

Carcinogenicity Studies of Tobacco Extract in Vitamin A-deficient Sprague-Dawley Rats

Sumati V. Bhide,¹ N. Ammigan, Urmila J. Nair, and V. S. Lalitha

Carcinogenesis Division [S. V. B., N. A., U. J. N.] and Cell and Developmental Pathology Division [V. S. L], Cancer Research Institute, Tata Memorial Centre, Parel, Bombay 400 012, India

ABSTRACT

Long-term carcinogenicity studies were carried out in male Sprague-Dawley rats maintained on vitamin A-sufficient (SLO⁺) and vitamin A-deficient (SLO⁻) diets and treated with tobacco extract (TE). Three-week-old rats received by gavage a total dose of 860 mg of TE at a daily dose of 3 mg/rat over a period of 21 months. Besides tumorigenicity, drug-metabolizing phase I and phase II enzymes in lung and liver as well as vitamin A and C levels in plasma and liver were measured at 12 and 21 months of age. The cumulative tumor incidence in TE-treated SLO⁻ rats was significantly higher (77-100%) than that observed in TE-treated SLO⁺ rats (20-22%). Furthermore, SLO⁺ rats treated with TE showed lung and forestomach tumors, whereas TE-treated SLO⁻ rats showed a preponderance of pituitary adenomas (87%). It was observed that TE treatment increased the activity of the hepatic and pulmonary phase I enzymes and decreased the glutathione/glutathione *S*-transferase detoxification system at both time points in SLO⁻ rats. On TE treatment the vitamin A levels in the liver and plasma were significantly decreased with a concurrent increase in vitamin C levels. The data show that a vitamin A-deficient diet renders male Sprague-Dawley rats more susceptible to TE treatment than the vitamin A-sufficient diet, an effect which was associated with the augmented induction of P-450 content and activities and depletion of the glutathione/glutathione *S*-transferase pathway by TE.

INTRODUCTION

Tobacco use poses the hazards of disease and death. In most developed countries for which data are available, the proportion of cancer attributable to tobacco use in men and women is strikingly high (1). Tobacco chewing forms an important part of the cultural life style in both urban and rural Indian populations. In India, the predominant cancer type is that of the oropharyngeal region, which accounts for approximately 40% of the total cancer incidence (2) and has been attributed to the various tobacco habits practiced in the peninsula. Together with tobacco chewing, malnutrition is a universal malady affecting the third world countries seriously, and the incidence of vitamin A deficiency in the Indian population is alarmingly high (3). This micronutrient is an important factor involved in a myriad of functional processes (4). A wealth of data is available on its biochemical properties, its modulatory role in chemical carcinogenesis, and its "cancer chemopreventive property" (5, 6). However, the exact mode of its action is still unclear. An attempt is made in the present study to understand the nutritional modulation of tobacco carcinogenesis by dietary vitamin A in rats.

MATERIALS AND METHODS

Chemicals

All chemicals used were of the highest purity available. Commercially available benzo(*a*)pyrene, 5,5'-dithio-bis-(2-nitrobenzoic acid), and 1-

chloro-2,4-dinitrobenzene were purchased from Sigma Chemical Co. (St. Louis, MO), and 3-hydroxybenzo(*a*)pyrene was a generous gift from Dr. J. N. Keith (Chemical Repository, IIT Research Institute, Chicago, IL).

Preparation of Tobacco Extract

TE² was prepared by shaking 100 g of commercial tobacco with 1 liter of dichloromethane at room temperature for 72 h. The mixture was then filtered and dried in a vacuum. The residue was dissolved in DMSO at a concentration of 3 mg/0.05 ml and stored at -20°C where it was stable for more than 2 months. The tobacco extract used in this study contained appreciable amounts of tobacco-specific *N*-nitrosamines (μg/g): *N*-nitrososnonnicotine, 25; 4-(*N*-nitrosomethylamino)-1-(3-pyridyl)-1-butanone, 2.8; and *N*-nitrosoanatabine, 9.4 (7).

Experimental Designs

Animals. The experiments were conducted on 19- to 21-day-old inbred male Sprague-Dawley rats obtained from the animal colony of the Cancer Research Institute. The animals were housed in plastic cages (3-4 animals/cage). The temperature of the animal colony was maintained at 21 ± 1°C (SD) and 60% humidity, and a 12-h light/dark cycle was used.

