Effect of food products on endogenous generation of *N*-nitrosamines in rats

BY V. K. ATANASOVA-GORANOVA[1], P. I. DIMOVA[2]
AND G. T. PEVICHAROVA[3]

[1]*Department of Hygiene and Ecological Medicine and* [2]*Department of Histology and Embryology,
Higher Medical Institute, Plovdiv, Bulgaria*
[3]*Department of Vegetable Quality, 'Maritsa' Vegetable Crops Research Insititute, Plovdiv, Bulgaria*

An experiment was conducted to study the efficacy of two tomato pastes and aronia nectar (fruit juice + pulp from the black chokeberry, *Aronia melanocarpa* Elliot) as inhibitors of nitrosamine production in cancer prophylaxis programmes. White male rats of the Wistar strain were employed in an acute trial. Aminopyrin + sodium nitrite (APSN) were used as precursors for generation of endogenous nitrosamine. The animals were allocated to different dietary groups and fed by intubation with APSN or APSN + food products. Introduction of tomato paste (TP), high-β-carotene tomato paste (HCTP) and aronia nectar (AN) as inhibitors of *N*-nitrosamine formation exerted a positive effect on blood and liver variables which was demonstrated by decreased concentrations of glutamic-oxaloacetic transaminase (*EC* 2.6.1.1), glutamic-pyruvic transaminase (*EC* 2.6.1.2) and uric acid in serum and lipid content in hepatocytes. Animals treated with APSN developed dystrophic changes in liver such as centrolobular necrosis, intense exangia, and enlarged cells with two, often large, pyknotic nuclei, while the structure of livers of rats fed with TP, HCTP or AN was well protected and almost normal. TP had a particularly beneficial effect on serum total protein and albumin concentrations as had AN on the urea value. The inhibitory effect of the food products used is explained by their chemical nature including pH, ascorbic index (ascorbate : nitrate), lycopene and β-carotene contents.

Cancer prophylaxis: Nitrosamines: Tomato paste: Aronia nectar

Among various cancer prophylactic measures an important place is taken by inhibitors of nitrosation which generates the carcinogenic *N*-nitrosamines (Mitrov & Chernozemski, 1985; Boyadjiev & Markova, 1987; Lathia *et al.* 1988; Tannenbaum *et al.* 1991).

It has been proved that, in the stomach of animals and man, nitrosamines may be synthesized from non-carcinogenic precursors, i.e. nitrites and amines (Holmes, 1971; Rubenchick, 1977; Zhukova *et al.* 1990; Shahidi & Pegg, 1993). The carcinogenic nitrosocompounds (NC) are formed mostly from secondary amines and amides. Nitrosation agents appear to be either nitrites or nitrates as the latter can be reduced endogenously to nitrites. Until now the inhibitory effect of compounds such as vitamins C, E, and A, thiamin, riboflavin, niacin and tannins has been studied in detail and it has been shown that in their presence the nitrite anion is not able to react with secondary amines (Rubenchick, 1979; Tannenbaum, 1989; Zhukova *et al.* 1989; Mergens, 1992). The nitrosation process may be inhibited not only by isolated chemical compounds but also by the foodstuff which contains these inhibitors (Rubenchick *et al.* 1985). The object of the present experiment

was to study the inhibitory effects of tomato paste (TP), high-β-carotene tomato paste (HCTP) and aronia nectar (AN) on endogenously generated nitrosamines in rats under acute experimental conditions.

MATERIALS AND METHODS

Food products

TP and HCTP were produced in the 'Maritsa' Vegetable Crops Research Institute, Plovdiv. Both were processed according to Bulgarian State Standard no. 508-85 (Council of Ministers, 1985). Red and high-β-carotene tomato varieties bred in the same institute were used as raw materials. Black chokeberry (*Aronia melanocarpa* Elliot) nectar (AN) was prepared according to Bulgarian State Standard no. 5162-81 (Council of Ministers, 1981) and the technological instructions of the Food Processing Industry (National Agro-Industrial Union, 1985). As defined by this standard, the fruit nectar is the fruit juice + pulp.

