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Effect of Soybean Feeding on Experimental Carcinogenesis—III. Carcinogenecity of Nitrite and Dibutylamine in Mice: a Histopathological Study

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Abstract—*The potential carcinogenic effect of nitrosamine precursors, DBA (dibutylamine) and nitrite, was clearly demonstrated pathologically in the liver and bladder of male Swiss albino mice. Benign tumours were induced in the bladder with an incidence of* 40%, *and hepatomas were detected in the liver in* 27% *of the cases. The protective effect of soybean and ascorbic acid, added separately to the diet or to the drinking water respectively, was demonstrated by a marked reduction in dysplastic features and absence of tumour in both the liver and the urinary bladder.*

INTRODUCTION

N-ALKYL-*N*-NITROSO compounds include a large number of chemical carcinogens. It is well known that they produce tumours that differ in type and site according to species, route of administration and dosage [1] through alkylation of DNA [2]. Histologically, nitrosamines can produce liver necrosis, degeneration and tumours after prolonged administration [3]. Carcinoma of the bladder can be produced in rats having squamous, transitional or adenocarcinoma pattern [4]. However, in mice papillomas and hyperkeratosis were induced [5].

The protective effect of soybean on tumour formation has been reported [6]. The antitumour properties of ascorbic acid and its oxidation products have also been well documented [7].

The aim of the present work is to demonstrate the pathological changes induced experimentally by nitrosamine precursors, namely nitrite and dibutylamine (DBA) in young male Swiss albino mice and to study the possible protective role of soybean and ascorbic acid.

MATERIALS AND METHODS

i. *Animals*

Young male adults of non-inbred Swiss albino mice, 45 days old, and each weighing about 20 g were used. Animals were kept under normal laboratory conditions for 10 days before the initiation of the experiments. They were fed a standard commercial diet (Egyptian Company of Oils and Soaps, Egypt) composed of 20% casein, 15% corn oil, 55% corn starch, 5% salt mixture, 5% vitaminized starch and vegetables such as lettuce and carrots. They were given water *ad libitum*. Groups under investigation were classified according to the type of treatment as follows:

1. Normal control group
2. Dibutylamine (Merk) + sodium nitrite (Sigma) treated mice.
3. Dibutylamine + sodium nitrite + soybean (General Poultry Co.) treated mice.
4. Dibutylamine + sodium nitrite + ascorbic acid (Sigma) treated mice.

ii. *Chemicals*

(a) *Nitrosamine precursors.* Animals were fed the control diet and drank tap water containing 1000 ppm dibutylamine and 2000 ppm sodium nitrite. The amine and nitrite were mixed together with the drinking water using a magnetic stirrer for a period of 15 min.

(b) *Soybean.* Animals were fed the control diet well mixed with 30% of autoclaved soybean (107°C, 15 min) (Egyptian Poultry Co.). The soybean used contained: 1–2% fats, 44.4% protein, 10–11% moisture, 8.2% ash, 3.2% fibres and trypsin inhibitor 10.36 unit/mg.

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Table 1. *Pathological changes in mice liver after different periods of treatment*

Group	Period of treatment (months)	No. of animals	Lymphocytic infiltrate	Dysplasia	Necrosis	Hepatoma
Control	—	60	—	—	—	—
Nitrite	1–3	18	+	+	—	—
+ DBA	4–6	22	++	++	+	—
	7–9	20	+++	+++	++	3 mice (15%)
	10–12	15	+++	+++	++	4 mice (27%)
Nitrite	1–3	20	—	—	—	—
+ DBA	4–6	20	+	+	—	—
+ soybean	7–9	22	++	+	—	—
	10–12	21	++	+	—	—
Nitrite	1–3	20	—	—	—	—
+ DBA	4–6	20	+	—	—	—
+ ascorbic acid	7–9	20	++	++	—	—

+ Mild; ++ moderate; +++ marked.

(c) *Ascorbic acid.* Animals received ascorbic acid in their drinking water at a concentration of 5000 ppm, freshly prepared daily (Sigma Co.).

The animals were supplied with soybean and ascorbic acid added either to the diet or to the drinking water respectively, at the same time of starting nitrite and DBA treatment.

iii. *Histopathological study*

Tissues of the whole bladder and parts of the liver from all groups were fixed in formalin and Bouin's solution, embedded in paraffin and cut and stained with haematoxylin and eosin.