Diet. The standard cereal-based diet fed to control animals had the following composition (g/100 g): wheat, 70; Bengal gram, 20; fish meal, 5; yeast powder, 4; ground nut oil, 0.75; SLO, 0.25. SLO is the principal source of vitamin A in this diet; hence the deficient diet was prepared without SLO. The vitamin A content of the diets was 5000 IU/kg (SLO⁺) and 1200 IU/kg (SLO⁻) and the vitamin C content was 3.75 mg/kg diet.

Treatment. Rats maintained on standard diet (SLO⁺) and vitamin A-deficient diet (SLO⁻) received a daily dose of 3 mg TE in 0.05 ml DMSO five times a week by gavage for a period of 21 months. Control animals from each group received 0.05 ml DMSO for the same period as the treated groups (Table 1). All the animals were weighed periodically.

Pathology. Autopsies were performed on all dead/killed animals. All organs were examined for gross abnormalities. Liver, lung, stomach, brain, and pituitary were fixed in 10% formalin and processed by routine histological methods. Histopathological examination was carried out on 6-μm-thick paraffin sections stained with hematoxylin and eosin.

Biochemistry. At 12 and 21 months 5 animals were killed in each group, and hepatic and pulmonary enzyme activities and vitamin A and C levels in liver and plasma were monitored. Animals were killed by cervical dislocation, and the livers and lungs were perfused with chilled potassium chloride (1.15%). The organs were excised, rinsed with the above solution, and homogenized in 4 volumes of ice-cold 0.25 M sucrose in 0.01 M phosphate buffer, pH 7.4. This homogenate was centrifuged at 12,000 × *g* for 30 min, and the resulting supernatant was recentrifuged at 100,000 × *g* for 60 min. The microsomal pellet was suspended in 0.25 M sucrose in 0.01 M phosphate buffer, pH 7.4. Microsomal cytochrome *b*₅ and P-450 were determined by the method of Omura and Sato (8) using extinction coefficients of 171 cm⁻¹ mM⁻¹ and 91 cm⁻¹ mM⁻¹, respectively. Microsomal B(*a*)PH activity was assayed by the method of Dehnen *et al.* (9). Microsomal benzphetamine demethylase was measured according to the method of Lu *et al.* (10).

² The abbreviations used are: TE, tobacco extract; SLO⁺, vitamin A-sufficient (with shark liver oil); SLO⁻, vitamin A-deficient (without shark liver oil); B(*a*)PH, benzo(*a*)pyrene hydroxylase; GST, glutathione *S*-transferase; GSH, glutathione; DMSO, dimethyl sulfoxide.

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¹ To whom requests for reprints should be addressed.

Table 1 Dietary regimen and treatment of experimental groups

| Treatment groups | No. of animals | Total dose | | | | | |
|----------------------------|----------------|------------|---------|-----------|--------|---------|--------|
| | | Daily dose | | DMSO (ml) | | TE (mg) | |
| | | DMSO (ml) | TE (mg) | 12 mos | 21 mos | 12 mos | 21 mos |
| SLO ⁺ (control) | 31 | 0.05 | | 8.3 | 14.3 | | |
| SLO ⁺ + TE | 29 | 0.05 | 3 | 8.3 | 14.3 | 500 | 860 |
| SLO ⁻ (control) | 30 | 0.05 | | 8.3 | 14.3 | | |
| SLO ⁻ + TE | 31 | 0.05 | 3 | 8.3 | 14.3 | 500 | 860 |

GST was measured in the cytosol using 1-chloro-2,4-dinitrobenzene and glutathione as substrates (11). Cytosolic reduced GSH was measured as reported by Moron *et al.* (12). Protein was estimated by the method of Lowry *et al.* (13) using bovine serum albumin as the standard. Vitamins A and C were assayed as reported by Roels and Mahadevan (14) and Roe and Kuether (15), respectively.

Statistical Analysis. Student's *t* test was used to calculate the statistical significance for enzyme activities and vitamin content.

In the carcinogenicity studies the χ^2 test was used to check the statistical significance.

