The incidence of spontaneously occurring benign hepatomas in CSH male mice is strikingly lower among animals fed a semi-purified ration containing 9 per cent casein than among those fed a diet containing 18 per cent casein as the principal source of protein (11). From the viewpoint of nutrition, the main differences between the diet containing 9 per cent and that with 18 per cent casein are (a) the total amount of protein and, more particularly, (b) the amount of specific essential amino acids, chiefly methionine and cystine; in a diet with 9 per cent casein these sulfur-containing amino acids are not present in amounts optimal for the growth of mice or the efficient utilization of the ingested ration. The following experiments were performed to determine whether either of these factors was responsible for the observed difference in hepatoma formation.

METHODS

The mice were inbred strain C3H males raised in our laboratories, and those used in a particular experiment were born within a 2-week period. They were distributed at random, approximately 50 in each group. The animals were housed in sets of five in cages with solid bottoms and fed Purina Laboratory Chow until the experimental diets were instituted.

The diets were composed of semi-purified known components and in general were prepared, stored, and fed as previously described (11). To achieve a desired level of dietary protein the proportions of casein and cornstarch were reciprocally varied. Constituents other than protein and cornstarch—fat, salt mixture, and vitamin supplements—were present in the same amounts in the several diets of an experiment. The diets contained 2 per cent of gelatin as a binder, except for one ration in which it was present at the level of 11 per cent (2 per cent as binder plus 9 per cent as supplement). When D,L-methionine and L-cystine were employed to increase the proportion of sulfur-containing amino acids of a low protein diet to that of a higher protein diet, they were added in excess of the calculated amounts (primarily to obviate the possible effects of more rapid absorption of the supplementary amino acids in comparison with those of the protein). The caloric values of the rations were computed from data supplied by the manufacturers; the dietary content of protein and of sulfur-containing amino acids was calculated from the following data: casein—93 per cent protein, 3.4 per cent methionine, and 0.3 per cent cystine; gelatin—88 per cent protein, 1.0 per cent methionine, and 0.06 per cent cystine.

Each week the mice were inspected and weighed. The incidence of hepatomas was determined at autopsy when the mice were 15–14 months of age. The tumors were recognized grossly; a considerable number was examined microscopically, including the few questionable lesions.

In one experiment analyses were performed on the livers and hepatomas of a number of animals. Protein-nitrogen was determined by a micro-Kjeldahl method (7) as the nitrogen insoluble in 5 per cent trichloroacetic acid; glycogen was determined by the method of Good, Kramer, and Somogyi (1).

EXPERIMENTS

Experiment 1.—Five groups of approximately 50 mice, 18 weeks of age, were given the rations shown in Table 1. Groups CA 1, CA 2, and CA 3 were fed diets in which the casein contents were 9, 18, and 45 per cent, respectively. The rations for groups CA 4 and CA 5 were modifications of that given group CA 1. In ration CA 4, 9 per cent gelatin replaced 9 per cent cornstarch; thus, it contained approximately the same amount of protein as the diet for group CA 2, but an amount...
of sulfur-containing amino acids similar to that of the diet for group CA 1. The ration for group CA 5 was the same as that for group CA 1, except that it was supplemented with 0.9 per cent DL-methionine and 0.1 per cent L-cystine; i.e., it had approximately the same amount of protein as the diet fed group CA 1 but an amount of sulfur-containing amino acids greater than that present in ration CA 2. The proportions of protein, methionine, and cystine in the five diets are given in Table 2.

The diets were fed at a restricted level (10.2 Calories daily) to ensure complete consumption of the daily rations and thereby equicaloric intakes among the several groups. The body weights of the mice changed from an initial average value of 31 gm. to the levels shown in Table 2; given are the ranges of individual body weights of the mice and the mean values for the several groups during the last 4 months of the experiment.

When the mice were 14 months old, they were killed and examined for hepatomas. The results (Table 2) may be summarized as follows: The augmentation was not of a statistically significant magnitude, and it is concluded that increasing the proportion of dietary casein from 18 to 45 per cent results in no great effect on the incidence of spontaneously occurring hepatomas.

The data of this study suggest that the proportion of dietary sulfur-containing amino acids may be a critical factor in the rate of formation of spontaneous hepatomas in the mouse. The comparisons can be made from the values in Table 2.