RESULTS

Liver (Table 1)

Control group. All mice showed normal liver parenchymal cells with eosinophilic granulated cytoplasm and small uniform nuclei radically arranged around the central vein.

Dibutylamine–sodium nitrite group. The liver showed focal changes in the form of mild peri-vascular lymphocytic infiltrate, fatty change and mild dysplasia. The nuclei showed a slight irregularity in shape and chromatin pattern but were still radially arranged around blood vessels. Dysplasia increased from mild to moderate to severe as the period of treatment was increased.

In moderate dysplasia (4–6 months) the nucleo-cytoplasmic ratio increased with chromatin condensation in the nuclei. Sinuses were slightly dilated with normal Kuppfer cells. Necrosis appeared only in few foci. Lymphocytes appeared as small aggregates in between liver cells (Fig. 1). After 6 months, marked dysplasia was seen in the liver in which there was loss of polarity, nuclei became hyperchromatic with appearance of bizarre shaped cells (Fig. 2). Sinuses were greatly dilated and Kuppfer cells were hyperplastic. Necrosis was seen in multiple areas with dense lymphocytic collections. Three cases of hepatoma appeared in this group after a period of treatment of 7–9 months (15%). There was massive necrosis of tumour cells and increased vascularity. In the group treated for 10–12 months, gross changes were noticed in the liver in the form of tumour nodules, marked softening and necrosis. Four cases of hepatoma (27%) were confirmed histologically (Fig. 3).

Dibutylamine–sodium nitrite–soybean group. The protective effect of soybean appeared as an absence of marked dysplastic changes. Only mild dysplasia was noticed (Fig. 4) after the 3rd month. No liver necrosis or tumours appeared in this group (83 mice). Moderate lymphocytic infiltrate was seen between liver cells and around the central vein.

Dibutylamine–sodium nitrite–ascorbic acid group. In this group 60 mice were included. Only one animal showed a moderate degree of liver dysplasia in the group treated for 7–9 months (5%). Mild lymphocytic infiltrate around portal vein was seen in the group treated for 4–6 months. Few lymphocytic aggregates were seen between liver cells in the group treated for 7–9 months. Experiments with 10–12 months treatment were not done. No liver necrosis or tumours appeared in any animals of this group.

