The Influence of Dietary Protein on Gulonolactone Hydrolase, Gulonate NADP Oxidoreductase, and Tissue Ascorbate in Male and Female Rats (37556)

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Conditions interfering with the translational facility of liver ribosomes to synthesize protein have been associated with decreased ability of rats to synthesize, store and excrete L-ascorbic acid. Hypophysectomized rats whose hepatic ribosomes fail to incorporate amino acids into protein at a normal rate (1, 2), also show a substantial diminution in rate of biosynthesis, body pool size and urinary excretion of L-ascorbic acid (3). Growth hormone administration, which has been shown to restore the amino acid incorporating function of ribosomes from hypophysectomized animals (4), has been found to enhance the biosynthesis, storage and excretion of ascorbate (3). This enhancing effect on ascorbate production appears to depend primarily on an induced increase in the activity of gulonolactone hydrolase (EC3.1.1.18) (5), an enzyme in the biosynthetic pathway between D-glucuronic acid and L-ascorbic acid specifically diminished by hypophysectomy and apparently the rate-limiting enzyme in ascorbate synthesis in hypophysectomized rats (6). The specific induction of gulonolactone hydrolase by growth hormone in hypophysectomized rats has been shown to be inhibited by administration of the metabolic inhibitor of protein synthesis, puromycin (7).

The decreased ribosomal function after hypophysectomy bears resemblance to decreased translational function of liver ribosomes in conditions of starvation and protein deficiency (8–11), conditions like hypophysectomy associated with decreased biosynthesis, tissue accumulation and excretion of L-ascorbic acid (12). Since hepatic enzymes are dependent on ribosomal protein synthesis, as are other proteins of liver, it is possible that the changes in ascorbate metabolism in these conditions are the result of decreased production of an enzyme or enzymes acting within the biosynthetic pathway to L-ascorbic acid. Unfortunately, the effects of starvation or protein deficiency on specific enzymes in the biosynthesis of L-ascorbic acid from D-glucuconic acid have not been clear. Apart from concurrence that the activity of gulonolactone O_2 oxidoreductase (EC1.1.3b) is decreased, agreement about other changes in enzymic activities within this part of the biosynthetic pathway in starved or protein-deficient rats is lacking (13–16). Furthermore, none of the published studies on effects of starvation or protein depletion on ascorbate synthesis includes reported data on the activity of gulonolactone hydrolase.

Materials and Methods. Animals and experimental procedure. The 48 animals used in this study were intact male and female rats of the Charles River CD strain. Initially rats received were 28 days of age and were placed on a normal protein test diet (27% protein) for 2 wk. At the end of this period the animals were divided into three groups. One group of eight males and eight females were placed on a low protein (8%) diet and another group of eight males and eight females placed on a protein-free (0%) diet. A third group of eight males and eight females were left on the normal protein test diet (27%) to serve as controls. After a period of 9 days half of the males and females of each group were sacrificed for comparison of enzymic activities and tissue concentrations of total ascorbic acid. The remaining half of males and females in each group were sacrificed for these comparisons after an additional week on the three diets (at 16 days). Differences were analyzed for significance by

Student's t test.

Diets. All diets were purchased from Nutritional Biochemicals Corp., Cleveland, OH. The normal protein test diet consisted of 27% casein, 59% sucrose, 10% vegetable oil and 4% salt mixture and ascorbic acid-free vitamin diet fortification mixture. The low protein diet contained 8% casein, 78% sucrose, 10% vegetable oil and 4% salt mixture and ascorbic acid-free vitamin diet fortification mixture. The protein-free diet was composed of 71% sucrose, 15% cellulose, 10% vegetable oil and 4% salt mixture and ascorbic acid-free vitamin diet fortification mixture. All animals were given free access to food and water *ad libitum*.

Enzyme assays. At time of sacrifice animals were decapitated, exsanguinated and tissues rapidly removed. Part of the liver was utilized for analysis of tissue ascorbate and part for enzymic assays. The liver sample for enzymic assays was weighed to the nearest milligram and homogenized in cold isotonic KCl solution. The homogenate was centrifuged for 1 hr at an average centrifugal force of 54,000g to remove nuclei, mitochondria, microsomes and other particulate elements. The cytoplasmic supernatant fraction was drawn off and assayed for activities of gulonolactone hydrolase (EC3.1.1.18) and gulonate NADP oxidoreductase (EC1.1.1.19). Gulonolactone hydrolase activity was measured manometrically by a modification of the method of Winkelman and Lehninger (17). Gulonate NADP oxidoreductase was determined spectrophotometrically by a modification of the method of Ul-Hassan and Lehninger (18). The specific modifications and conditions of these assays were essentially as described elsewhere (7).

