the causation of gastric cancer in man is questionable.

These experiments are demonstrations of a two-stage mechanism of carcinogenesis, a process which has not been demonstrated in man, though it has been suggested in bronchial carcinoma (*13*). If one assumes that such a mechanism exists, then it is probable that man is exposed to sufficient BP and other carcinogens to cause initiation. The per capita consumption of citrus oils in Britain, a country that grows no citrus fruit, can be calculated from import and export figures. Over a period of 40 years the average consumption is 600 to 700 ml plus oil derived from fresh fruit. On the basis of comparative body weight and lifespan, the dose given to the mice in the experiments reported here is about 6 times the average consumption per person in Britain. Thus, at present levels of consumption, it seems unlikely that the citrus oils contribute significantly to the incidence of gastric cancer. However, the fact that citrus drinks are now sometimes mixed in the home by pulverization of the entire fruit in a food-mixing machine could lead to a greatly increased intake of citrus oils by some people.

There is no epidemiological evidence to corroborate or refute the experimental finding that citrus oils may act as tumor promoters. Wynder *et al.* (*14*) found that the consumption of citrus fruits had no consistent correlation with the incidence of esophageal cancer. At our suggestion, a large organization in California examined the health records of men involved in picking and packing citrus fruits, but found no excess incidence of cancer. However, there were few permanent employees in this group, since pickers and packers are usually itinerant laborers.

It seems that only a very detailed survey of diets, with special study of citrus fruit-flavored food, on a large sample of the population could settle the question of whether the citrus oils contribute to the incidence of human cancer. Since there are many more urgent problems, such a survey in connection with the citrus oils does not, on present evidence, deserve high priority.

REFERENCES

(*1*) Roe, F. J. C.: Oil of sweet orange: A possible role in carcinogenesis. Brit J Cancer 13: 92–93, 1959.

(*2*) Roe, F. J. C., and Peirce, W. E. H.: Tumor promotion by citrus oils: Tumors of the skin and urethral orifice in mice. J Nat Cancer Inst 24: 1389–1403, 1960.

(*3*) Berenblum, I., and Haran, N.: Influence of croton oil and of polyethylene glycol 400 on carcinogenesis in forestomach of mouse. Cancer Res 15: 510–516, 1955.

(*4*) Bock, F. G., and King, D. W.: A study of the sensitivity of the mouse forestomach toward certain polycyclic hydrocarbons. J Nat Cancer Inst 23: 833–839, 1959.

(*5*) Berenblum, I., and Haran, N.: The influence of dose of carcinogen, emptiness of stomach and other factors on tumor induction in forestomach of mouse. Cancer Res 15: 504–509, 1955.

(*6*) Ekwall, P., Ermala, P., Setälä, K., and Sjöblom, L.: Gastric absorption of 3,4-benzpyrene. II. The significance of the solvent for the penetration of 3,4-benzpyrene into the stomach wall. Cancer Res 11: 758–763, 1951.

(*7*) Norden, G.: The uptake of 3,4-benzpyrene in the stomach of the mouse. Acta Path Microbiol Scand 40: 188–196, 1957.

(*8*) Peirce, W. E. H.: Tumor-promotion by lime oil in the mouse forestomach. Nature (London) 189: 497–498, 1961.

(*9*) Stewart, H. L., Snell, K. C., Morris, H. P., Wagner, B. P., and Ray, F. E.: Carcinoma of the glandular stomach of rats ingesting *N,N'*-2,7-fluorenylenebisacetamide. Nat Cancer Inst Monogr 5: 105–139, 1961.

(*10*) Schoental, R.: Induction of tumors of the stomach in rats and mice by N-nitroso-N-alkylurethans. Nature (London) 199: 190, 1963.

(*11*) Roe, F. J. C.: The development of malignant tumors of mouse skin after "initiating" and "promoting" stimuli. I. Brit J Cancer 10: 61–69, 1956.

(*12*) Hulse, E. V.: Gastric erosions: A terminal phenomenon in starved and in aged mice. J Path Bact 81: 526–528, 1961.

(*13*) Roe, F. J. C., Salaman, M. H., and Cohen, J.: Incomplete carcinogens in cigarette smoke condensate: Tumor-promotion by a phenolic fraction. Brit J Cancer 13: 623–633, 1959.

(*14*) Wynder, E. L., and Bross, I. J.: A study of etiological factors in cancer of the esophagus. Cancer 14: 389–413, 1961.

FIGURE 1.—Stomachs of mice photographed by transmitted light. *Above:* Halves of stomach from mouse of group 2 (expt. 1) that received a single dose of 100 μg dimethylbenz[*a*]anthracene (DMBA) by stomach tube and no further treatment. Stomach was fixed by distention with formol saline. Forestomach is at *top* and glandular stomach and pylorus at *bottom*. No tumors are visible in the forestomach. *Below:* Stomach from mouse of group 1 (expt. 1) treated with DMBA once and then lime oil once weekly for 40 weeks. The 4 opacities, 2 in each half of the forestomach, are benign papillomas. Vessels supplying the tumors are clearly visible. × 2

FIGURE 2.—Papilloma of forestomach from mouse of group 1 (expt. 1) showing abundant keratin. × 40

FIGURE 3.—Carcinoma of forestomach of mouse of group 1 (expt. 1). Tumor had invaded adjacent parts of liver, spleen, diaphragm, and wall of body. Invasion of liver is clearly visible. × 13

FIGURE 4.—Same tumor as shown in figure 3. Invasion of muscle layers of wall of stomach by anaplastic carcinoma is shown. Hematoxylin and eosin. × 160

FIGURE 5.—Squamous carcinoma of forestomach invading liver of mouse of group 1 (expt. 2). Mouse received a single dose of 50 μg dimethylbenz[*a*]anthracene followed by doses of lime oil once weekly for 40 weeks. Hematoxylin and eosin. × 50

FIGURE 6.—Deposit of undifferentiated carcinoma of forestomach in pancreas of mouse of group 1 (expt. 2). Mouse given same treatment as that described in previous legend. Hematoxylin and eosin. × 50