The Influence of Dietary Protein on Ascorbic Acid Metabolism in Rats

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1. D-Glucuronolactone reductase, L-gulonolactone oxidase, uronolactonase, dehydroascorbate, L-gulonate dehydrogenase and L-gulonate decarboxylase have been measured in the tissues of rats fed on diets containing variable amounts of protein. Rats fed on a protein-free or a 2% casein diet for 15 days showed a marked decline in the activities of D-glucuronolactone reductase, L-gulonolactone oxidase, uronolactonase and dehydroascorbate in the liver, and no change in L-gulonate dehydrogenase and L-gulonate decarboxylase activities in the kidney when compared with rats fed on diets containing 9%, 18% or 25% casein. Giving diets containing 60% or 88% casein to rats did not appreciably alter the activities of uronolactonase, dehydroascorbate, L-gulonate dehydrogenase and L-gulonate decarboxylase, but inhibited considerably the activities of D-glucuronolactone reductase and L-gulonolactone oxidase in the liver, resulting in decreased synthesis of ascorbic acid. 2. Rats fed on a 25% casein diet showed maximal weight gain, higher tissue reserve of ascorbic acid and higher urinary excretion of both ascorbic acid and glucuronic acid when compared with rats fed on diets containing lower or higher amounts of protein.

The interrelationship between protein metabolism and ascorbic acid metabolism has been widely studied during recent years, and this has been extensively reviewed by Chatterjee (1967). Scurvy is known to bring about alterations in protein metabolism, as is reflected in the imbalance of serum proteins such as albumin and globulins (Chalopin, 1955). Under these conditions there is a lowering of albumin with a simultaneous increase in the globulin content in serum. Kieckebusch (1964) studied the effect of high ascorbic acid supplement on guinea pigs fed on 100% protein, protein-deficient and scorbutic diets. It has been shown that a high vitamin supplement has no regular effect on growth, food intake, protein efficiency or survival rate during the first 20 weeks, and the scorbutic incidents during the remaining period of test are adopted as criteria. It has been reported that in rats the excretion of ascorbic acid in urine increases progressively with the increase of protein in the diet. Ascorbic acid contents in liver, adrenal and blood also rise with increase in protein intake (Srinivasan, Rao & Bhagaban, 1960). Similarly, lower concentrations of the vitamin have been found in liver and other tissues with low protein intakes (Yanovskaya, 1952) and also during protein depletion in guinea pigs (Esh, Bhattacharya & Basu, 1966).

The pathways of biosynthesis of L-ascorbic acid and of L-xylulose and of the degradation of ascorbic

acid in the animal system have now been established in detail (Burns, 1961) and these are schematically represented in Scheme 1. In view of the above findings regarding the possible interrelationship of protein and ascorbic acid metabolism, a series of investigations have been undertaken. The present study was planned to provide data on the response of several liver and kidney enzymes connected with ascorbic acid metabolism in rats to various proportions of protein in the diet.

MATERIALS AND METHODS

Determination of total ascorbic acid in tissues. Rats were killed by decapitation and the tissues (liver, kidney, brain, spleen and adrenal gland) were taken. Each tissue was weighed and was ground in an all-glass mortar and pestle with a small amount of sea sand and cold 10% (w/v) metaphosphoric acid; the metaphosphoric acid extracts were then centrifuged. The supernatant liquids were collected and were used for the estimation of total ascorbic acid by the dinitrophenylhydrazine method of Roe & Kuether (1943).

Preparation of rat liver and kidney homogenates. A portion of the liver removed from the decapitated animal was swabbed with a blotting paper to absorb the blood, immediately frozen, weighed and then homogenized with 4 vol. of cold iso-osmotic (0.25 M) sucrose solution in an all-glass Potter-Elvehjem homogenizer for 2–3 min. at 5°.

Kidney homogenates were prepared in the same way but 0.15 M-KCl (3 vol.) was used as the homogenizing medium.

