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INFLUENCE OF DIETARY PROTEIN ON THE CARCINOGENIC ACTIVITY OF TANNIC ACID*

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Among the chemical carcinogenic substances the azocompounds are remarkable 1. owing to their selective potency in producing liver tumours, and, 2. because of the fact that the development of these tumours can be highly influenced by dietary factors [3, 43]. We have recognized a new carcinogenic substance in the tannic acid [21, 22]. Tannic acid is also noteworthy among the chemical carcinogenic substances, partly because, according to our investigations, when administered parenterally it had the same selective potency on the liver as the azo-dyes [22], though its chemical composition differs from that of every other carcinogenic substance hitherto known. On the other hand it must not be overlooked that certain foods and drinks playing a considerable part in human nutrition contain tannic acid.

In order to study tannic acid carcinogenesis on the basis of the similarity of its selective effect to that of azo-dyes, it seemed first of all necessary to investigate the possible influence of dietary factors. In one of our earlier experiments we have noticed a certain protective effect of a high casein diet [23]. In the present experiments we have examined principally the influence of a low and high casein diet. A preliminary communication of these investigations has already been published [24].

Experiments

120 young white rats have been divided into three equal groups, each containing 17 males and 23 females. The rats of group I. at the beginning of the experiment weighed from 48 gm. to 89 gm., the average being 63 gm. This group was fed a high casein low fat diet. The weight of the rats in group II. varied from 45 gm. to 100 gm., the average being 68,6 gm. This group was kept on a low casein high fat diet. The initial weight of the rats of group III. amounted from 51 gm. to 105 gm., on an average 66 gm. Group III. was fed also a low casein high fat diet. The composition of the diets was the following.

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I.			II. and III.		
casein	25	per cent	casein	3	per cent
sunflower oil	5	« «	sunflower oil	20	« «
cod liver oil	1	« «	cod liver oil	1	« «
Sós' salt mixture	4	« «	salt mixture	4	« «
yeast powder	3	« «	yeast powder	3	« «
dextrine	12	« «	dextrine	19	« «
maize-barley grits	50	« «	grits	50	« «

The diet of groups II. and III. has been changed from the 150th day of the experiment, inasmuch as 44 per cent of dextrine and 25 per cent of maize-barley grits have been given. The grits were cooked in water until thick and then cooled, then about 10 per cent unboiled milk was added together with the other constituents. Group II. and III. received pyridoxine (»Benadon«) until the 210th day of the experiment, per animal daily 1,25 mg. mixed into the diet. Ten g. of the above mixture was given daily to every rat, in the summer months in two portions. Water was not restricted. Each rat of groups II. and III. was kept in a separate cage.

Groups I. and II. were treated with tannic acid. All the rats received in the beginning on every fifth day 150 mg., from the 48th day on 200 mg., from the 87th day on 225 mg. and from the 128th day on 200 mg. of tannic acid per kg. of body weight in the form of an 1,5, 2,0, and 2,25 per cent aqueous solution respectively injected subcutaneously in the back. Thus up to the 180th day every rat received altogether 6975 mg. of tannic acid per kg. body weight. The diet was begun on October 23, 1950, and the tannic acid treatment on October 30, 1950

The body weight was regularly recorded. The increase in weight was roughly equal in each group.

	I.	II.	III.
1st day of the treatment	$91,9 \pm 10,5$	$82,1 \pm 11,1$	$85,5 \pm 7,39$
90th « « « «	141,9	121,9	168,7
180th « « « «	193,3	168,4	188,3

Up to the 100th day of the treatment, 2 males and 1 female died in group I., 1 male and 2 females in group II.; until the 180th day another 8 males and 14 females in group I., and 5 males and 13 females in group II. In group III. no animal died spontaneously up to 360th day, 4 were sacrificed on the 145th and 4 on the 180th day, 2 on the 226th and 2 on the 315th day.