The chemical composition of these three products is presented in Table 1. The ascorbic index (AI) denotes the ratio ascorbic acid : nitrate content in the food product.

Animals

An acute experiment similar to those described by Kamm *et al.* (1975) was performed. White male rats of the Wistar strain with average weight about 200 g were randomly allocated to five dietary groups of ten animals each: group 1, control; group 2, aminopyrin + sodium nitrite (APSN); group 3, APSN + TP; group 4, APSN + HCTP; group 5, APSN + AN.

Operative procedure

On three consecutive days the animals were fed by intubation as follows: group 1, distilled water, 1 ml/100 g body weight (BW); group 2, *N*-nitrosamine precursors (12·5 mg aminopyrin + 11 mg sodium nitrite (APSN) per ml water), 1 ml/100 g BW. Groups 3, 4 and 5 were given the same volume of APSN plus 1 ml/100 g BW of TP, HCTP or AN respectively.

The trial was conducted at 21°, 40–60 % relative humidity with a 12 h light–dark cycle. The rats were killed by cervical dislocation on the 4th day, 24 h after the last intubation.

Tissue preparation

Blood was collected from cervical blood vessels. A portion of it was immediately centrifuged and frozen at −20°.

The serum was studied by Kone Dynamic apparatus (Kone Instruments Oy, Espoo, Finland) on the next day to determine total protein, albumin, non-protein N compounds (urea, creatinine, uric acid), and the enzymes glutamic-pyruvic transaminase (*EC* 2.6.1.2; GPT) and glutamic-oxaloacetic transaminase (*EC* 2.6.1.1; GOT). They were determined by means of the colorimetric methods of the Technicon RA-1000™ system (Technicon Instruments Corporation, 1985).

Table 1. *Chemical composition of the food products*

Food products	DM* (g/kg)	Total sugars (g/kg)	pH	Organic acid† (g/kg)	Ascorbic acid (mg/kg)	β-Carotene (mg/kg)	Lycopene (mg/kg)	AI‡
Tomato paste	349	172·0	4·6	18·0	451·2	11·9	235·7	0·35
High-β-carotene tomato paste	320	131·5	4·8	16·4	564·0	89·7	33·0	0·45
Aronia nectar§	108	38·2	3·3	3·9	1100·0	–	–	1·64

* Assessed by refractometry.
† As citric acid.
‡ Ascorbic index = ascorbate : nitrate.
§ Fruit juice + pulp from black chokeberry (*Aronia meanocarpa* Elliot).

Another portion of the blood was used for analysis of haematological values: haemoglobin, erythrocytes, leucocytes, differential leucocyte count and erythrocyte sedimentation rate assessed by the Panchenko method (Todorov, 1962).

The liver was removed immediately and samples were taken for histopathological and histochemical examination. Paraffin specimens were prepared, which, after staining with haematoxylin–eosin, were studied by light microscopy. A test for total lipids was performed on cryostatic segments, 5–6 μm thick, by staining with Sudan III-H.

Statistical analysis

The data were evaluated by coefficient of variability and Duncan's multiple-range test.

RESULTS

Effect of food products on some serum and haematological variables

The inhibitory effect of the food products on some serum variables in rats is shown in Table 2. Groups 3 and 4 had significantly lower serum total protein and albumin concentrations in comparison with groups 1, 2 and 5.

The variables most indicative of liver damage are GOT and GPT. Substantial differences were found between group 2, in which values were very high, and the other four groups.

Non-protein N compounds showed a different pattern in each group. AN, the diet with highest ascorbic acid content, was the only treatment which reduced the urea concentration. There were no differences among groups in serum creatinine content. Diets including TP, HCTP and AN led to lower serum levels of uric acid than in controls whilst APSN alone increased uric acid. The inhibitory effects of food products on the haematological indices are shown in Table 3. Haemoglobin was not affected by APSN but in group 4 (given HCTP) a value significantly below the control was obtained. The value for erythrocytes in the AN group was very similar to that in the control group while both groups receiving tomato paste had lower erythrocyte levels. The leucocyte count of the AN group was also close to that of the control. No significant differences were found among the five groups in erythrocyte sedimentation rate (Table 3) nor in differential blood count (Table 4).