RESULTS

Fig. 4 depicts the growth rate of the Sprague-Dawley rats in various groups from 3 months. At 3 months the SLO⁺ group had gained 8% more weight than the SLO⁻ group, while TE had had no effect on the weights of either group up to that point.

DMSO-treated SLO⁺ animals gained weight continuously for up to 12 months as compared to 10 months in the SLO⁻ group. The weight reached a plateau thereafter. TE treatment stopped the growth of SLO⁺ animals and resulted in a decrease in body weight in SLO⁻ rats. All treated animals were sacrificed between 16 and 21 months as they approached death or developed paralysis. Only in the SLO⁻ TE group did rats develop paralysis of upper and lower limbs, and those were found to have pituitary tumors at autopsy, compressing the brain. Control animals remained apparently healthy but were also sacrificed at 21 months.

Tables 2 and 3 summarize data on tumor incidence. Twenty % of the SLO⁺ TE-treated animals developed lung adenomas and forestomach papillomas (Table 2). As revealed histologically in the forestomach papillomas, the mucosal lining developed papillary projections which consisted of a core of connective tissue lined by hyperplastic and hyperkeratinizing squamous epithelium (Fig. 1).

Besides forestomach papillomas, lymphomas of the lung were also observed with a high incidence (77 and 71%, respectively) in the SLO⁻ group. The tumors were composed of an admixture of larger reticular cells containing bizarre nuclei with mitotic activity and lymphocytes (Fig. 3). The tumor cells were loosely arranged, a characteristic feature of certain lymphomas; there were no plasma cells, eosinophils, or neutrophils. The absence of these inflammatory cells ruled out the possibility of pseudolymphomas.

A high incidence (61%) of pituitary adenomas was observed only in the SLO⁻ TE group (Table 3). This type of tumor has not been reported in the inbred male Sprague-Dawley rats of our animal house colony, although there is a low incidence of spontaneous pituitary tumors in males of randomly bred strains. The tumors were well circumscribed, compressing the normal pituitary which was seen at the periphery. In the hematoxylin and eosin stained sections the tumors were seen in most places

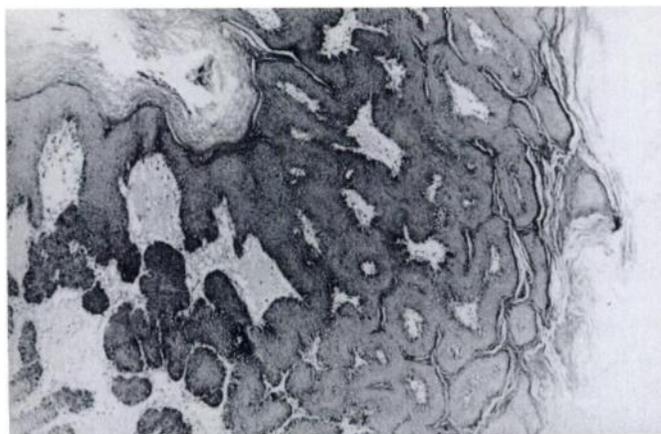


Fig. 1. The lining of forestomach papilloma showing hyperplasia of stratified squamous epithelium. H & E, x 62.

Table 2 Tumor incidence of SLO⁺ animals treated with TE

| | Tumor incidence at | | Total incidence |
|--------------------------|---------------------------|--------------|-----------------------------|
| | 9-15 mos | 16-21 mos | |
| Total tumor incidence | | | |
| SLO ⁺ control | 0/7 | 0/24 | 0/31 |
| SLO ⁺ + TE | 2/10 (20) ^b | 4/19 (21) | 6/29 ^a (20.7) |
| Tumor type | | | |
| Lung adenomas | 1/10 | 2/19 | 3/29 (10.3) |
| Stomach papillomas | 1/10 | 2/19 | 3/29 (10.3) |
| Pituitary adenomas | Nil | Nil | Nil |

^a Values are significant as compared to SLO⁺ control at *P* < 0.001.

^b Numbers in parentheses, percentages.

