of free arsenate, were characterized in arsenate-fed birds. None of the products of arsenate metabolism could be detected, even in trace amounts in arsanilic acid-fed chicks. *p*-Aminophenylarsenoxide, the reduced form of arsanilic acid, could not be detected in tissues or excreta. The tissue "bound-forms" of arsanilic acid appeared to be excreted via the liver and kidney, and then converted to free arsanilic acid in the intestine and excreta. The chromatographic and electrophoretic characteristics of the major bound arsanilic acid component suggested that it was a conjugate with a small acidic molecule via the arsenic. The other component was chemically very similar to arsanilic acid itself.

Tissue-bound arsanilic acid from swine liver or chick muscle has been fed to second animals, including rats (*5*), chickens (*6*), and man (*7*). The results are best characterized by saying that residual arsanilic acid in edible animal tissues is handled in second animals very much like authentic arsanilic acid.

In the study with rats, residual arsanilic acid was almost completely excreted via the liver and kidney during a 14-day feeding cycle. For comparison, rats excreted only about5 0% of As_2O_3 arsenic. Table II shows the average excretion of the two forms of arsenic. In another study, actual retention of residual arsanilic acid was studied in blood and tissues of chickens (*6*). The residual arsanilic acid in swine liver was partially absorbed by the chicks and appeared in blood and organs of excretion. However, as shown in Table III, the level of arsenic in muscle did not rise commensurate with the increased blood levels. On the other hand, the arsenic of As_2O_3 did appear in muscle and other tissues in proportion to the blood levels.

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FEED ADDITIVES

A Carcinogenicity Evaluation of Potassium Arsenite and Arsanilic Acid

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Tests were made to determine whether $KAsO_2$ might act as an incomplete carcinogen; thus it was tested in conjunction with agents able either to initiate (DMBA) or to promote (croton oil) skin tumors in mice. No activity for $KAsO_2$ was detected. Both benign and malignant skin tumors were induced in skin tumor-susceptible mice by means of a single application of DMBA followed by repetitive applications of croton oil. Both agents were used in amounts that did not produce maximal tumor yields. The influence of arsanilic acid and $KAsO_2$ on the yield of tumors was tested by including one of these arsenicals in the diet of the mice concurrently with exposure to DMBA or to croton oil. No change was found in the tumor incidence of the test mice attributable to the arsenicals.

Massive amounts of potassium arsenite ($KAsO_2$) have been reported to be carcinogenic for man with a special propensity to induce skin cancer (*9, 11*). In contrast, several investigators have concluded that there is no decisive evidence that exposure to arsenic compounds is causally related to ensuing cancer in man. Kenaway (*10*) rejected the evidence for arsenic carcinogenicity while Boyland (*3*) wrote ". . . no unequivocal evidence has ever been presented that arsenic is carcinogenic." More recently, Vallee *et al.* (*14*) stated that although a number of cases of cancer due to arsenic have been officially reported, "It is still questionable that a direct relationship was established." Frost (*6*) reached a similar conclusion.

With respect to the question of the experimental production of cancer by arsenicals, it is clear that no established method exists whereby one can produce tumors at will in laboratory animals (*5*). As the experiments with arsenic alone did not cause tumors, other ways were tried. Neubauer (*11*) has stated that Ciechanowski, Morozowa, and Wilhelmi (1925) observed an accelerating effect of oral ingestion of potassium arsenite on the development of tar carcinoma in rabbits, although both Schiller (1926) and Pucinelli (1930) reported a protective effect of arsenic on the appearance of tumors in the tarred skin of mice.

Further carcinogenicity evaluations of two arsenicals, $KAsO_2$ and arsanilic acid (AA), were undertaken using tests designed to reveal the smallest possible contribution of the test compound to tumor incidence. Because palmer and planter hyperkeratoses are sometimes observed in man after chronic systemic poisoning with arsenic (*7*), and in consideration of the hyperkeratotic lesions that develop on the skin of mice exposed to a single application to the skin of 7,12-dimethylbenz(a)anthracene (DMBA) followed by repetitive applications of croton oil (*1*), the role of $KAsO_2$ on the induction of skin tumors in mice by the two-stage process (*1, 2, 13*) was investigated. Even if the arsenicals were not complete carcinogens but

were able to contribute to only one phase of the process, this activity should be revealed by the technique used.