Effect of food products on liver structure and quantity of lipids in hepatocytes

The histological examination of liver samples showed substantial differences in structure and lipid content in animals given APSN only (Fig. 1(c) and (d)) and those given APSN + TP or AN (Fig. 1(e) and (f)).

Animals treated only with APSN (group 2) developed dystrophic changes in the liver (compare Fig. 1(a) and (c)) while those treated also with TP or AN (groups 3, 4 and 5) had a well-preserved liver structure (Fig. 1(e)).

The results from histochemical analysis showed that total lipid content in hepatocytes of group 2 animals was greatly increased (suggesting fatty degeneration; compare Fig. 1(b) and (d)). Conversely all groups given TP or AN had a decreased lipid content in liver cells; this was most pronounced in groups 3 and 5 (Fig. 1(f)).

Table 2. *Effect of tomato paste (TP), high-β-carotene tomato paste (HCTP) and aronia nectar (AN) on some serum variables in rats given nitrosamine precursors by oral intubation**

(Mean values and coefficients of variation for ten rats per group)

Groups	Total protein (g/l) Mean	CV	Albumin (g/l) Mean	CV	GPT (U/l) Mean	CV	GOT (U/l) Mean	CV	Urea (mmol/l) Mean	CV	Creatinine (μmol/l) Mean	CV	Uric acid (μmol/l) Mean	CV
1: Control	66[a]	6·5	12[a]	19·6	61[b]	19·6	269[b]	25·0	8·2[a]	31·0	68	18·9	45[ab]	40·0
2: APSN	62[a]	17·2	11[a]	14·9	570[a]	64·6	940[a]	51·4	8·3[a]	33·2	66	12·0	57[a]	39·0
3: APSN + TP	51[b]	10·7	8[b]	19·4	95[b]	47·6	316[b]	17·3	8·4[a]	36·3	60	10·8	30[b]	39·8
4: APSN + HCTP	48[b]	14·8	8[b]	13·2	104[b]	54·6	455[b]	37·2	8·5[a]	23·0	68	10·0	33[b]	37·4
5: APSN + AN	62[a]	7·8	11[a]	12·4	126[b]	27·0	324[b]	33·6	5·5[b]	12·6	64	3·8	32[b]	43·1

GPT, glutamic-pyruvic transaminase (*EC* 2.6.1.2); GOT, glutamic-oxaloacetic transaminase (*EC* 2.6.1.1); APSN, aminopyrin + sodium nitrite.

[a,b] Mean values within a column not sharing a common superscript letter were significantly different, $P < 0.05$.

* For details of food products and procedures, see Table 1 and p. 336.

Table 3. *Effect of tomato paste (TP), high-carotene tomato paste (HCTP) and aronia nectar (AN) on some haematological variables in rats given nitrosamine precursors by oral intubation**

(Mean values and coefficients of variation for ten rats per group)

Groups	Hb (g/l)		Er ($\times 10^{12}$/l)		Leucocytes ($\times 10^9$/l)		ESR (mm/h)	
	Mean	CV	Mean	CV	Mean	CV	Mean	CV
1: Control	139[a]	9·7	6·82[a]	15·4	5·4[a]	20·2	1·3	38·7
2: APSN	128[ab]	8·3	5·82[ab]	18·8	3·1[bc]	31·9	1·7	31·0
3: APSN + TP	123[ab]	5·5	5·20[b]	13·8	4·0[bc]	10·5	1·3	38·7
4: APSN + HCTP	113[b]	9·4	4·64[b]	7·0	3·0[c]	9·9	1·5	36·5
5: APSN + AN	140[a]	23·8	6·80[a]	27·0	4·7[ab]	29·6	1·5	36·5

Hb, haemoglobin; Er, erythrocytes; ESR, erythrocyte sedimentation rate; APSN, aminopyrin + sodium nitrite.
[a,b,c] Mean values within a column not sharing a common superscript letter were significantly different, $P < 0.05$.
* For details of food products and procedures, see Table 1 and p. 336.