Scheme 1. Schematic representation of the pathways of biosynthesis of L-ascorbic acid and L-xylose and of the catabolism of L-ascorbic acid in animals. (a) D-Glucuronolactone reductase; (b) L-gulonolactone oxidase; (c) dehydroascorbic acid; (d) L-gulonolactone dehydrogenase and L-gulonate decarboxylase.

Incubation media for studying the biosynthesis of L-ascorbic acid. The test system contained sodium phosphate buffer, pH 7.4 (20mm), D-glucuronolactone (10mm), liver homogenate (0.50ml., equivalent to 100mg. of fresh liver) and KCN (50mm). The total volume was 2.5ml., and the mixture was incubated at 37° for 90 min.

When L-gulonolactone (5mm) was used as substrate, KCN (1mm) was added to the test system to prevent the oxidation of L-ascorbic acid in the medium. Though cyanide at a higher concentration is inhibitory to the synthesis of L-ascorbic acid, a lower concentration of cyanide (1mm) is not (Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1960b). The experiments were performed in the presence of sodium pyrophosphate (50mm) unless otherwise mentioned (Kar, Chatterjee, Ghosh & Guha, 1962).

The synthesized ascorbic acid was determined titrimetrically against 2,6-dichlorophenol-indophenol (Chatterjee, Chatterjee, Ghosh, Ghosh & Guha, 1960a).

Test system to study catabolism of L-ascorbic acid. The test system contained dehydroascorbic acid (10 μmoles, freshly prepared by Br₂ oxidation of ascorbic acid), liver homogenate (equivalent to 100mg. of tissue), GSH (0.3 μmole), tris-maleate buffer, pH 6.8 (200 μmoles), and MgCl₂ (20 μmoles). The final volume was 3.0ml. The system was incubated at 37° for 15 min. The reaction was stopped by the addition of 1ml. of 20% (w/v) metaphosphoric acid containing 2% (w/v) SnCl₂. The remaining dehydroascorbate was rapidly reduced with H₂S and filtered. Excess of H₂S was removed from the filtrate by bubbling a stream of CO₂. A sample (0.1 ml.) was taken for the estimation of 2,3-dioxogulonic acid thus formed, according to the method reported by Kagawa, Hisashi & Shimazono (1961). Another system was also prepared in the same way as described, but the reaction was stopped at 0 min. to serve as a zero-time control.

Incubation system to study synthesis of L-xylulose. The test system contained sodium phosphate buffer, pH 7.8 (40mm), NAD (1mm), nicotinamide (30mm), sodium L-gulonate (10mm) and kidney homogenate (0.25ml.) in a total volume of 2.5ml. The mixture was incubated for 20 min. at 37° in air. The reaction was stopped with 0.5ml. of 30% (w/v) metaphosphoric acid and the mixture filtered.

The synthesized L-xylulose was determined by the cysteine-carbazole method (Dische & Borenfreund, 1951).

Determination of uronolactonase activity. The activity of uronolactonase, which catalyses the hydrolysis of D-glucuronolactone to its free acid, was determined by the method described by Eisenberg & Field (1956).

Determination of protein. Protein was determined by the biuret method (Gornall, Bardawill & David, 1949).

Determination of glucuronic acid. Glucuronic acid in the urine was determined by the carbazole reaction (Dische, 1947).

Animal experiments. Male albino rats (50–65g.), which had been maintained on a stock diet, were divided into several groups and fed on the experimental diets *ad libitum*. The diets contained 0–88% casein, 91.0–3.4% corn starch, 5% groundnut oil and 4% Steenbock salt mixture no. 40 (Steenbock & Nelson, 1923). Variations in protein content were made at the expense of starch. All the rats were given an oral supplement of water-soluble vitamins daily. Six animals were used in each group. The animals were weighed half-weekly. Rats were killed 2 or 4 weeks after being given the experimental diets.

RESULTS

Body-weight gain and organ weights. It can be seen from Table 1 that the weight gain increased as the proportion of casein increased from zero to 25% in the diet. The maximum growth was obtained by feeding on the 25% casein diet. Further increase in the amount of dietary casein did not result in a progressive increase in growth rate. Rather, the 60% casein diet produced a slightly lower weight gain. Rats maintained on the 88% casein diet showed a very much lower weight gain.