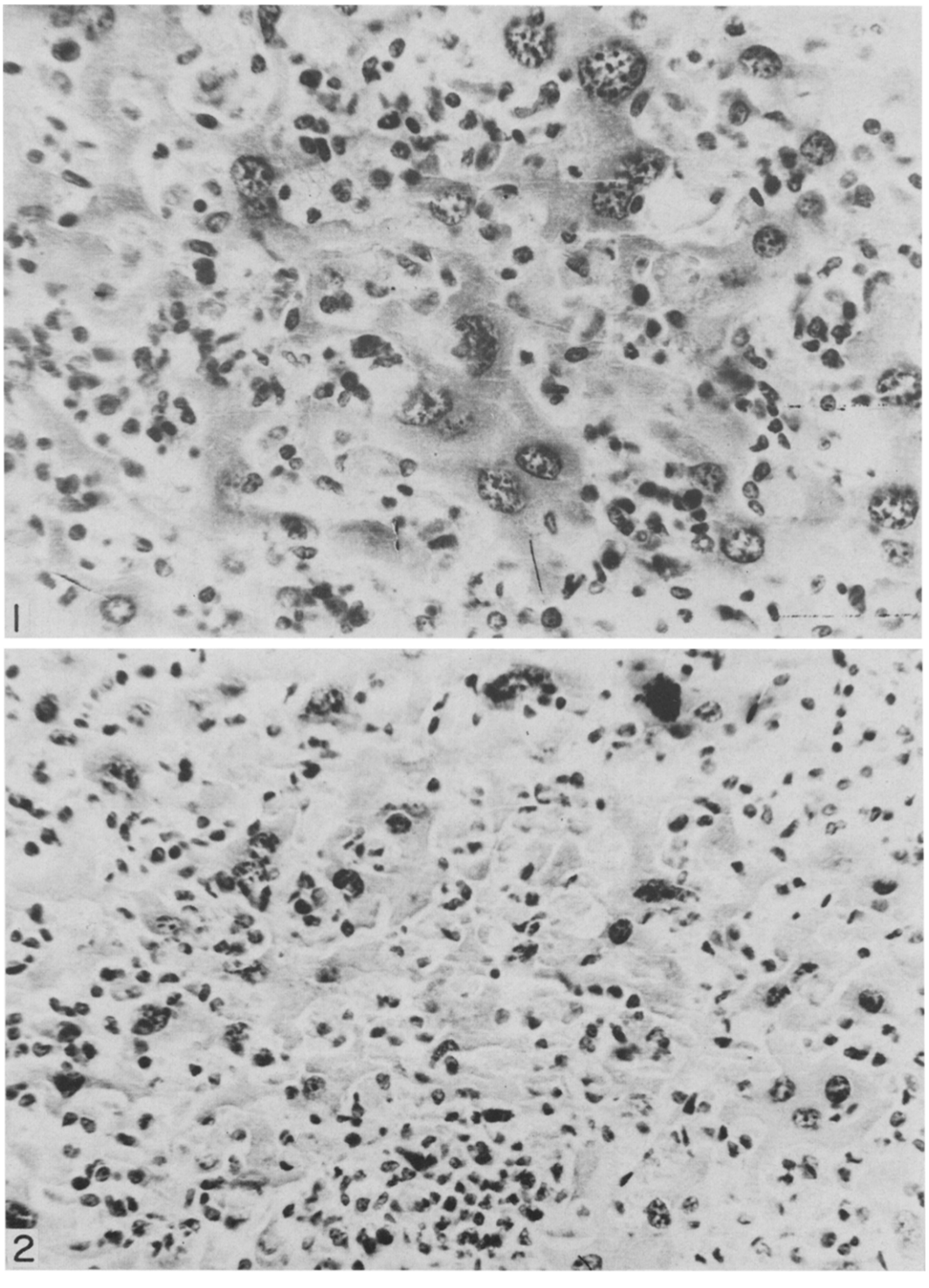