In the first of four experiments, $KAsO_2$ was tested for its ability to initiate the formation of skin tumors as manifest by subsequent applications of croton oil. The $KAsO_2$ was administered either locally or by mouth. The second experiment was designed to test $KAsO_2$ for tumor-promoting activity following initiation of the process by a single application of a carcinogenic hydrocarbon to the skin of the mouse. Because no tumors attributable to treatment with $KAsO_2$ resulted in these experiments, two additional tests were designed in which the mice were treated with one or the other of two agents responsible for the two-stage process in amounts that did not elicit maximal tumor yields. Either $KAsO_2$ or AA was administered in combination with these levels of initiator or promoter.

Materials and Methods

In the first two experiments, female mice of the Rockland all-purpose strain, 2 to 3 months of age, were used. Skin tumor–susceptible (STS) female mice, 95 to 102 days of age, were used in the third and fourth experiments. The animals were housed in units of 10 each in screen-bottomed metal cages at 75° ± 3° F. Water and a basal diet of Purina Laboratory Chow were given *ad libitum*. The animals were weighed at weekly intervals and inspected twice weekly. The chow was ground for the last two experiments and fed from feeders designed to minimize spillage (*12*) so that food consumption records could be made.

The chemicals used were: arsanilic acid, Abbott Laboratories; benzene, reagent-grade, thiophene-free, and redistilled in this laboratory; croton oil, S. B. Pennick and Co.; 7,12-dimethylbenz(a)anthracene (DMBA), Distillation Products Co.; potassium arsenite, reagent-grade, Coleman and Bell. Solutions of DMBA and croton oil were made on a weight:volume basis using benzene as the solvent. The DMBA solution was freshly made before use. Appropriate amounts of $KAsO_2$ or AA were added to batches of ground Purina Chow and thoroughly blended.

About 1 week prior to the first application of the test substance, the fur was shaved from the test area of the back with surgical clippers. Because of the possibility of mechanical irritation and damage to papillomas, the mice were not shaved after the experiment started. The solutions to be tested were applied as a single drop of 25 μl. in volume to the mid-dorsal region of each mouse at the times specified in each experiment.

Gross identification of both benign and malignant tumors was confirmed peri-

Table I. Papilloma Incidence in Groups of Mice Treated with Croton Oil (CO) Alone or with Croton Oil Preceded by $KAsO_2$; a Test for Tumor Initiation

Treatment	Time, Weeks 6	10	14	18
Control + CO	1/20[a]	4/20	6/20	7/20 (15)
Oral $KAsO_2$ + CO	1/19	3/18	7/17	9/15 (12)
Local $KAsO_2$ + CO	1/20	4/20	6/20	7/20 (11)

[a] Results are expressed as number of papilloma-bearing mice over number of surviving mice in each group at specified time after start of exposure to $KAsO_2$. Figures in parentheses represent total number of papillomas existing in each group at 18th week.

Table II. Papilloma Incidence in Groups of Mice Treated with DMBA Alone or with DMBA Followed by Phenol or $KAsO_2$; a Test for Tumor Promotion

Treatment	Time, Weeks 12	18	24	30
DMBA alone	0/20[a]	0/18	0/18	0/17
DMBA + phenol	4/19	6/17	7/14[b]	7/14[b]
DMBA + $KAsO_2$	0/20[c]	0/20	0/19	0/18

[a] Results are expressed as the ratio of the number of papilloma-bearing mice to the number of surviving mice at specified time after exposure to a single application of DMBA.
[b] One of the seven mice bore a malignant skin tumor.
[c] At the 12th week, five of 20 mice showed gross skin hyperplasia; at 18 weeks, three showed hyperplasia and two had wounds; at 24 weeks, three showed hyperplasia; and at 30 weeks, one showed hyperplasia and three had wounds in the area of $KAsO_2$ treatment.