At the site of the tannic acid injections skin necroses, later ulcers arose during the treatment. These, however, did not influence the general condition of the rats in any way. Ulcers usually healed without treatment.

The rats were dissected immediately after death and the organs were fixed in 4 per cent formaldehyde. Gross hepatic tumours and cirrhotic changes were microscopically controlled in every case.

Rats used in this and in our previous experiments [21, 22, 23] were of the same strain of our own stock bred for years. No spontaneous tumour could be noted even in older rats of this strain.

Results

Taking the first day of tannic acid treatment for the beginning of the experiment, in group I. (high casein, low fat diet) 25 rats died and in group II. (low casein, high fat diet) 21 rats until the 180th day. In group III. (untreated animals fed on a low casein, high fat diet) not a single rat was lost.

Hepatic tumours of varying size were formed far more frequently in the animals of group II. than in those of group I. (Fig. 1, 2, 3). In order to have a proper picture of the state of the experiment, on the 180th day of treatment an explorative laparotomy was performed on the 15 surviving rats of group I., on the 19 of group II. and on all the animals of group III., and a thorough gross examination of the liver was made. As, according to our previous experience, the liver lesions of non-tumorous nature which rarely occurred in the rats of our own breeding, can be well differentiated by the naked eye from those produced by tannic acid, and as produced tumours appear almost invariably immediately below the capsule, this method seemed to be adequate.

Three rats, each from the first two groups, which had died before the 100th day of treatment were not included in the statistics to be discussed as in those animals only initial lesions were found. Data referring to both laparotomized animals and those which had died between the 100th and 180th day of the experiment show that the frequency of hepatic tumours in animals fed a low casein, high fat diet was twice as much (26 of the 37 animals, i. e. $70 \cdot 3 \pm 7{,}5$ per cent) as in those kept on a high casein low fat diet (11 of the 37 animals, i. e. $29{,}7 \pm 7{,}5$ per cent).

The error of the 40,5 per cent difference in the frequency of the tumours produced in groups I. and II., on the basis of the formula $m_{diff} = \pm \sqrt{m_1^2 + m_1^2}$ (where m_1 signifies the standard error in the percentage of group I.; m_2 the standard error in the percentage of group II.), amounts to $\pm$ 10,62, thus the threefold error of the difference is still considerably lower than the difference between the percentual frequencies of the two groups. Consequently, the difference between the two groups in the frequency of tumours produced is significant [5].

In group III., which was not treated with tannic acid and was kept on the same low casein high fat diet as group II. no changes worth mentioning could be found either with the naked eye or microscopically (8 animals were sacrificed before the 180th day).

The tannic acid treatment lasted 280 days and during this time the rats received further 14 subcutaneous injections of tannic acid. Eight animals of group

I., and 7 of group II., survived the 280th day. After discontinuing the tannic acid treatment, the diets were left unchanged until the 360th day when the