Table 4. *Influence of tomato paste (TP), high-β-carotene tomato paste (HCTP) and aronia nectar (AN) on differential blood count of rats given nitrosamine precursors by oral intubation**

(Mean values for ten rats per group)

Groups	Ne	Eo	Mo	Ly
1: Control	0·34	0·02	0·04	0·61
2: APSN	0·44	0·02	0·02	0·53
3: APSN + TP	0·42	0·03	0·04	0·53
4: APSN + HCTP	0·46	0·02	0·03	0·50
5: APSN + AN	0·43	0·04	0·04	0·50

Ne, neutrophils; Eo, eosinophils; Mo, monocytes; Ly, lymphocytes; APSN, aminopyrin + sodium nitrite.
* For details of food products and procedures, see Table 1 and p. 336.

DISCUSSION

These results have established an inhibitory effect of the three food products on *N*-nitrosamine production. TP exerted a positive effect on serum total protein and albumin content. It also sharply decreased GOT and GPT values which are the most important indices related to liver damage. Similar results were reported by Rubenchick *et al.* (1985) in experiments with apple pectin alone or enriched with ascorbic acid, as well as with drugs made from plantain (plantastin) and lime blossom (tiliaflan).

Neither of the TP diets affected serum urea or creatinine content but both significantly reduced the uric acid content.

In comparison with the two TP products AN showed a different pattern as far as serum variables are concerned. No changes were found in total protein or albumin content. Liver enzymes showed the same positive effect as for the two TP diets. AN exerted the strongest effect on the urea value which was reduced by 34 %. This demonstrates improved kidney function which is also evident when the urea value is compared with the control. With respect to haematological indices the inhibitory effect of AN was similar to that of both TP products.
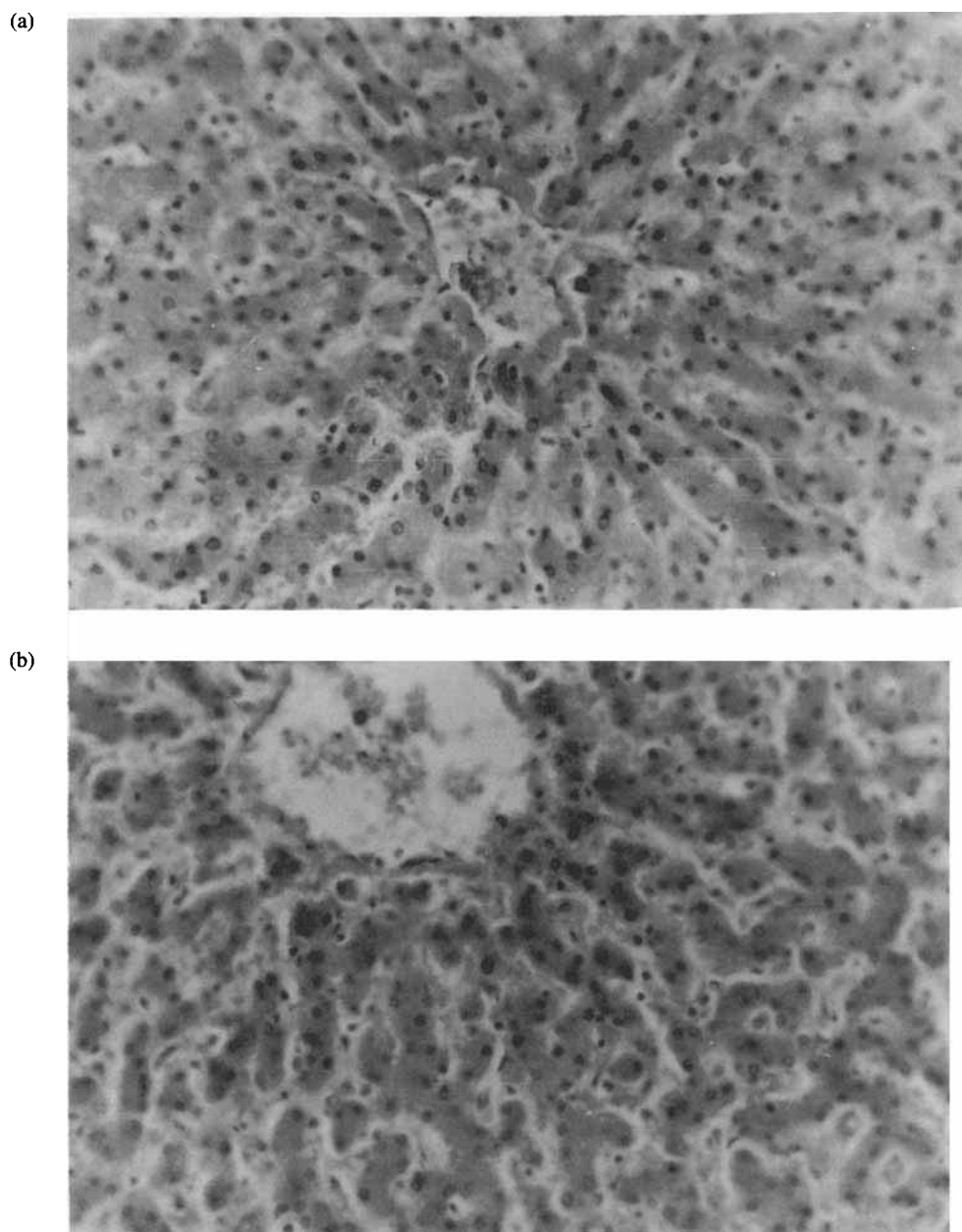
(a)