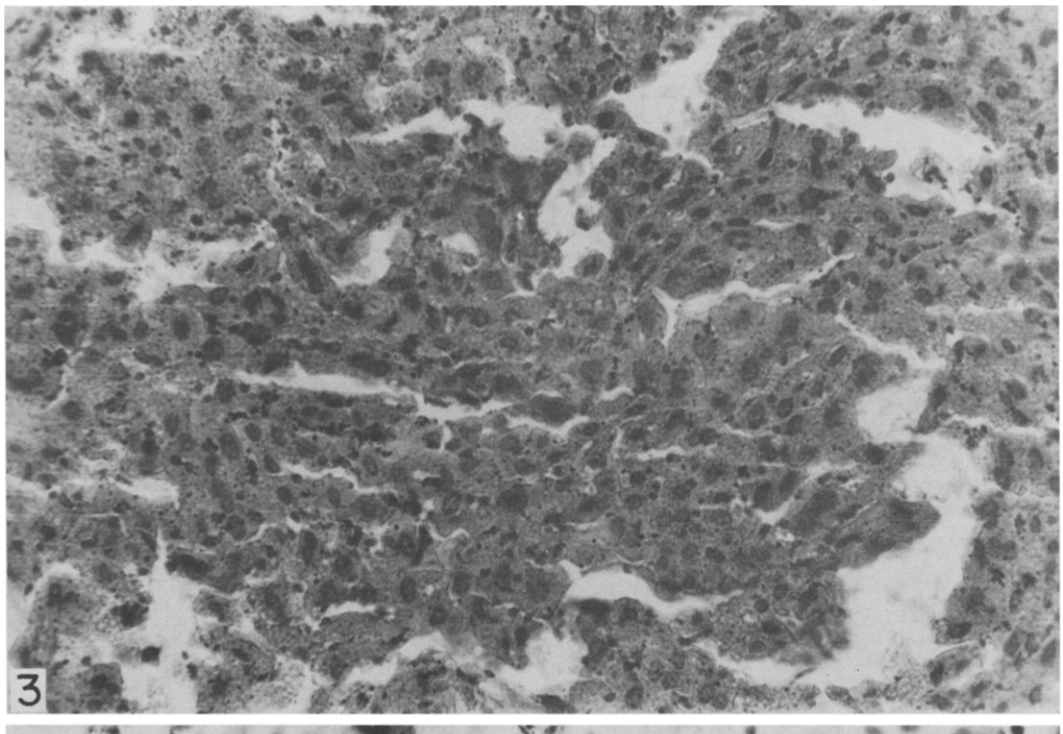