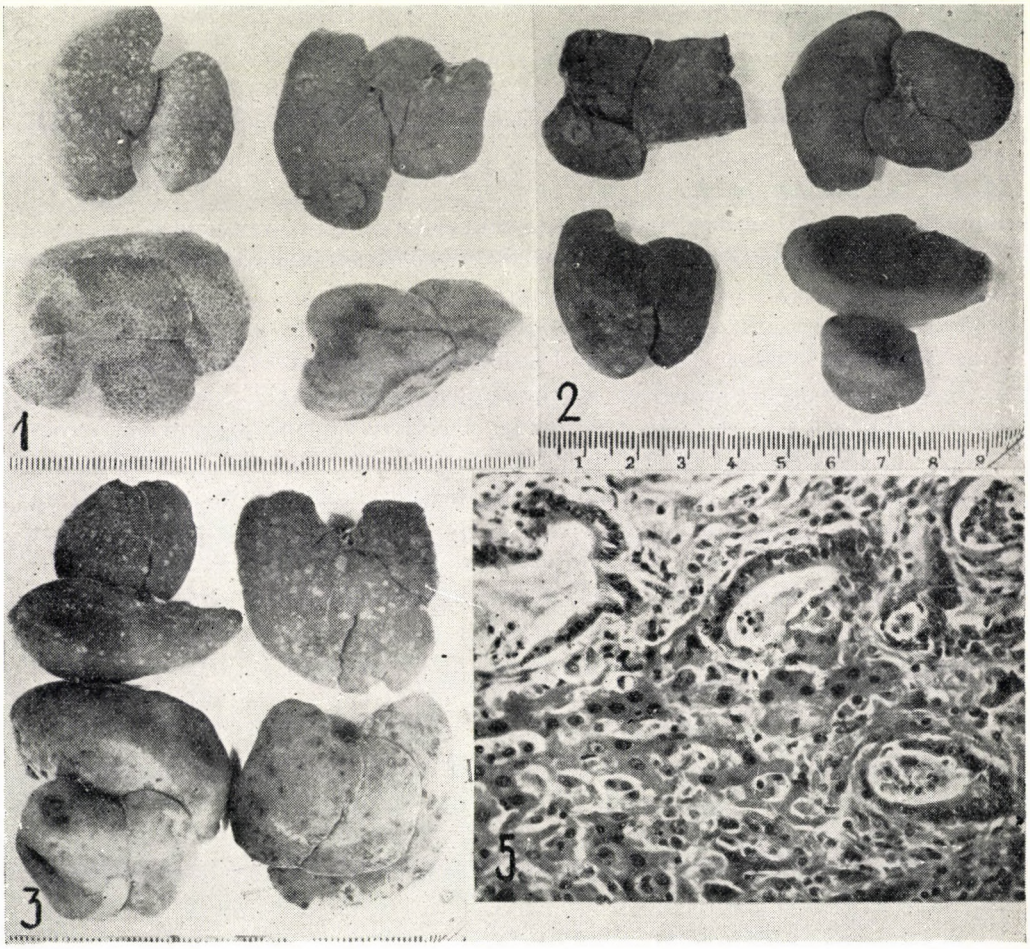

Fig. 1. Upper row, livers of rats of group II. (low casein high fat diet) died on the 117th and 127th day ; lower row, livers of those of group I. (high casein low fat diet) died on the 114th and 126th day. — Tumours in the upper row.

Fig. 2. Upper row, livers of rats of group II. died on the 130th and 144th day ; lower row, livers of those of group I. died on the 129th and 143d day. Tumours in the two upper and in one of the lower.

Fig. 3. Upper row, livers of rats of group II., died on the 193th and 203th day ; lower row, livers of those of group I., died on the 192nd and 197th day. Tumours in the upper row.

Fig. 5. Rat CTSz/39, killed on the 360th day. Low grade adenocarcinoma. x400.

few surviving animals were sacrificed. The same significant difference between the groups which were fed the high casein low fat and the low casein high fat diets in the frequency of the tumours which had been found by explorative laparotomy on the 180th day, could be stated also at the end of the experiment

(see Fig. 4). In group III., neither cirrhosis nor tumour has developed in any of the rats up to the 360th day.

Remarkable are the findings referring to *liver cirrhosis*. At the end of the experiment, in 16 (43 per cent) from among the rats of group I. surviving the 100th day, kept on high casein and low fat diet, and in 14 (37 per cent) of the 37 rats of group II., fed the low casein and high fat diet, a diffuse nodular