(b)

Fig. 1. Photomicrographs of liver samples taken from rats given nitrosamine precursors by oral intubation for 3 d, either alone or together with tomato paste or aronia nectar (fruit juice + pulp of black chokeberry (*Aronia melarocarpa* Elliot)). Samples were prepared in paraffin wax, stained with haematoxylin and eosin and studied for structural change by light microscopy (a, c and e) or prepared by cryostatic segmentation and stained with Sudan III-H for total lipids (b, d and f). (a) Normal hepatic structure from an untreated animal (magnified 10×40). (b) Normal lipid inclusion in hepatocytes from an untreated animal (magnified 10×40). (c) Severely dilated *vena centralis* and dystrophic changes in the hepatic cells resulting from ingestion of nitrosamine precursors (magnified 10×25). (d) Severely increased lipid inclusion in hepatocytes resulting from ingestion of nitrosamine precursors (magnified 10×40). (e) Hepatic structure preserved in animals receiving tomato paste with nitrosamine precursors (magnified 10×40). (f) Almost normal lipid inclusion in hepatocytes of animals given aronia nectar with nitrosamine precursors (magnified 10×40).
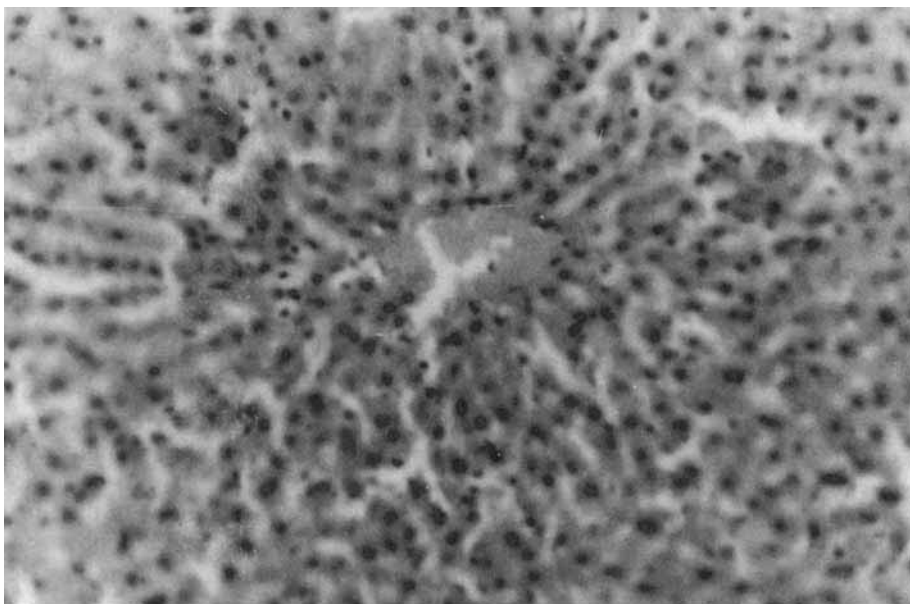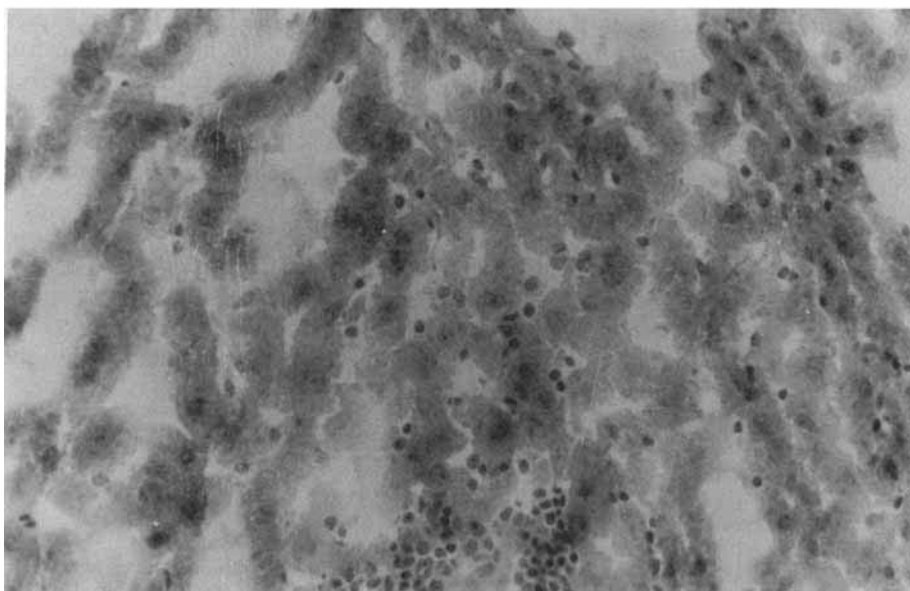
(c)