Table 3 Tumor incidence of SLO⁻ animals treated with TE

| | Tumor incidence at | | Total incidence |
|--------------------------|---------------------------------------|-----------------------------|------------------------------|
| | 9-15 mos | 16-21 mos | |
| Total tumor incidence | | | |
| SLO ⁻ control | 0/9 | 0/21 | 0/30 |
| SLO ⁻ + TE | 7/9 ^a (77) ^b | 22/22 ^a (100) | 29/31 ^a (93.5) |
| Tumor type | | | |
| Lung lymphomas | 5/9 | 17/22 | 22/31 (71) |
| Stomach papillomas | 4/9 | 20/22 | 28/30 (77) |
| Pituitary adenomas | 0/9 | 19/22 (86) | 19/31 (61) |

^a Values are significant compared to corresponding age groups and total incidence of SLO⁻ controls at *P* < 0.001.

^b Numbers in parentheses, percentages.

to consist of cells without granules in the cytoplasm, intersected by tenuous fibrovascular stroma giving rise to the classical trabecular pattern. The cell types could not be identified because immunostaining was not done. In some places the tumor cells were disposed around blood vessels, giving rise to a papillary pattern (Fig. 2). Occasionally, large vascular spaces (sinusoids) were seen among tumor cells. Mitotic activity was very modest, as shown by high-power field.

The SLO⁻ diet resulted in vitamin A deficiency, and continuous long-term treatment with TE significantly decreased the hepatic pool of vitamin A with a concurrent increase in vitamin C levels (Table 4). No plasma vitamin A could be detected in SLO⁻ groups at 12 or 21 months.

Figs. 5-7 depict the modulation by vitamin A deficiency and

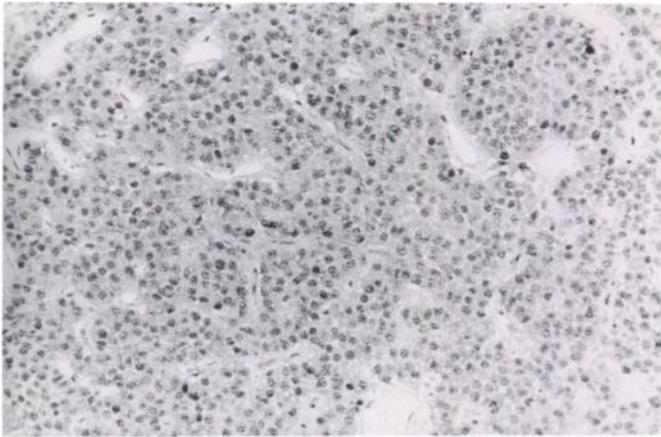


Fig. 2. Pituitary adenoma showing perivascular disposition of tumor cells. H & E, $\times 160$.

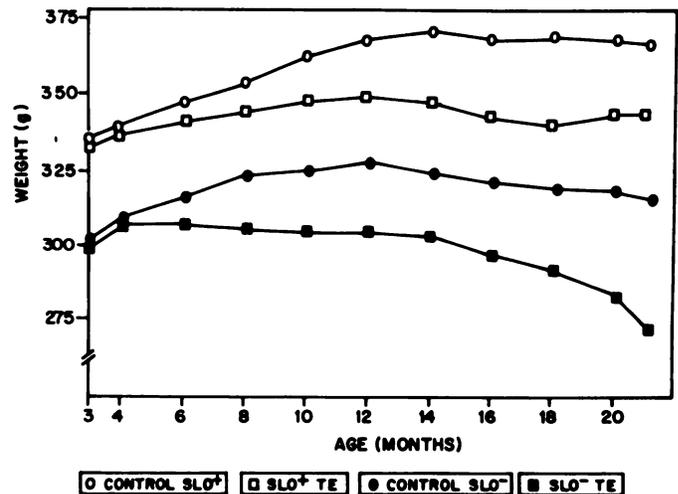


Fig. 4. Growth curves of Sprague-Dawley rats maintained on SLO⁺ and SLO⁻ diets and treated with TE.