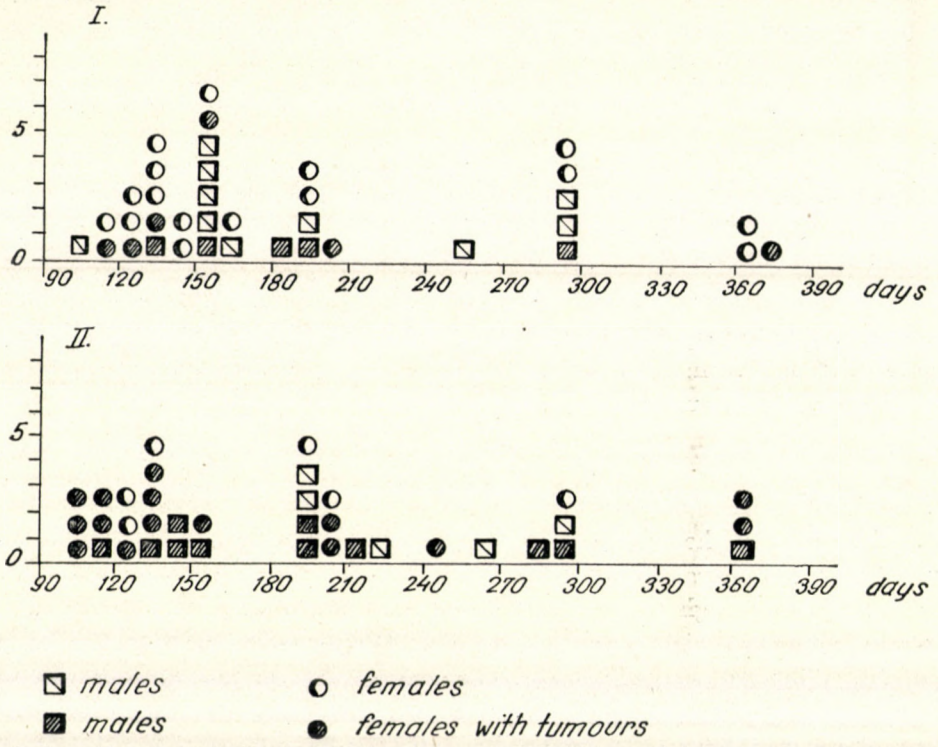

Fig. 4. Above are shown the rats fed the high casein diet, underneath those which had been fed the low casein diet and which died during treatment. On the abscissa the time of the experiment is given in months and on the ordinata the number of animals dead.

cirrhosis occurred. Cirrhosis and liver tumour occurred simultaneously in 5 rats of group I., and in 8 rats of group II. In 6, and 18 rats, respectively, liver tumours were formed without cirrhotic changes.

As regards the *influence of sex*, liver tumours occurred in 5 of the 15 male rats surviving the 100th day, in 6 of the 22 females of group I., and in 11 of the 16 males and in 15 of the 21 females of group II. Thus there was no definite difference between males and females. As to *the survival time* of the animals in group I., it was identical both with males and females (191 and 192 days, respectively) ; it was the shortest with the males of group II. (156 days), while the life of females was somewhat longer (175 days).

The tumours were usually well circumscribed and paler than the surrounding liver tissues. Some of the larger ones had a moderately lobulated appearance. No metastases were seen.

Upon *microscopic* examination, the growths proved to be hepatomas or cholangiomas, as it had been described in detail in our previous publications [22, 23]. The hepatomas were composed of fairly regular or irregular cords of large hepatic cells. It was difficult to differentiate between adenoma and low grade carcinoma. The latter showed a greater irregularity of the liver cell cords, decreased oxyphylia of liver cells, more mitotic figures and an invasive tendency at the margins.

In the cholangiomas, the tubules considerably varied in shape and width, and were surrounded by a small amount of connective tissue only. Still more marked irregularities were present in the epithelial lining and not infrequently small islets of liver cells could be found incorporated in the mass of proliferating bile ducts (Fig. 5).

Tumours that probably originate from hepatic or bile duct epithelial cells were roughly equally distributed in the groups fed the two different diets (group I., 7 hepatomas, 4 cholangiomas ; group II., 13 hepatomas, 7 cholangiomas ; in 6 cases, tumours of both types).