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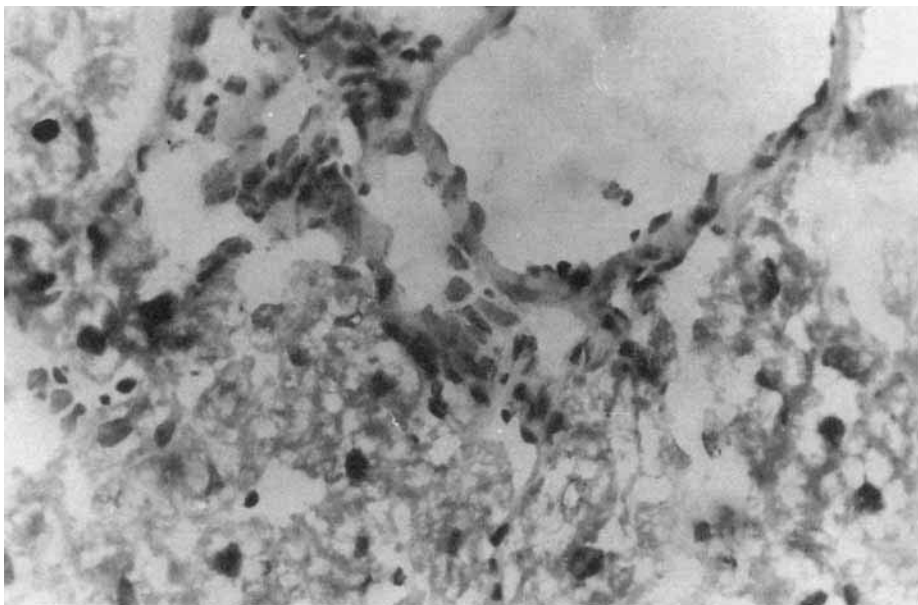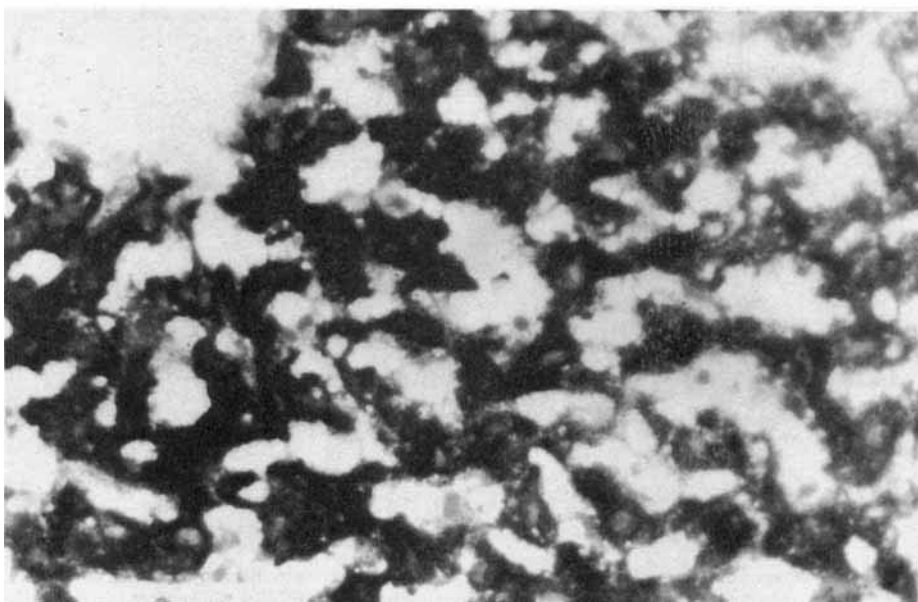
(e)