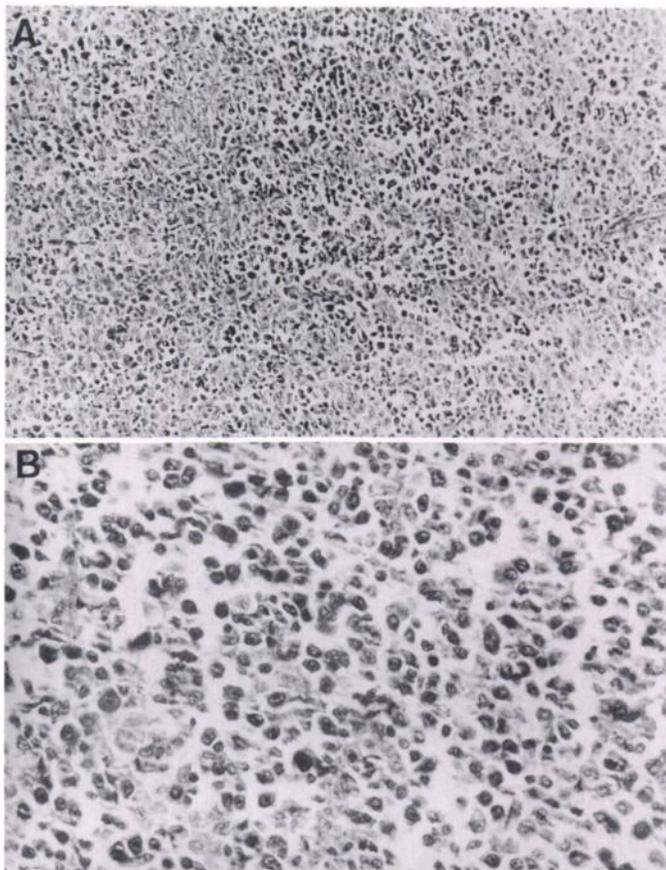


Fig. 3. A, primary lymphoma of lung, showing admixture of pale reticular cells and lymphocytes. H & E, $\times 160$. B, closer view of tumor cells showing pleomorphic nuclei. H & E, $\times 400$.

TE treatment of the hepatic and pulmonary phase I activating enzymes assayed at 12 and 21 months. Cytochrome *b*₅ levels were not affected (data not presented). TE significantly increased cytochrome P-450 and benzphetamine demethylase levels in the lung of the SLO⁻ group at 21 months. Pulmonary cytochrome P-450 was significantly higher in the SLO⁻TE group at 21 months as compared to the SLO⁺TE group.

SLO⁻ animals had lower basal levels of hepatic and pulmonary B(a)PH than did the SLO⁺ animals. No significant difference in basal levels between 12 and 21 months was observed in the SLO⁺ control group, and only hepatic B(a)PH was signifi-

cantly lower in SLO⁻ controls at 21 months. TE treatment significantly increased both the liver and lung B(a)PH in the SLO⁺ and SLO⁻ groups. Despite lower basal levels in the SLO⁻ group, there was no significant difference between SLO⁺TE and SLO⁻TE groups at 12 months, while at 21 months SLO⁻TE levels of B(a)PH were significantly higher in the liver but lower in the lungs.

As seen in Figs. 8 and 9, the animals fed the SLO⁻ diet had lower GSH and GST levels than those maintained on the SLO⁺ diet. However, treatment with TE led to an interesting pattern of modulation of GSH and GST. At 12 months, TE treatment did not significantly alter hepatic and pulmonary GSH and GST levels in SLO⁺ animals, while a significant decrease was observed in both the lung and liver tissues in the SLO⁻ TE-treated group at the same time point. However, at 21 months, TE treatment significantly increased the GSH/GST levels in SLO⁺ groups, while a drastic decrease was observed in the SLO⁻ animals. At 21 months, the pulmonary GSH/GST levels in the SLO⁻TE groups were significantly lower than in any other group.

DISCUSSION

The main aim of this study was to see whether vitamin A status affects the tumorigenic potency of tobacco extract. Tobacco chewing has long been associated with oral cancer in India which has the highest rate of oral cancer in the world.

This study yielded a number of important observations. The most important finding was that gavage feeding of tobacco extract induced tumors in almost 100% of SLO⁻ rats as compared to 20% in the SLO⁺ group. In a previous study, reported from this laboratory, an alcoholic extract of tobacco was found to induce lung adenocarcinomas and hepatocellular carcinomas in male Swiss mice (16). However, the present study is the first to report pituitary tumors induced by tobacco extract in male rats rendered vitamin A deficient.