Discussion

The data of the experiment have shown that liver tumours could be produced with parenteral administration of tannic acid in 70 per cent of the rats fed a low casein high fat diet with pyridoxine added while in 29 per cent of the rats kept on a high casein low fat diet. The difference in frequency of the tumours produced was significant. Considering that in our previous investigations liver tumours could be produced with this same treatment in 56 per cent of the rats kept on a mixed diet, our results suggest that using tannic acid as a carcinogenic substance a low casein high fat diet accelerates, to a certain extent, the production of liver tumours while a high casein low fat diet has a retarding effect. The acceleratory effect of the diet, at least during the short time of the experiment, became evident merely in the increased frequency of the tumours produced, while it had no demonstrable effect either on the structure of the tumours produced or on the degree of malignancy.

As cirrhosis was equally frequent in both groups, it may be stated that under the above experimental conditions the cirrhogenic effect of tannic acid has not been influenced by the casein or fat content of the diet. This fact, in addition to the opinion of numerous authors [29, 30, 32, 16, 13] with reference to azo-compounds, is in favour of the assumption that cirrhosis is not a necessary precursor of liver tumours, though not infrequently the two conditions appear simultaneously.

The sex of the animals in this experiment did not influence either the cirrhogenic or the carcinogenic effect of tannic acid. Though tannic acid is a strongly toxic substance, chiefly when parenterally administered, both the treated animals and the untreated ones developed satisfactorily. The average survival time of the rats (chiefly males) kept on a low casein diet was shorter than of those fed a high casein diet. In the untreated animals which were fed the low casein high fat diet neither cirrhosis nor tumour was formed within a year.

The relation of dietary factors and carcinogenesis, with regard to the effect of the carcinogenic azo-compounds, offered a wide field for study. It became evident that several significant constituents of the food, and also other factors exert an accelerating or retarding effect on tumour formation. From the important role played by proteins in the structure and function of tissues it may be concluded that the protein content of the diet is a very important modifying factor in the growth and development of tumours. Retarding factors are, casein and riboflavin together [29, 19, 39, 14]; yeast and ether extract of yeast [29, 31, 40, 41]; dried milk [38]; fresh milk [16]; tocopherol in large doses [42]. It is worth mentioning that although it is possible to delay the azo-carcinogenesis by these and other [3] retarding factors so far no diet is known that would prevent it altogether if the carcinogenic substance is administered for a sufficient time.

Accelerating factors are a high fat diet [32, 20]; biotin [7, 15]; pyridoxine [30, 28]; vitamin B_{12} [6]. There are, however, contradictory data, e. g. according to *Silverstone* [35] a high fat diet would have no accelerating effect. The type of the fat used is not indifferent either [20]. *Baumann* [2] attributes a smaller part to caloric changes in azo-carcinogenesis.

We owe to *Kensler* et al. [18] the recognition of the importance of riboflavin in the carcinogenic effect of azo-compounds. They have established that the riboflavin content in rats' liver fed a basic diet of rice and beet is quickly reduced. The most intensive carcinogenic effect is shown by those of the azo-compounds which produce the greatest decrease in the riboflavin content of the liver [9]. Addition of riboflavin to the incomplete diet did not influence tumour formation but administering the substance together with casein proved to exert a strong retarding effect [19].

It was assumed that the diets accelerating tumour formation (low casein and high fat) would act by increasing the lipoid content of the liver. *György*, *Poling* and *Goldblatt* [12] attributed the protective effect of casein to its lipotropic activity. According to others, casein increases the utilization of riboflavin. *Sarett* et al. demonstrated that the ability of the liver to store riboflavin is augmented by the protein of the food [34]. *Griffin* et al. have shown that casein and methionine retard the formation of azo-tumours owing to the retention of the riboflavin content of the liver [10, 11].

As to the influence of the diet on acetylaminofluorene carcinogenesis, views are divergent. In *Harris'* opinion [13] the protein content of the diet and liver extracts are ineffective. *Engel* and *Copeland*, on the other hand, recently demonstrated a certain protective effect of natural foodstuffs [8].