It was noted that the incidence of hepatomas among the 5 groups was directly correlated with the mean body weights; these varied, even though the groups had been fed and had consumed equicaloric rations. However, the differences in body weight were probably not sufficient to account for more than a small part of the differences in tumor formation (12). Nevertheless, in
the following experiment undertaken to confirm the significance of sulfur-containing amino acids, the mean body weights of the groups were kept approximately equal by adjustment of the food intake.

Experiment 2.—Three groups of 50 mice, 24 weeks of age, were fed diets similar in composition to those for groups CA 1, CA 2, and CA 5 of Experiment 1. Group CH 1 was fed ad libitum a ration containing 9 per cent casein, and group CH 2 a ration with 19 per cent casein. Group CH 3 was given a ration with 9 per cent casein and 0.6 per cent DL-methionine and 0.03 per cent L-cystine; diet CH 3, therefore, contained approximately the same amount of protein as diet CH 1, but higher proportions of methionine and cystine than diet CH 2. At the beginning of the study the individual animals ranged from 26 to 44 gm., each group averaging 35 gm. The mice were weighed once a week, and, when necessary, the amounts of rations for groups CH 2 and CH 3 were adjusted to achieve the same mean body weight as that of the ad libitum-fed group CH 1; the range of food intake and the mean for each group are given in Table 3. As it happened, the groups maintained equal average body weights between 34 and 36 gm. during the entire experiment. The investigation proceeded smoothly, and the mice were examined for hepatomas when they were 15–14 months of age.

The results (Table 3) confirm the data obtained in the previous experiments: as compared to the ration containing 9 per cent (CH 1), the augmenting effect on the incidence of hepatomas of the ration with 19 per cent casein (CH 2) was entirely duplicated by the one containing 9 per cent casein supplemented with sulfur-amino acids (CH 3). Furthermore, the incidence was not related to the mean food intake, and the results of both experiments indicate that the effect obtained was not due to differences in caloric intake or body weight.

At the end of the experiment the mice were anesthetized with nembutal so that the livers, in addition to being inspected for hepatomas, could be analyzed for protein and glycogen. Approximately half the mice were sacrificed before fasting, the others after being deprived of food for 24 hours (during which time they lost approximately 15 per cent of their body weight). Whenever a hepatoma more than 6 mm. in diameter was found, both the hepatoma and nearby normal liver were analyzed, the samples for the latter being taken first. The livers of a number of mice without tumors were also studied to provide reference values.

Among the mice with hepatomas, the normal portions of the livers were similar in protein and glycogen content to those of the nontumor-bearing mice. However, the hepatomas invariably contained a higher percentage of glycogen and, except in one case, a lower level of protein than the adjacent normal liver. This is shown by the ratios of the glycogen and protein concentrations in the hepatoma to the concentrations in the liver given in Table 4. In both normal hepatic tissue and hepatoma a 24-hour fast resulted in a decrease in glycogen and an increase in protein, but comparison of the ratios for mice deprived of food with those for mice not deprived of food for 24 hours suggests that during fasting the hepatoma did not lose as great a proportion of glycogen as the normal liver, nor did the protein concentration increase as rapidly.

**DISCUSSION**

The present studies confirm our previous report (11) that a decrease in the proportion of dietary casein from approximately 18 per cent to 9 per cent results in a striking retardation in the rate of formation of spontaneously occurring benign hepatomas in C3H mice. The inhibition is due to a deficiency of sulfur-containing amino acids in the 9 per cent casein diet and not to the difference in the proportion of total protein. This is indicated by the finding that the supplementation of the 9 per cent casein diet with methionine and cystine

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### Table 3

<table>
<thead>
<tr>
<th>Dietary*</th>
<th>Daily Food Intake (Gm.)</th>
<th>Number*</th>
<th>Mice with Hepatomas per Cent</th>
<th>Ration*</th>
<th>Range</th>
<th>Mean</th>
<th>Mice per Cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH 1</td>
<td>9</td>
<td>3.8—4.0</td>
<td>3.9</td>
<td>49</td>
<td>7</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>CH 2</td>
<td>19</td>
<td>3.8—5.8</td>
<td>3.2</td>
<td>50</td>
<td>22</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>CH 3</td>
<td>9§</td>
<td>2.9—3.8</td>
<td>3.4</td>
<td>48</td>
<td>22</td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

*See text for description of diets.

†Amounts of ration were adjusted weekly to maintain equal average body weights, which varied from 34 to 36 gm. during the experiment.