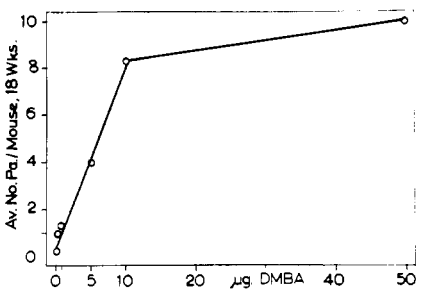

Figure 1. Relationship of tumor incidence to increasing quantities of a single application of DMBA

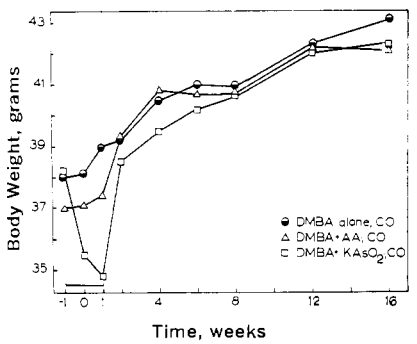

Figure 2. Body weight curves of mice fed 0.1% arsanilic acid or an equivalent amount of As as $KAsO_2$ during the period of initiation shown by the horizontal line

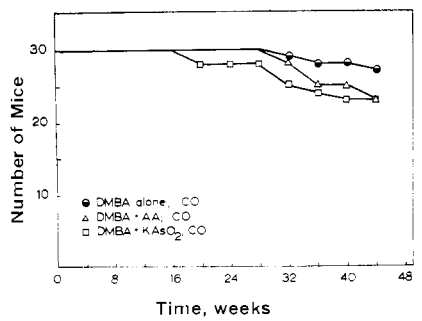

Figure 3. Total number of mice during the course of the experiment

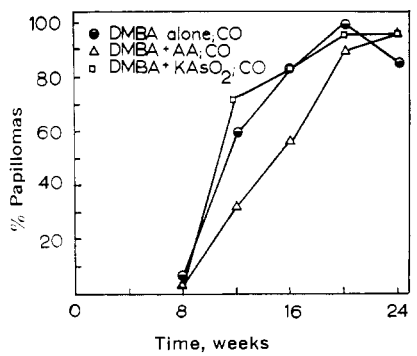

Figure 4. Percentage of mice bearing papillomas after feeding arsenicals at the time of tumor initiation

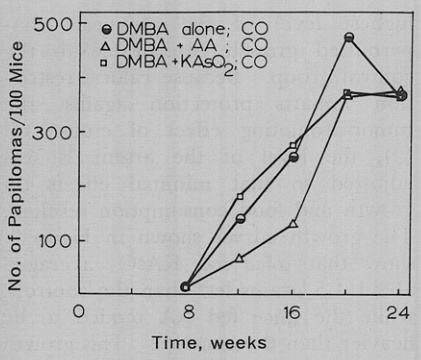

Figure 5. Effect of feeding arsenicals during tumor initiation on the total number of papillomas

Figure 6. Appearance of typical papilloma-bearing mice

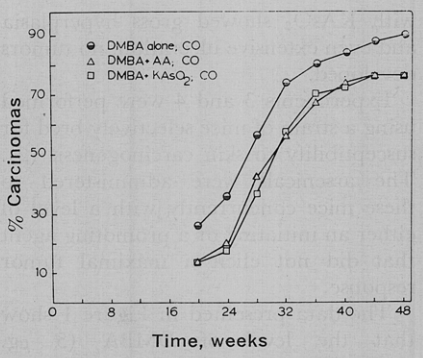

Figure 7. Incidence of carcinomas in groups of mice fed arsenicals during tumor initiation