The weights of certain organs, such as liver, kidney, spleen and adrenal gland, were found to increase with the increase of dietary casein from zero to 25%. The tissue weights were roughly maintained on the 60% casein diet, but the 88% casein-fed group showed significantly lower organ weights. The results are shown in Table 1.

Tissue concentration of ascorbic acid. The concentrations of ascorbic acid in liver, kidney, brain, spleen and adrenal gland are shown in Table 2.

The data show that with the increasing proportion of casein in the diet from 2 to 25%, ascorbic acid concentrations in the above-mentioned tissues increased progressively. The 60% casein-fed group, however, had a slightly lower tissue ascorbic acid concentration.

Synthesis of L-ascorbic acid from D-glucuronolactone and L-gulonolactone by rat liver. The synthesis of L-ascorbic acid from D-glucuronolactone and L-gulonolactone by the liver homogenates of rats has been studied. The results are shown in Table 3. The activities of liver D-glucuronolactone reductase and L-gulonolactone oxidase increased with increasing proportion of dietary protein until the casein content was 9% of the diet. At this point their activities were maximal. Diets with increased proportions of protein from 9 to 25% casein produced no further increase in the activities of these enzymes. Feeding the rats on diets containing 60% or 88% casein resulted in a decrease in the activities of both the enzymes compared with the maximum activities obtained with rats fed on 9–25% casein diets.

Table 1. *The influence of various proportions of dietary protein on the weight gain and various tissue weights of the rat*

All values are means of six rats.

Casein in diet (%)	Body-weight gain (g.)	Total tissue weights (g.)				
		Liver	Kidney	Brain	Spleen	Adrenal gland
0	—20	1.831	0.492	1.289	0.124	0.015
2	— 6	1.850	0.581	1.274	0.119	0.016
9	40	3.234	0.837	1.271	0.260	0.017
18	48	3.226	0.961	1.368	0.302	0.027
25	61	4.152	1.127	1.411	0.442	0.028
60	53	4.283	1.335	1.324	0.428	0.026
88	15	3.008	0.952	1.289	0.230	0.020

Table 2. *Effect of various proportions of dietary protein on the tissue concentration of ascorbic acid in the rat*

All values are means of six rats. The numbers in parentheses denote the amount of ascorbic acid (mg.) in the whole tissue.

Casein in diet (%)	Concn. of ascorbic acid in tissue (mg./100 g.)				
	Liver	Kidney	Brain	Spleen	Adrenal gland
2	21.6 (0.399)	17.0 (0.098)	30.2 (0.385)	42.9 (0.051)	256.0 (0.040)
9	29.3 (0.950)	17.8 (0.148)	35.2 (0.447)	46.4 (0.120)	310.6 (0.052)
18	33.6 (1.084)	18.7 (0.179)	38.3 (0.524)	48.7 (0.147)	323.9 (0.087)
25	35.4 (1.469)	18.8 (0.212)	38.9 (0.549)	49.6 (0.219)	342.1 (0.096)
60	32.1 (1.374)	15.5 (0.207)	38.2 (0.506)	49.0 (0.209)	339.7 (0.088)

Table 3. *Effect of dietary protein on the synthesis of L-ascorbic acid by rat liver homogenates*

Incubation media and the method of estimation of ascorbic acid are given in the Materials and Methods section. Each result is expressed as the mean $\pm$ S.D. of four experiments, each on a different animal. When the differences between the means were found to be statistically significant, *P* values are given.

Casein in diet (%)	L-Ascorbic acid formed (μ moles/g. of protein)	
	From D-glucuronolactone	From L-gulonolactone
0	8.2 $\pm$ 2.4	8.4 $\pm$ 1.7
2	9.7 $\pm$ 4.3	10.6 $\pm$ 3.2
9	15.9 $\pm$ 4.0*	11.2 $\pm$ 2.1
18	12.7 $\pm$ 3.5	11.3 $\pm$ 2.0
25	13.1 $\pm$ 1.6	11.0 $\pm$ 1.2**
60	10.2 $\pm$ 1.8†	10.1 $\pm$ 1.7
88	9.3 $\pm$ 1.5†	8.3 $\pm$ 1.6

* and ** Mean values significantly different from the casein-free diet group (*P* values $<0.01^*$ and $<0.05^{**}$ respectively).