(f)

The histological and histochemical investigations of liver confirmed an inhibitory effect on nitrosamine formation of the food products used. Animals treated with APSN (group 2) showed dystrophic changes in liver that have been described by many authors (Rubenchick, 1979; Donchev & Petrova, 1982; Rubenchick *et al.* 1983; Lin & Ho, 1992; Tziganenko, 1993). These include centrolobular necrosis, intense exangia, hepatocytes showing an increased oxyphilic activity in the cytoplasm, and pyknotic nuclei in many cells. There were also enlarged cells with large nuclei and frequently with two nuclei (compare Fig. 1(a) and (c)). Addition of TP or AN to the diet resulted in a considerable protection of liver structure. Very few dystrophic changes were noticed, there was no centrolobular necrosis, nor were there any pyknotic nuclei or enlarged cells. These findings show the protective effect of the three food products in relation to liver structure; this was particularly evident in the diet containing TP (Fig. 1(e)).

A large quantity of lipid in hepatocytes causing adipose degeneration was found in animals of group 2 (compare Fig. 1(b) and (d)), while a considerable reduction in lipid content of liver cells was found with the rats of groups 3, 4, and 5. This is further evidence of the inhibitory effect of the food products employed, and was most pronounced in the animals given AN (Fig. 1(f)).

The positive histological and histochemical profiles in the rats fed with the three food products correlated with the levels of serum GOT and GPT. There are a number of factors in these food products which may afford this protection against the harmful effects of nitrosamines. First, they may have changed the pH of the digestive tract; it has been shown that a pH of 3·3–4·8 of the food product or the reaction mixture is likely to inhibit nitrosamine production (Rubenchik, 1979, 1990). Second, the AI, an indicator of the relative concentrations of ascorbate and nitrate in vegetable foods, may be one reason for the protective effects observed. A high AI, together with a high tannin content, is believed to be of benefit in preventing nitrosamine production (Petrova, 1987; Hristov *et al.* 1988; Mitov *et al.* 1990; Rooma & Kann, 1993; Seidemann, 1993*a,b*). In the experiments described here, the beneficial effects of AN seem to be due to the high AI of this fruit. It is interesting to note therefore that this treatment appeared to be particularly effective in reducing plasma urea concentration. The effect of TP may be due to its high content of lycopene. In the case of the third food product, HCTP, it is the high β-carotene content that is the most important factor. All four of these factors appear to be important in the protection against the carcinogenic effects of nitrosamines.

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