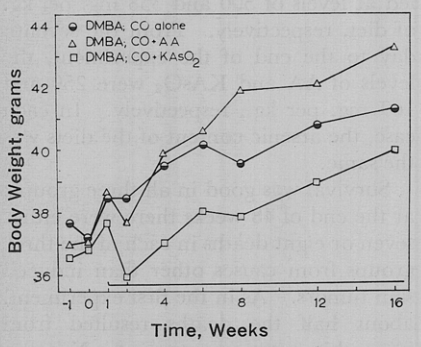

Figure 8. Body weight curves of mice fed arsenicals during the period of promotion shown by the horizontal line

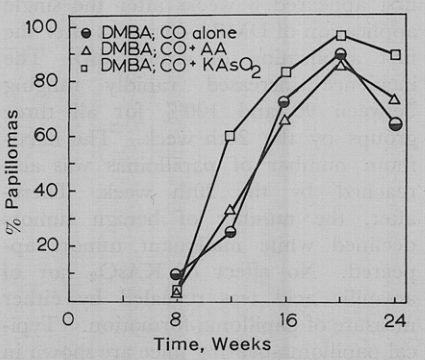

Figure 9. Percentage of mice bearing papillomas after feeding arsenicals during the period of tumor promotion

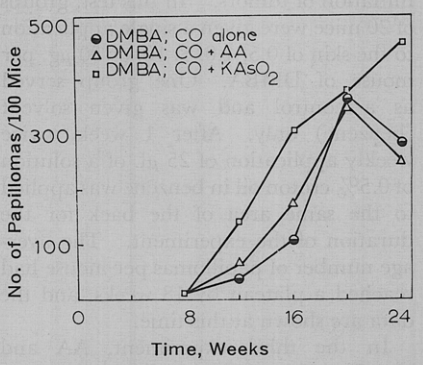

Figure 10. Total number of papillomas per 100 mice fed arsenicals during the period of tumor promotion

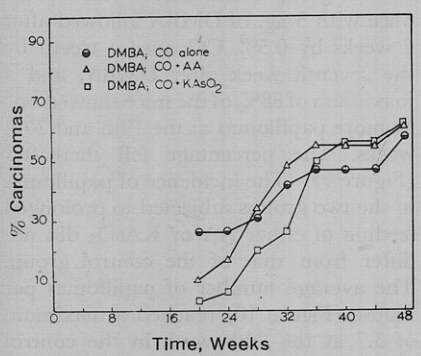

Figure 11. Incidence of carcinomas in groups of mice fed arsenicals during tumor initiation

odically by microscopic examination. Only typical papillomas larger than about 1 mm. in diameter were recorded, care being taken to exclude hyperplasias and other miscellaneous lesions.

Experiments and Results

The first experiment was designed to test $KAsO_2$ for tumor-initiating activity. Groups of 20 female mice were treated as follows: Group 1 served as a control, and the mice were not exposed to an initiator but were treated twice weekly with 25 μl. each of 2.0% croton oil in benzene. Mice of group 2 were given $KAsO_2$ by stomach tube. A total of 2.4 mg. of $KAsO_2$ per mouse was administered in seven feedings totaling 1.2 ml. of a 2 mg. per ml. H_2O solution over a 5-day period. The skin of the mice of group 3 was tested for initiation by the direct application of a solution of $KAsO_2$ to a shaved area of the back. Each mouse received a total of 1.24 mg. of $KAsO_2$ divided into eight applications over a 5-day period totaling 0.31 ml. of a 4 mg. per ml. solution of $KAsO_2$ in 80% ethanol. Beginning 2 days after completion of the arsenic treatments, croton oil was applied to the mice of groups 2 and 3 just as was done in group 1.

The first skin papillomas appeared in the area of croton oil treatment on the mice of all three groups at the sixth week. The incidence continued to increase until the 18th week when the experiment was terminated, as it was apparent that previous exposure to $KAsO_2$ had not made the mice of groups 2 and 3 more responsive to croton oil than the control mice (group 1) treated with croton oil alone. The data are shown in Table I.