† Mean values significantly different from the 9% casein-fed group (*P* values <0.05).

Table 4. *Effect of dietary protein on the catabolism of L-ascorbic acid by rat liver homogenates*

Incubation medium and the method of estimation of 2,3-dioxogulonic acid are given in the Materials and Methods section. Each result is expressed as the mean $\pm$ S.D. of six experiments, each on a different animal.

Casein in diet (%)	2,3-Dioxogulonic acid formed (μ mole/mg. of protein)
0	0.16 $\pm$ 0.042
2	0.23 $\pm$ 0.028*
9	0.25 $\pm$ 0.029*
18	0.27 $\pm$ 0.024*
25	0.27 $\pm$ 0.028*
60	0.24 $\pm$ 0.026*
88	0.29 $\pm$ 0.020**

* and ** Mean values significantly different from the casein-free-diet group (*P* values $<0.01^*$ and $<0.001^{**}$ respectively).

Catabolism of L-ascorbic acid by rat liver. Table 4 shows that the activity of liver dehydroascorbate catalysing the conversion of dehydroascorbic acid into 2,3-dioxogulonic acid was lowest in the protein-free group. It increased markedly when the diet was supplemented with only 2% casein ($P < 0.01$). Thereafter there was slight influence from increasing dietary protein above 18% casein.

Synthesis of L-xylulose from L-gulonate by rat kidney. The activities of L-gulonate dehydrogenase

Table 5. *Effect of dietary protein on the synthesis of L-xylulose from sodium L-gulonate by rat kidney homogenates*

Each result is expressed as the mean $\pm$ S.D. of six experiments, each on a different animal.

Casein in diet (%)	L-Xylulose formed (μ mole/mg. of protein)
0	0.54 $\pm$ 0.068
2	0.48 $\pm$ 0.055
9	0.49 $\pm$ 0.020
18	0.51 $\pm$ 0.022
25	0.56 $\pm$ 0.032
60	0.50 $\pm$ 0.023
88	0.63 $\pm$ 0.118*

* Mean value not significantly different from the casein-free-diet group, but significantly different from the 2% casein-fed group ($P < 0.02$).

Table 6. *Effect of dietary protein on rat liver uronolactonase activity*

Each result is expressed as the mean $\pm$ S.D. of four experiments, each on a different animal.

Casein in diet (%)	D-Glucuronolactone hydrolysed (μ mole/mg. of protein)
0	0.19 $\pm$ 0.043
9	0.37 $\pm$ 0.025*
18	0.53 $\pm$ 0.025*
60	0.50 $\pm$ 0.066†

* Mean values significantly different from the casein-free diet group (*P* values <0.001).

† Mean value significantly different from the 9% casein-fed group ($P = 0.01$).

and L-gulonate decarboxylase of rat kidney were not affected appreciably by the proportion of protein in the diet. The results are presented in Table 5.

Conversion of D-glucuronolactone into D-glucuronic acid by rat liver. Table 6 shows that the activity of liver uronolactonase, catalysing the hydrolysis of D-glucuronolactone to its free acid, increased with increasing proportion of protein in the diet until the casein formed 18% of the diet. There was little influence from increasing dietary casein above this proportion.

Urinary excretion of ascorbic acid and glucuronic acid. The data on the urinary excretion of ascorbic acid and glucuronic acid, as observed in a 24 hr. collection of urine, are shown in Table 7. Increasing the casein content of the diet from zero to 25% resulted in a progressive increase in the urinary excretion of ascorbic acid and glucuronic acid.

Table 7. *Effect of dietary protein on the urinary excretion of ascorbic acid and glucuronic acid in rats*

Results are expressed as mean $\pm$ S.D. values and the numbers in parentheses denote the number of rats used in each experiment.