Fig. 1. *Liver tissue from a mouse treated with DBA and nitrite showing moderate dysplasia and dilated sinuses filled with lymphocytes. Period of treatment* 6 *months* (× 320).

Fig 2. *Liver tissue from a mouse treated with DBA and nitrite showing marked dysplasia, bizzare cells, dilated sinuses and focal lymphocytic aggregates. Period of treatment* 9 *months* (× 320).



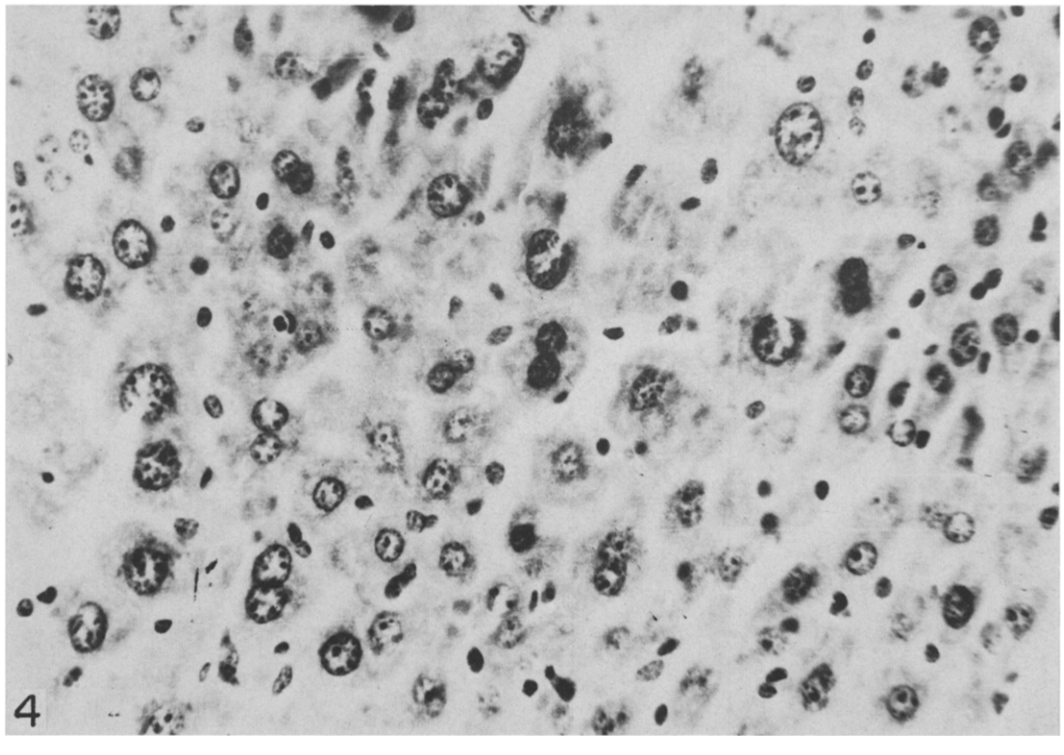


Fig. 3. *Liver tissue from a mouse treated with DBA and nitrite showing hepatoma. Period of treatment* 9 *months* (× 200).

Fig. 4. *Liver tissue from a mouse treated with DBA, nitrite and soybean showing mild dysplasia, binucleated liver cells and normal sinuses. Period of treatment* 9 *months* (× 320).