The second experiment was designed to test $KAsO_2$ for possible tumor-promoting activity. A single application of 75 μg. of DMBA in 25 μl. of acetone was applied to a shaved area on the skin of the back of 60 Rockland female mice. Twenty of these mice were left without further treatment for the duration of the experiment (group 1). The appearance of skin tumors was elicited in 20 mice of group 2 by means of twice weekly applications to the initiated area of the back of a 10% solution of phenol in acetone (2.5 mg. of phenol per mouse, twice a week). The phenol applications were begun 1 week after the single application of DMBA and were continued for the duration of the experiment. A third group of 20 mice were treated twice daily (11 times a week) with 50 μl. of a 0.4% solution of $KAsO_2$ in 80% ethanol. Each mouse received 2.2 mg. of $KAsO_2$ per week. Treatment was continued for the duration of the experiment. No tumors developed on the skin of the mice treated with DMBA alone. In contrast, six of 17 surviving mice treated with DMBA and phenol had developed papillomas by 18 weeks. By the 24th week seven of 14 surviving mice had developed tumors, and one of these had a malignant skin tumor (Table II). In contrast to the tumor-promoting activity of phenol, $KAsO_2$ showed none. Although the skin of several mice treated

with $KAsO_2$ showed gross hyperplasia and even extensive ulceration, no tumors developed.

Experiments 3 and 4 were performed using a strain of mice selectively bred for susceptibility to skin carcinogenesis (*2*). The arsenicals were administered to these mice concurrently with a level of either an initiating or a promoting agent that did not elicit a maximal tumor response.

The data presented in Figure 1 show that the level of DMBA (5 μg. per mouse) used to initiate tumors in experiments 3 and 4 resulted in an intermediate yield of tumors and therefore was a proper level to test for a possible influence of arsenic compounds on the initiation of tumors. In this test, groups of 20 mice were given a single application to the skin of 0.5, 1.0, 5, 10, or 50 μg. per mouse of DMBA. One group served as a control and was given solvent (benzene) only. After 1 week, twice weekly application of 25 μl. of a solution of 0.5% croton oil in benzene was applied to the same area of the back for the duration of the experiment. The average number of papillomas per mouse had reached a plateau by 18 weeks, and the data are shown at this time.

In the third experiment, AA and $KAsO_2$ were tested by feeding concurrently with a single application of 5 μg. per mouse of DMBA. Three groups of 30 STS mice each were treated as follows. The mice of group 1 were treated once with 5 μg. each of DMBA. After an interval of 2 weeks, 25 μl. of a 1% solution of croton oil (CO) in benzene was applied to the same area of the skin of each mouse twice weekly for the duration of the experiment. The mice of group 2 were treated the same as those of group 1 with the addition of 1 gram of arsanilic acid per kg. of diet beginning 1 week before and continuing until 1 week after the single application of DMBA. Thereafter, these mice were maintained on the same diet (Purina Chow ground) as group 1. The third group of mice were also treated the same as group 1 with the addition of 676 mg. of $KAsO_2$ per kg. of diet beginning 1 week before until 1 week after the single application of DMBA. The arsenic content of the diets fed to groups 2 and 3 was the same.

The body weight curves of the three groups of mice in the first experiment are shown in Figure 2. Inclusion of arsanilic acid at 0.1% of the diet (10 times the recommended level) for 2 weeks had no appreciable effect on growth, but $KAsO_2$ at a level furnishing an equivalent amount of arsenic caused a sharp fall in body weight that was reversed within a week upon change to the control diet. Otherwise, the weight curves closely paralleled that of the control group.

In addition to body weight, survival of the mice is an important consideration in evaluating an experiment in carcinogenesis. Figure 3 shows the number of mice in each group that constituted the effective population at risk during the course of the experiment. The decrease in the number of mice with time was the result of deaths from causes other than induced skin malignancies. About half the deaths were attributable to spontaneous mammary tumors. Survival was good, and so the data on tumor incidence are not invalidated by the stress of intercurrent illness (*8*) nor by variations from group to group in the number of deaths. There was no difference between the groups in the incidence of tumors of organs other than the skin.