It is possible that vitamin A deficiency has altered the hormonal milieu (probably due to stress) which is conducive to tumor development in the pituitary during exposure to TE. TE-induced systemic tumors in SLO⁺ rats were benign, while a high incidence of malignant tumors of the lung, namely, lymphomas, was observed in the SLO⁻TE group. It is believed that the immune system is impaired in vitamin A deficiency states

Table 4 Effect of long term TE treatment on the hepatic and circulating levels of vitamins A and C

Results are means ± SE of 5 animals.

| Parameters | Dietary groups | Treatment | 12 mos | | 21 mos | |
|----------------|------------------|-----------|----------------------|-------------------------|------------------------|---------------------------|
| | | | Vitamin A | Vitamin C | Vitamin A | Vitamin C |
| Hepatic (µg/g) | SLO ⁺ | Control | 180 ± 14 | 182 ± 11 | 228 ± 12 ^a | 234 ± 14 ^a |
| | | Treated | 114 ± 3 ^b | 231 ± 14 ^b | 171 ± 4 ^{a,b} | 316 ± 10 ^{a,b} |
| | SLO ⁻ | Control | 32 ± 4 ^a | 235 ± 11 ^c | 15 ± 4 ^{a,c} | 285 ± 12 ^{a,c} |
| | | Treated | ND | 345 ± 12 ^{b,d} | ND ^e | 430 ± 10 ^{a,b,d} |
| Plasma (µg/ml) | SLO ⁺ | Control | 71 ± 11 | 22 ± 4 | 84 ± 13 | 28 ± 7 |
| | | Treated | 50 ± 9 | 30 ± 9 | 63 ± 5 | 45 ± 5 ^b |
| | SLO ⁻ | Control | ND | 27 ± 8 | ND | 30 ± 4 |
| | | Treated | ND | 41 ± 5 | ND | 50 ± 4 ^b |

^a Values significant compared to corresponding value at 12 months at *P* < 0.05.
^b Values are significant compared to respective control groups at *P* < 0.05.
^c Values significant compared to corresponding SLO⁺ control groups at *P* < 0.05.
^d Values significant compared to corresponding SLO⁻ TE group at *P* < 0.05.
^e ND, not detected.

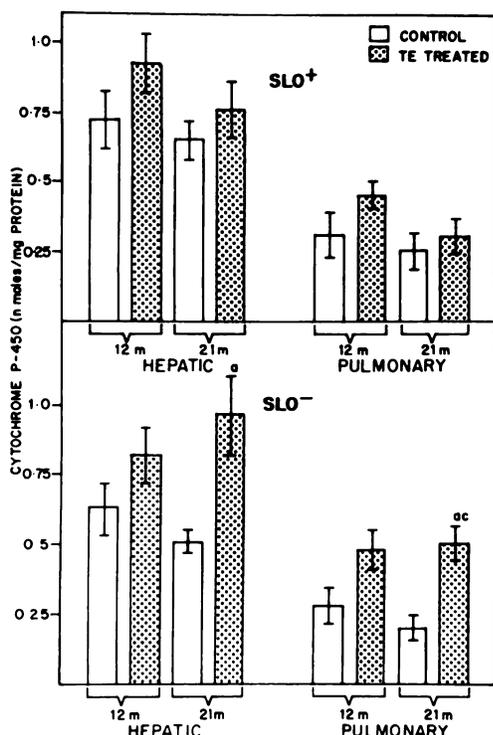


Fig. 5. Effect of TE on hepatic and pulmonary cytochrome P-450 in Sprague-Dawley rats fed SLO⁺ and SLO⁻ diets. Columns, means of 5 animals; bars, SE. *P* < 0.05. *a*, significant compared to corresponding controls; *c*, significant compared to corresponding SLO⁺TE groups. *m*, months.