As to the influence of sex, it is known that male rats fed 2-acetylaminofluorene develop liver tumours with increased frequency, while females are more resistant [4, 37, 27]. With azo-compounds such differences were not observed for long. Recently, however, *Rumsfeld* et al. [33], employing 3-methyl-4-dimethylaminoazobenzene and 4-fluoro-4-dimethylaminoazobenzene, demonstrated that tumours are more frequently formed in the liver of male rats.

The effect of dietary factors on tumours both transplantable and produced by other chemical carcinogenic substances has been studied by several workers. Noteworthy is the statement of *Sós* and al. [36] according to which the growth of the Guérin-cancer of rats is greatly inhibited when methionine is completely lacking in the diet, although extensive metastases may occur in the animals.

On the basis of our investigations, the influence of dietary factors on tannic acid carcinogenesis may be justly assumed. Both accelerating and retarding effects seem to depend primarily on the protein content of the diet. Whether the retarding effect of casein on the formation of tumours produced by tannic acid is exercised through an influence on the riboflavin content of the liver, is to be decided by further investigations. In our opinion, however, there are still other factors to be considered in the problem of tannic acid carcinogenesis. *Kovács* and *Korpássy* [26] have demonstrated that in tannic acid stress [26] a high casein diet increases the activity of the hypophyseal-adrenocortical system, furthering in this manner the non-specific resistance of the organism. The change in neuro-endocrine regulations during prolonged tannic acid administration can not be a negligible factor in tumour formation either. Hepatocerebral relations deserve also special attention. The relation between the liver and the nervous system, well known from Wilson's disease [17] and from the encephalitis of dogs with an Eck fistula [1], may offer a new view in studying the mechanism of hepatogenous carcinogenic substances.

Summary

1. A high casein low fat diet retarded the carcinogenic effect of tannic acid on the liver, while a low casein high fat diet accelerated it.

2. The accelerating effect of the diet on tumour formation in the liver has been manifested merely in the frequency of the tumours produced.

3. Neither the casein nor the fat content of the diet have influenced the cirrhogenic effect of tannic acid under the experimental conditions.

4. No parallelism could be found in this experiment between the cirrhogenic and carcinogenic effects of tannic acid.

5. Sex had no influence either on the cirrhogenic or on the carcinogenic effect of tannic acid.

6. The survival time of the rats was somewhat longer on a high casein low fat diet than on a low casein high fat diet.

7. A low casein high fat diet without tannic acid administration did not show to have either a cirrhogenic or a carcinogenic effect during the 360 days of the experiment.

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О ВЛИЯНИИ СОДЕРЖАНИЯ КАЗЕЙНА НА КАРЦИНОГЕННУЮ АКТИВНОСТЬ ДУБИЛЬНОЙ КИСЛОТЫ

Б. Корпаши и М. Мошоньи

Резюме

1. Пищевой режим, богатый казеином и бедный жиром задержал бластомогенное влияние дубильной кислоты на печень, а наоборот — бедный казеином, богатый жиром пищевой режим усиливал это действие.

2. Усиливающее влияние пищевого режима на образование опухолей в печени проявлялось только в том, что опухоли возникали более часто.

3. Содержание пищевого режима в казеин или же в жир при нашей обстановке опытов не оказало влияния на циррогенное действие дубильной кислоты.

4. Нельзя на основании этого опыта установить параллельность между циррогенным и карциногенным действиями дубильной кислоты.

5. Пол крыс не оказал влияния ни на циррогенное, ни на бластомогенное действия дубильной кислоты.

6. При пищевом режиме, богатом казейном и бедным жиром, крысы жили более длительное время по сравнению с крысами поставленными на пищевой режим бедный казейном и богатый жиром.

7. Пищевой режим, бедный казейном и богатый жиром сам по себе, — без лечения дубильной кислоты в течение всего опыта, длившегося 360 дней не проявлял ни циррогенное, ни бластомогенное действия.