The incidence of benign epithelial tumors (papillomas) is plotted in terms of percentage of mice in each group bearing one or more papillomas (Figure 4) and in terms of the total number of papillomas per 100 mice (Figure 5). Papillomas first appeared 8 weeks after the single application of DMBA (6 weeks after the first application of croton oil). The incidence increased rapidly ranging between 90 and 100% for all three groups by the 20th week. The maximum number of papillomas was also reached by the 20th week. Thereafter, the number of benign tumors declined while malignant tumors appeared. No effect of $KAsO_2$ nor of arsanilic acid was revealed by either measure of papilloma formation. Typical papilloma-bearing mice are shown in Figure 6.

The incidence of malignant tumors (almost exclusively epithelial carcinomas) is shown in Figure 7. Although inclusion of the arsenicals in the diet at the time of initiation appeared to offer some protection against the formation of carcinomas, upon analysis the difference proved to be not significant at the 1% level.

In the fourth experiment, AA and $KAsO_2$ were tested in combination with the tumor-promoting agent, croton oil. The level of croton oil was such that the resulting tumor incidence was not maximum. Three groups of mice were treated as follows. Group 1 was comprised of 20 mice, and 5 μg. of DMBA was applied once to the skin of the back of each mouse. After an interval of 2 weeks, 25 μl. of a 0.5% solution of croton oil in benzene was applied to the same area of the skin only once weekly for the duration of the experiment. There were 30 mice in group 2, and they were treated the same as those in group 1 with the addition of AA to the diet beginning 1 week before the applications of croton oil were begun and continuing until the end of the experiment. The third group of 30 mice was also treated the same as group 1 with the addition of $KAsO_2$ to the diet beginning 1 week before croton oil treatments were begun and continuing until the end of the experiment. The intent was to feed the highest level of the arsenicals that permitted growth comparable to the control group. Because caloric restriction imparts protection against the tumor-promoting effect of croton oil (*2*), the level of the arsenicals was adjusted so that minimal effects on growth and food consumption resulted. The growth curves shown in Figure 8 show that mice fed $KAsO_2$ averaged about 1.5 grams less than the controls, while the mice fed AA tended to be heavier than the controls. This growth pattern was maintained throughout the 48 weeks of the experiment, and was judged to be acceptable. It was achieved by the following regimen. For the first 6 days, AA and $KAsO_2$ were fed at levels of 500 and 338 mg. per kg. of diet, respectively. From the seventh day to the end of the experiment, the levels of AA and $KAsO_2$ were 250 and 169 mg. per kg., respectively. In each case, the arsenic content of the diets was the same.

Survival was good in all three groups; at the end of 48 weeks there were either seven or eight deaths in each of the three groups from causes other than induced skin tumors. As in the first experiment, about half the deaths resulted from idiopathic mammary tumors. No relationship was apparent between the occurrence of tumors of organs other than skin and exposure to arsenicals.

Papillomas began to appear in the control group (each mouse was treated once with 5 μg. of DMBA followed after 2 weeks by 0.5% CO once a week) by the seventh week after DMBA, and a maximum of 88% of the mice showed one or more papillomas at the 19th and 20th weeks. The percentage fell thereafter (Figure 9). The incidence of papillomas in the two groups subjected to prolonged feeding of either AA or $KAsO_2$ did not differ from that of the control group. The average number of papillomas per mouse (Figure 10) reached a maximum of 3.7 at the 20th week in the control group and fell thereafter as malignant tumors appeared. Feeding AA during tumor promotion had no effect on the total number of papillomas. In contrast, the number of papillomas per mouse reached a maximum of 4.9 in the group fed $KAsO_2$ during croton oil treatment and did not decline until after the 32nd week. This difference between the control group and that fed $KAsO_2$ was inversely related to the development of carcinomas; while the incidence of carcinomas in the control group was 26% at the 20th week, it was not until the 32nd week that a similar incidence was reached in the group fed $KAsO_2$ concurrently with the croton oil treatments (Figure 11). It is unlikely that these differences in the pattern of the curves of tumor incidence shown in Figures 9, 10, and 11 were the result of the experimental regimen. In fact, statistical

analyses revealed no significant difference in the incidence of carcinomas among the three groups at the 5% level.