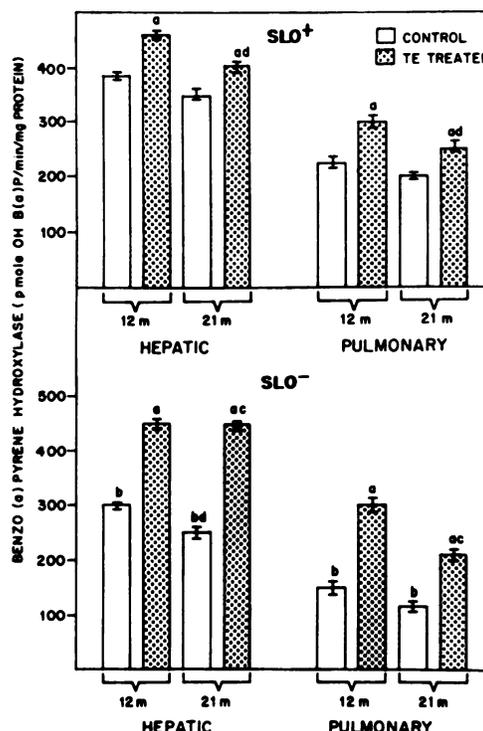


Fig. 6. Effect of TE on hepatic and pulmonary benzo(a)pyrene hydroxylase in Sprague-Dawley rats fed SLO⁺ and SLO⁻ diets. Columns, means of 5 animals; bars, SE. *P* < 0.05. *a*, significant compared to corresponding controls; *b*, significant compared to SLO⁺ controls; *c*, significant compared to SLO⁺TE group; *d*, significant compared to corresponding values at 12 months. *m*, months.

(17), and the most common cancers encountered in immunosuppressed and immunodeficient patients are lymphomas (18). It may be possible that the vitamin A-deficient rats of this particular strain (SD) are prone to develop lung lymphomas when TE is administered. The increased tumor incidence in vitamin A-deficient mice treated with diethylnitrosamine has been reported by Santhanam *et al.* (19). Several studies have demonstrated an apparent association between low dietary intake of vitamin A and the development of cancer, specifically lung, oropharyngeal, laryngeal, esophageal, gastric, colorectal, and prostate cancers (20). Interestingly, there was an inverse association between hepatic and plasma vitamin A and vitamin C levels. Padma *et al.* (21) and Kulkarni and Bhide (22) have also reported an increase in vitamin C levels when vitamin A levels were depleted upon treatment with tobacco-specific nitro-

samines or masher (a pyrolyzed tobacco product). Whether this response plays a role in the carcinogenic process is difficult to say at present, although various studies supporting the effect of vitamin C in reducing cancer risk have been reported (20, 23). Boyland and Grover (24) have reported a marked stimulation of ascorbate synthesis in rats and mice on exposure to various noxious compounds. *N*-Nitrosamines are the major known carcinogens in chewing tobacco (25). The tobacco-specific *N*-nitrosamines are known to be metabolized by the cytochrome P-450 system which in turn can be modulated by the vitamin A status of the animal. In the present study, vitamin A deficiency resulted only in an augmented induction by TE treatment of total P-450 levels in the lung and B(a)PH activity

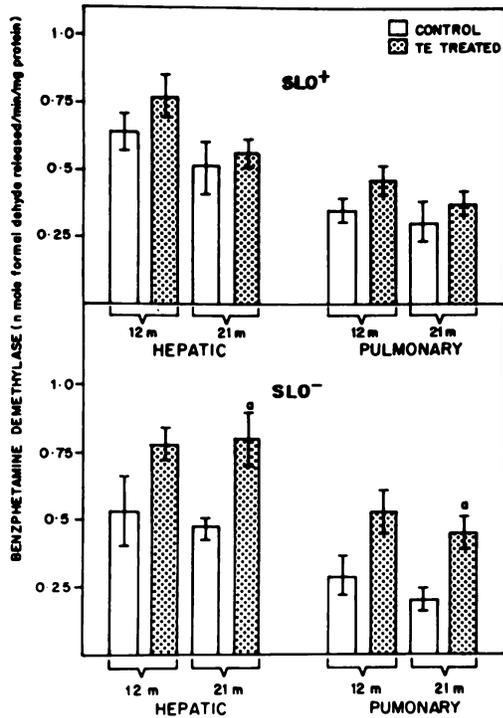


Fig. 7. Effect of TE on hepatic and pulmonary benzphetamine demethylase in Sprague-Dawley rats fed SLO⁺ and SLO⁻ diets. Columns, means of 5 animals; bars, SE. a, significant compared to corresponding control. *P* < 0.05. m, months.