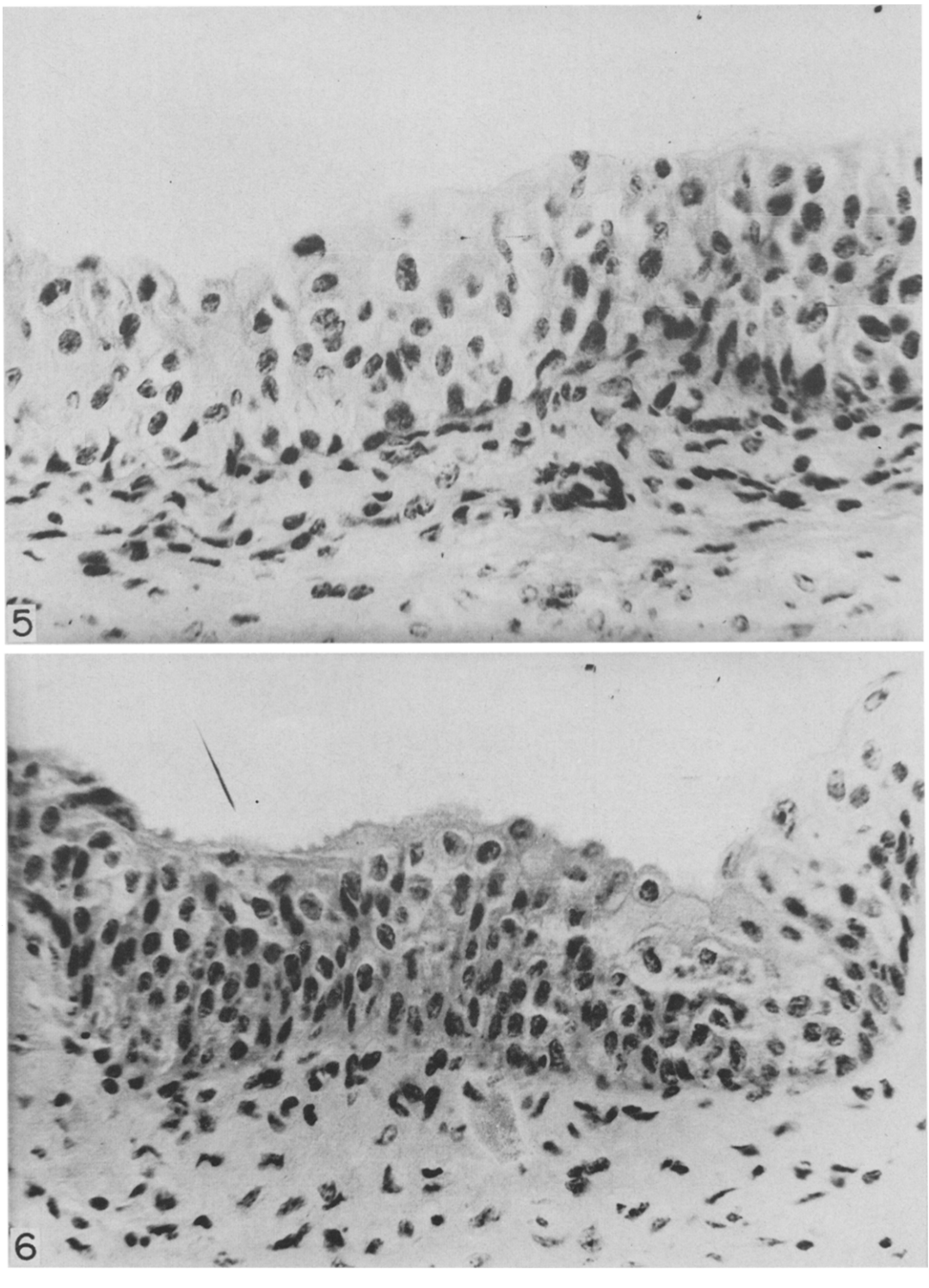
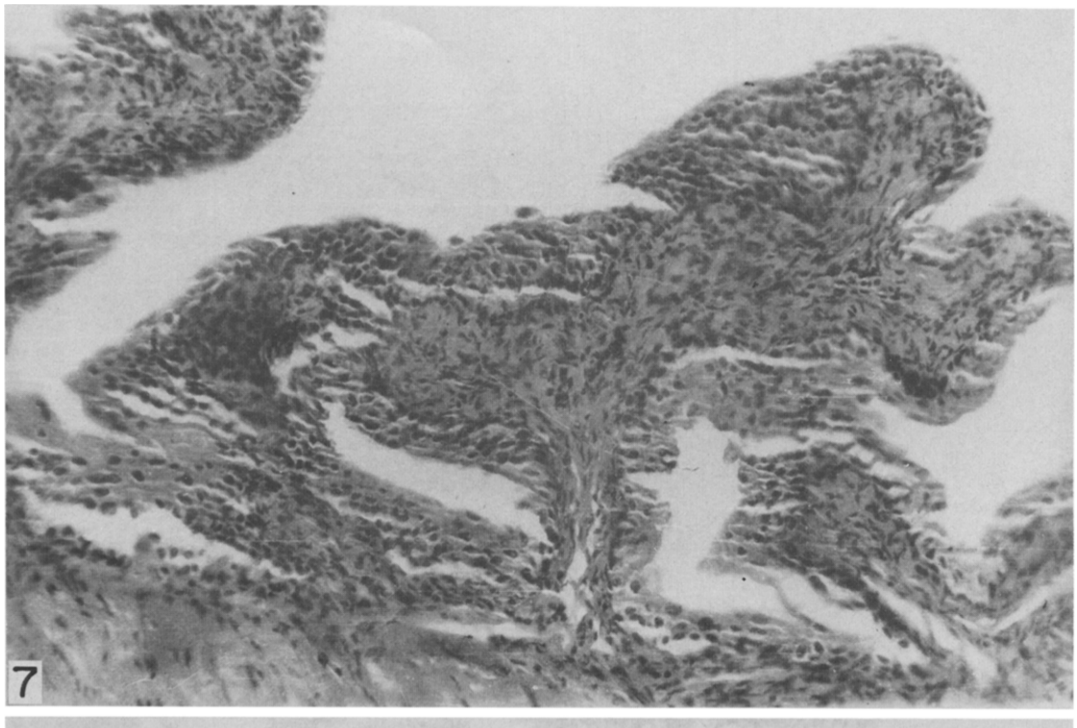