‡Number of mice when sacrificed at 15–14 months of age.

§Supplemented with 0.6 per cent DL-methionine and 0.03 per cent L-cystine.
resulted in a hepatoma incidence equal to that produced by a diet containing 18 per cent casein; while only a minimal effect (of a magnitude compatible with its methionine content) followed supplementation with 9 per cent gelatin. Furthermore, the design and results of the experiments show that the differences in hepatoma formation were not dependent on inequalities in caloric intake and body weight.

The data also suggest that increasing the proportion of dietary casein from 18 per cent to 45 per cent does not greatly affect the incidence of hepatomas. It is, therefore, probable that the rate of formation of these tumors is dependent on an optimal or critical amount of nutritionally adequate protein, and that increases above this amount have little influence upon tumor formation.

With regard to other tumors of the mouse, a reduction of dietary casein from 18 per cent to 9 per cent had virtually no inhibitory action upon the formation of spontaneously occurring mammary carcinomas or induced skin tumors (11). It is possible that decreasing the casein content to a still lower proportion and the employment of moderate carcinogenic activity would result in effects comparable to those seen with the spontaneous hepatoma. The influence of the proportion of dietary protein on the formation of induced liver tumors in the rat is modified by other features of the regimen, such as the levels of riboflavin (5) and pyridoxine (8) and the carcinogen employed (3). While there have been occasional reports that the proportion of protein had little or no effect (4, 9), other experiments indicate that decreases in casein content from 24 per cent or more to 10 or 12 per cent augmented the formation of tumors induced by p-dimethylaminoazobenzene (2, 10). However, it is significant that, in contrast to the inhibitory action on the spontaneous hepatoma of the mouse, such a decrease in dietary protein has never been reported to result in a decreased incidence of induced hepatic tumors in rats.

Other investigators have examined the influence of the level of dietary sulfur-containing amino acids on the formation of various types of tumors of the mouse and rat (2, 4, 6, 14, 15, 16, 17); the findings vary with the kind of tumor, and in some instances interpretation of the results is obscured by the concurrent effects of caloric intake and body weight. Nevertheless, the experiments of White and associates (16, 17) suggest that a diet low in cystine inhibits the formation of methylcholanthrene-induced leukemia in mice; they interpret the effect as not being associated with the properties of cystine as an amino acid essential for growth.

The fact that supplementing a 9 per cent casein diet with methionine and cystine resulted in an incidence of hepatomas equal to that found in mice on an 18 per cent casein diet does not mean that sulfur-containing amino acids act specifically in the carcinogenic process. There remains another and more probable explanation centering about the likelihood that the genesis of hepatomas is influenced by the proportion of nutritionally adequate or "balanced" protein. Because casein is relatively deficient in methionine, the 9 per cent casein ration actually contained less than 9 per cent "balanced" protein. Supplementation with 9 per cent gelatin increased the total amount of protein as measured by amino nitrogen but only slightly affected the amount of nutritionally adequate protein. On the other hand, adding the sulfur-containing amino acids had little effect on the total protein but definitely augmented the level of nutritionally adequate protein. Further experimentation is necessary to demonstrate the exact factors responsible for the observed effects on hepatoma formation.

SUMMARY

C3H male mice ingesting a diet containing 9 per cent casein as the principal source of protein developed a significantly lower incidence of hepatomas than mice on a diet containing 18 per cent casein. This occurred whether the daily rations were isocaloric or were adjusted to maintain equivalent body weights. Changing the proportion of dietary casein from 18 to 45 per cent had no noteworthy effect on the incidence of hepatomas.

The amount of dietary protein per se was not the factor responsible for the striking difference in the rate of formation of hepatomas between mice on 9 per cent and those on 18 per cent casein diets. Adding 9 per cent gelatin to the 9 per cent casein ration had little effect. On the other hand, supplementing the 9 per cent casein ration with methionine and cystine increased the incidence of hepatomas to that of mice on an 18 per cent casein diet. Thus, the reduced incidence of hepatomas in the mice on the 9 per cent casein rations was a consequence of inadequate amounts of dietary sulfur-containing amino acids. The supplements of these amino acids may have acted directly in the carcinogenic process or, more likely, produced their effect by augmenting the nutritional adequacy of the dietary protein.

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Proportion of Dietary Protein and the Formation of Spontaneous Hepatomas in the Mouse

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