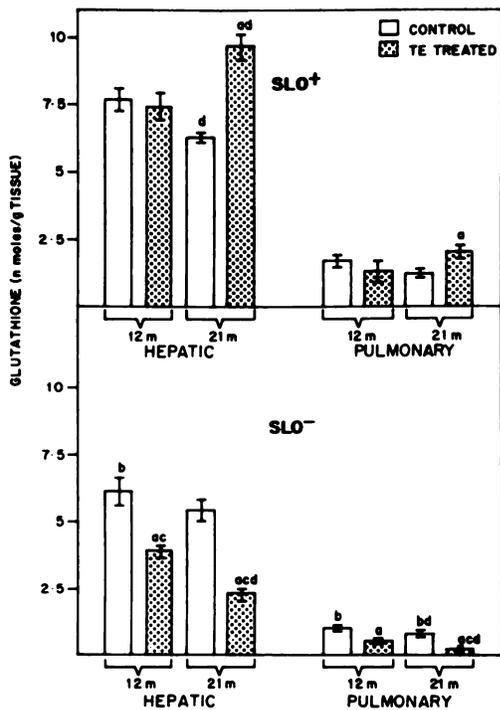


Fig. 8. Effect of TE on hepatic and pulmonary glutathione levels in Sprague-Dawley rats fed SLO⁺ and SLO⁻ diets. Columns, means of 5 animals; bars, SE. *P* < 0.05. a, significant compared to corresponding controls; b, significant compared to SLO⁺ controls; c, significant compared to SLO⁺TE rats; d, significant compared to corresponding values at 12 months. m, months.

in liver and lung and in an augmented depletion of the GSH/GST detoxification system.

Although the extent of tumorigenicity cannot be fully explained by the results of the metabolic studies, there appears to

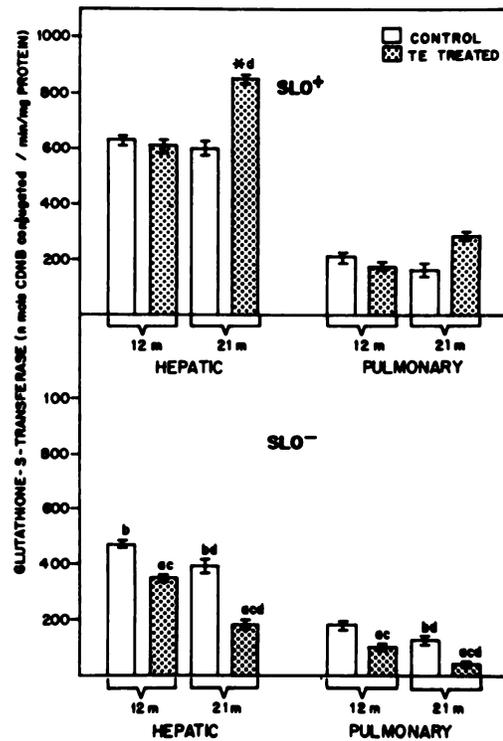


Fig. 9. Effect of TE on hepatic and pulmonary glutathione S-transferase levels in Sprague-Dawley rats fed SLO⁺ and SLO⁻ diets. Columns, means of 5 animals; bars, SE. *P* < 0.05. a, significant compared to corresponding controls; b, significant compared to corresponding SLO⁺ controls; c, significant compared to SLO⁺TE group; d, significant compared to corresponding values at 12 months. m, months.

be a significant pattern. That the lung is the target organ for tobacco-specific *N*-nitrosamines is well documented by various workers (25, 26). In our studies we find that pulmonary enzymes were most susceptible to modulation by TE. The activating enzymes were significantly higher in the SLO⁻TE groups at 21 months than in any other group. On the other hand the drastic depletion of the glutathione detoxification system in the treated groups, especially in the SLO⁻TE lung, perhaps led to the exposure of this organ to more activated metabolites, which in turn was reflected in the high incidence of lung tumors in this group. Although the enzyme pattern in the stomach was not determined in this study, earlier we had observed a similar pattern of induction of phase I enzymes and inhibition of GSH-GST system in the stomach when rats were treated with masher in the diet.³ Forestomach papillomas were also observed in the latter animals (27), a condition similar to what was observed in the present study.

In conclusion, the results of this study suggest that the enhanced tobacco carcinogenesis in vitamin A-deficient rats may be mediated by a decrease in detoxification (GSH/GST) and an increase in bioactivation.

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³ Unpublished data.

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