Discussion

The procedure used for evaluating the carcinogenicity of AA and $KAsO_2$ is unique (*4*, *13*) and requires some comment. Because the tumors attributed to arsenic are almost exclusively of the skin of man (*9*, *11*), and because the skin of man is exposed to many known carcinogenic stimuli, including both ionizing and ultraviolet radiations, as well as many chemical carcinogens, such as coal tar and petroleum products, it is of great interest to test arsenicals in conjunction with an incomplete carcinogenic stimulus.

The tests were based on the fact that the formation of skin tumors in mice may be divided into two stages called initiation and promotion, and that substances exist with potency to accomplish either one or the other stage (*1*, *2*). If $KAsO_2$ were found to possess predominantly either initiating or promoting power, exposure to $KAsO_2$ alone would not ordinarily result in tumor formation. Thus the contradictory nature of reports on the carcinogenicity of $KAsO_2$ might be resolved.

Second, the arsenicals were fed in conjunction with exposure of the animals to substances able to cause either the initiation or promotion of skin tumors. The existence of subthreshold levels of each of these agents is readily demonstrable as well as the additive nature of quantities of either initiators or promoters insufficient to elicit maximal tumor response (*2*). Therefore, it is likely that even if the arsenical possessed only a subthreshold initiating or promoting action, that property would be revealed by addition to a submaximal stimulus of the same kind provided by either DMBA or croton oil. The data in Figure 1 show that the initiating dose of 5 μg. of DMBA was submaximal while a comparison of the results shown in Figures 4, 5, and 7 with those in 9, 10, and 11 shows that the lower level of croton oil employed in the fourth experiment did not cause a maximal response (experiments 3 and 4 were run simultaneously with the mice obtained from a common pool). The higher incidence of carcinomas among the mice of experiment 3 compared to experiment 4 was significant at the 1% level.

Finally, although the procedure that was used is unique for carcinogenicity testing, its use in this respect has adequate precedent in research on dissection of the carcinogenic process (*1*, *2*, *13*) to justify application to problems of carcinogenicity testing where appropriate. These techniques utilizing mice selectively bred for susceptibility to skin tumor formation make a relatively rapid and highly sensitive test.

Acknowledgment

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FEED ADDITIVES

Metabolism of Selenium in the Mammalian Organism

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THE World Health Organization Expert Committee Report of 1961 (*35*) on "Evaluation of the Carcinogenic Hazards of Food Additives" makes the following statement regarding selenium: "It seems clear that too little selenium is harmful and gives rise to a deficiency state. However, too much selenium may cause toxic effects and may perhaps constitute a carcinogenic risk." Despite the proposal that selenium be included among the "essential" dietary trace elements (*26*, *28*, *29*), there are many unanswered questions about selenium metabolism in the mammalian organism. The function of selenium is not clear. The purpose of this paper is to review in part results from laboratory studies which may add to the general knowledge of the metabolism of selenium and assist in an eventual evaluation of dietary selenium.

Nearly all of the experiments employed the radioactive tracer technique which made possible the detection of submicro amounts of this element. The amount of Se^{75} and selenium in the single dose administered to the experimental animals was kept within trace amounts; thus, both radiation and toxic effects from selenium were avoided, and the metabolism under study was considered normal.

Retention of Selenium in Lactating Dog

One of the pathways by which selenium may be eliminated from the animal body other than through the kidney, gastrointestinal tract, and lung is through the mammary glands, into the milk of lactating animals (*1*). A lactating dog, having five 1-day-old pups, was injected subcutaneously with 0.64 mc. of $Se^{75}O_3^{-2}$ which contained 7 μg. of selenium. Milk samples were collected at various time intervals from 4 hours (*17*) throughout a period of 7 weeks and were separated into skim milk, casein, and milk serum. All the fractions including whole milk