Fig. 5. *Bladder of mouse treated with DBA and nitrite showing moderate hyperplasia* (6–8 *layers*), *loss of polarity and mild dysplasia. Period of treatment* 6 *months* (× 200).

Fig. 6. *Bladder of mouse treated with DBA and nitrite showing marked hyperplasia* (9 *layers*), *dysplasia, loss of polarity and basal activity. Period of treatment* 9 *months* (× 200).



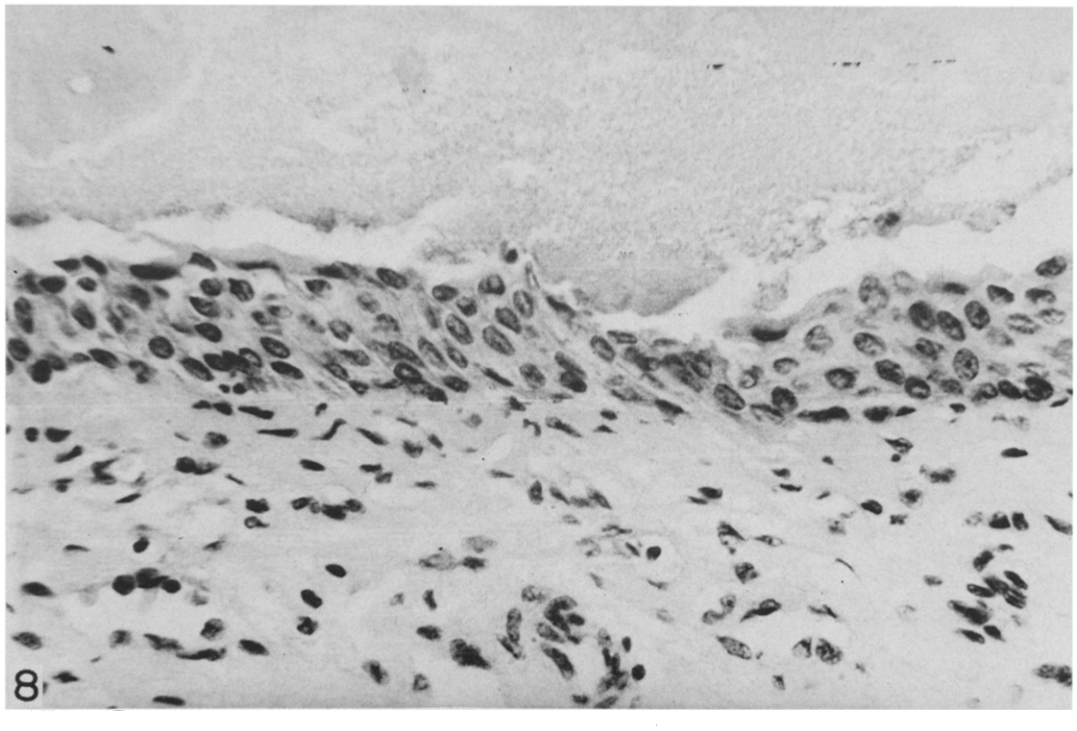


Fig. 7. *Bladder of mouse treated with DBA and nitrite showing benign papilloma. Period of treatment* 9 *months* (× 80).

Fig. 8. *Bladder of mouse treated with DBA, nitrite and soybean showing mild hyperplasia* (4–5 *layers*). *Period of treatment* 9 *months* (× 200).

Table 2. Pathological changes in mice bladder after different periods of treatment

Group	Period of treatment (months)	Hyperplasia	Dysplasia	Squamous metaplasia	Lymphocytic infiltrate	Atrophy	Papillary hyperplasia	Papilloma
DBA + nitrite	1–3	+	—	—	—	—	—	—
	4–6	++	+	+	+	+	—	—
	7–9	+++	++	++	++	+	+++	4 mice (20%)
	10–12	+++	++	++	++	—	+++	6 mice (40%)
DBA + nitrite + soybean	1–3	—	—	—	—	—	—	—
	4–6	+	—	—	—	—	—	—
	7–9	+	+	—	—	—	—	—
	10–12	+	+	—	+	—	—	—
DBA + nitrite + ascorbic acid	1–3	—	—	—	—	—	—	—
	4–6	—	—	—	+	—	—	—
	7–9	+	—	—	+	—	—	—

+ Mild; ++ moderate; +++ marked.

Bladder (Table 2)

Control group. This group included 20 mice. All showed a normal urothelium composed of three layers of transitional cells with a superficial umbrella layer.

Dibutylamine–sodium nitrite group. Epithelial hyperplasia appeared as an early manifestation from the first month of treatment. Transitional cell layering increased to 4–5 layers with no atypical nuclear features. After the 3rd month moderate hyperplasia was seen with urothelium reaching 6–8 cell layers, loss of surface maturation and polarity (Fig. 5). There was mild dysplasia, basal activity and focal areas of squamous metaplasia. From the 7th month onwards marked atypical hyperplasia with moderate dysplasia was observed. Epithelial layering included more than eight layers of cells with hyperchromasia of nuclei and loss of polarity in some cells (Fig. 6). With increased stratification there were areas of hypercellularity and a crowded pattern (papillary hyperplasia). Benign papillomas were detected in four mice (20%) in the group treated for 7–9 months, and in six mice (40%) in the group treated for 10–12 months. The tumours were characterized by papillary exophytic growth of epithelium with central fibro-vascular core and regular nuclear uniformity. Tumours arising during 10–12 months of treatment showed benign papillomas (Fig. 7) accompanied by surface squamous metaplasia in adjacent areas. The rest of the mice in this group showed marked hyperplasia, dysplasia and lymphocytic infiltrate in lamina propria.

Dibutylamine–sodium nitrite–soybean group. The protective effect of soybean was demonstrated by lack of marked dysplasia, squamous metaplasia or any tumour formation. Only mild dysplasia and hyperplasia were seen after the 7th month of treatment (Fig. 8). Focal aggregates of lymphocytes were also detected in the late months of treatment (10–12 months).

Dibutylamine–sodium nitrite–ascorbic acid group. Ascorbic acid also manifested protective effects very similar to those of soybean. Mild hyperplasia was seen after the 7th month of treatment but with no dysplastic features or tumours. Experiments with 10–12 months treatment were not done.