Casein in diet (%)	Ascorbic acid in urine (mg./24 hr.)	Glucuronic acid in urine (mg./24 hr.)
0	0.029 $\pm$ 0.008 (6)	0.644 $\pm$ 0.135 (6)
2	0.093 $\pm$ 0.042 (5)	1.83 $\pm$ 1.12 (5)
9	0.434 $\pm$ 0.198 (5)	1.94 $\pm$ 0.817 (5)
18	0.646 $\pm$ 0.243 (5)	2.11 $\pm$ 1.57 (6)
25	0.752 $\pm$ 0.105 (6)	3.04 $\pm$ 0.992 (6)
60	0.746 $\pm$ 0.156 (4)	1.68 $\pm$ 0.338 (4)
88	0.463 $\pm$ 0.183 (5)	1.56 $\pm$ 0.676 (5)

Further increase in the dietary protein resulted in striking decreases in the excretion of both metabolites compared with the maximum.

DISCUSSION

The data indicate that the weight gain and organ weights of rats increase as the proportion of protein in the diet increases in steps from zero to 25%. The weight gain and organ weights are maximal on the 25% casein diet. Rats fed on 60% and 88% casein display decreased growth rate compared with the maximum.

Tissue storage of the vitamin is highest on the 25% casein diet, decreasing slightly in the higher-protein-fed groups. Data on the urinary excretion of glucuronic acid and ascorbic acid follow a similar pattern. Contrary to the observations of Srinivasan *et al.* (1960), the results show that the urinary excretion of ascorbic acid reaches its maximal value on the 25% casein diet and then there actually occurs a slight decline in its excretion in the 60% and 88% casein-fed rats.

The studies reported here indicate that diet may be a significant factor in controlling the activity of specific liver enzymes involved in ascorbic acid biosynthesis and degradation and may contribute to the changes associated with growth. The liver enzymes studied include D-glucuronolactone reductase, L-gulonolactone oxidase, dehydroascorbate and uronolactonase. From a comparison of the response of specific liver enzymes for the synthesis of ascorbic acid and of the growth rate with the proportion of protein in the diet, it can be seen that possibly there exists a correlation between them. The liver D-glucuronolactone reductase and L-gulonolactone oxidase show maximal response on the 9% casein diet, remain almost unaffected up to 25% casein, and display markedly lowered activities with both lower and higher proportions of protein in the diet. Lowered synthesis of ascorbic acid from

both D-glucuronolactone and L-gulonolactone in high-protein-fed rats may be attributed to greater activities of lactonase in these cases. It is known that the enzymes catalysing the synthesis of ascorbic acid have a high degree of specificity for the lactone structure (Burns, 1961; Kar *et al.* 1962) and the lactonase, which favours the hydrolysis of the lactones to their respective free acids, inhibits the synthesis of ascorbic acid by limiting the substrate concentration.

The liver uronolactonase activity increases progressively with increasing dietary protein until the casein forms 18% of the diet. But unlike D-glucuronolactone reductase and L-gulonolactone oxidase, its activity does not seem to be much inhibited by diets containing a high proportion of protein. Muramatsu & Ashida (1962) made a careful study of the response of several liver enzymes in rats to various proportions of dietary protein, and found that the activities of xanthine oxidase, succinate dehydrogenase and glutamate dehydrogenase decreased in rats fed on a protein-free diet, but did not change greatly, after reaching the maximal value, with increasing proportion of dietary protein. They also postulated that there might be a correlation between the response curves of these enzymes and the growth response in rats, because the maximal growth was obtained in rats receiving the 25% casein diet, on which diet the activities of these enzymes each reached their maximal value. Our results indicate that liver D-glucuronolactone reductase, L-gulonolactone oxidase and uronolactonase may also belong to this class of enzymes.

The kidney enzymes L-gulonate dehydrogenase and L-gulonate decarboxylase, which catalyse the conversion of L-gulonate into L-xylulose, are found not to be appreciably altered by any proportion of protein in the diet. In this they resemble the cathepsin activity of rats (Muramatsu & Ashida, 1962).

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