DISCUSSION

The potential carcinogenic effect of the nitrosamine precursors dibutylamine and nitrite was clearly demonstrated in the present study by the pathological changes detected in the liver and bladder of Swiss albino mice. Nitrosamines exert their carcinogenic action through alkylation of DNA [2]. In the present material, benign tumours (papillomas) were induced in the bladder while malignant tumours (hepatomas) were induced in the liver with an incidence of 27% after 12 months of treatment. Using mice, similar results were obtained by Toth *et al.* [8] and Clapp *et al.* [9]. All tumours in the present study appeared after 9 months. Similarly with rats, since they have a longer life span, chronic feeding of sodium nitrite and aminopyrine in drinking water led to almost 100% incidence of liver tumours [10].

The effect of DBA and sodium nitrite on the bladder of mice produced marked hyperplasia of surface urothelium with increased transitional cell dysplasia in 60% of the mice after 4 months of treatment. Papillomas were also observed in 20% of the mice after 9 months, and increased to 40%

after 12 months. It should be pointed out that failure to induce tumours in some mice does not necessarily indicate that no nitroso compounds were formed but that the dose was not sufficient to induce tumours within the period covered by the experiment. Hirose *et al.* [11] suggested that the possible development of bladder tumours depends on the period of treatment and the dose of carcinogen received.

The protective effect of soybean was observed as a marked reduction of dysplastic features in the liver and bladder. In the group of mice treated with DBA and nitrite and fed soybean mixed with the diet their livers did not show any detectable changes during the first 3 months. The other groups (6, 9 and 12 months) showed only mild dysplasia in 9–14% of the animals. The rest of the animals did not show any significant pathologic features.

These results confirmed that feeding soybean in the diet overcomes the carcinongenic action of the nitrosamines formed. Several other investigators have shown a risk reducing effect of a soybean diet [6, 12]. The present study indicates that soybean can minimize the carcinogenecity of the nitrosamine formed from its precursors. Soybean contains at least five protease inhibitors [13]. Feeding a soybean diet rich in protease inhibitors was shown to delay the onset of 2-stage carcinogenesis in mouse skin [14]. Furthermore, ingestion of these protease inhibitors was found to suppress 7,12-dimethylbenz(a)anthracene (DMBA)-induced mammary tumours in rats [15]. The great value of the Bowman–Birk natural protease inhibitor is evident from the fact that it can survive inactivation by stomach digestion in rodents and can act as a protease inhibitor in the small intestine [12]. This protease inhibitor is an anti-carcinogen which has a specific inhibitor of transformation caused by ionizing radiation [16]. Also a synthetic protease inhibitor (amino caproic acid) supplied in the drinking water of experimental animals has been shown to block dimethylhydrazine-induced colon cancer in mice [17].

Soybean seems to minimize the chemical changes that usually occur after administration of a carcinogen. This may be attributed to the high nutritional value of soybean which can antagonize or overcome the destructive action of the carcinogenic nitrosamine. The mechanism of protection is not known; however, soybean may eliminate the carcinogen by binding or interacting with it. Further investigations are required to clarify this point.

Ascorbic acid also as a protector appeared to achieve its action by blocking the formation of nitrosamine *in vivo* by reacting with nitrite and nitrous acid [7], thus competing with amines for the available nitrosylating anions.

The role of ascorbic acid (vitamin C) in the inhibition of the formation of carcinogen *N*-nitroso compounds is well established [18], through a process of nitrosation inhibition. Ascorbic acid considerably reduces the yield of mononitrosopiperazine and sodium nitrite [19] and also prevents the nitrosation and transnitrosation of nornicotine by sodium nitrite and *N*-nitrosodiphenylamine respectively [20]. It was reported that when ascorbic acid was added to nitrite and amines immediately before nitrite addition, there was a 20–25% decrease in mutagenecity [21].

There is evidence to suggest that ascorbate affords protection only when it is present in the rat stomach simultaneously with the amine and nitrite [22]. The protective effect depends on the rapid reaction of ascorbate with nitrite to form nitroso-ascorbate to inhibit nitrosamine formation. Ascorbic acid has no effect on the previously formed ethylnitrosourea. This proves that ascorbic acid acts by removing nitrite from the reaction mixture before it reacts with the amines [23].

In conclusion this study as well as other reports from other laboratories strongly demonstrate the important role of soybean and ascorbic acid in counteracting the potential human hazards due to nitrosamine precursors in our environment. Also it supports the protective action of soybean and ascorbic acid against tumour incidence in animals used in experiments.

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