Tissue ascorbate. Samples of liver, lung, kidney and heart were removed following decapitation, weighed to the nearest milligram and homogenized in cold 10% trichloroacetic acid. Precipitated protein and debris were removed by centrifugation and aliquots of the supernates were analyzed for total ascorbic acid by a modification of the method of Lowry, Bessey and Burch (19).

Results and Discussion. The effects of feeding diets of differing protein content on gulonolactone hydrolase activity over periods of 9 and 16 days are summarized in Table I. The protein deficient diets (8 and 0%) produced marked decreases in this enzyme activity. After 9 days of the 8% protein diet both males and females had gulonolactone hydrolase activities less than half those of controls of the same sex and age ($p < 0.001$). These values did not decrease further after an additional week on the 8% protein diet. Despite the considerable decrease in both sexes, a significant sexual difference in activities remained ($p < 0.02$) with males having a higher activity than females. In contrast to the effects of the 8% protein diet, the 0% protein diet not only caused a much greater fall in gulonolactone hydrolase activities in both sexes, but continued to cause decreased enzymic activity between 9 and 16 days. Furthermore, the 0% protein diet completely obliterated any significant differences in enzymatic activities between males and females.

The enzymic data for gulonate NADP oxidoreductase activity from rats on the different diets are listed in Table II. It can be seen that no significant differences were

TABLE I. Gulonolactone Hydrolase Activity with Diets of Differing Protein Content.

Diet (% protein)	Males		Females	
Days:	9	16	9	16
27 (controls)	149.5 ± 0.6[a,c]	157.2 ± 11.1[c]	118.3 ± 3.9[c]	119.6 ± 5.0[c]
8	66.5 ± 2.5[b,c]	63.3 ± 1.3[b,c]	42.5 ± 2.7[b,c]	49.0 ± 3.4[b,c]
0	37.8 ± 11.0[b]	19.5 ± 3.7[b]	33.8 ± 9.6[b]	21.5 ± 3.1[b]

[a] Each entry is the average of 4 animals as microliters CO_2/15 min ± standard error of the mean. To obtain micromoles gulonolactone hydrolized per gram of liver per hr, multiply entries by 8.2.

[b] Significantly different from controls of same sex on same day of experiment ($p < 0.001$).

[c] Significant sexual difference from corresponding animals of opposite sex ($p < 0.02$).

TABLE II. Gulonate NADP Oxidoreductase Activity with Diets of Differing Protein Content.

Diet (% protein)	Males		Females	
Days:	9	16	9	16
27 (controls)	0.334 ± 0.013[a,b]	0.325 ± 0.010[b]	0.259 ± 0.016[b]	0.247 ± 0.007[b]
8	0.328 ± 0.008[b]	0.314 ± 0.016[b]	0.241 ± 0.010[b]	0.236 ± 0.006[b]
0	0.330 ± 0.011[b]	0.319 ± 0.003[b]	0.268 ± 0.009[b]	0.248 ± 0.007[b]

[a] Each entry is the average of 4 animals as change in absorbance of NADPH (340 ml) over 20 min ± standard error of the mean. To obtain micromoles NADPH oxidized per gram of liver per hour, multiply entries by 145.

[b] Significant sexual difference from corresponding animals of opposite sex ($p < 0.001$).

found within each sex for the different diets, and the sexual differences between males and females were maintained in each group.

The effects of the three diets on the ascorbate concentrations in several tissues are shown in Table III. The data for tissue ascorbate in Table III show the same trends and changes as those for gulonolactone hydrolase activity in Table I. The fall in tissue ascorbate concentrations was greatest for the animals on the 0% protein diet and intermediate for those on the 8% protein diet. The decrease in tissue ascorbate in animals on the 8% diet for 9 days was not further enhanced by an additional week on this diet. Additional parallels between changes in tissue ascorbate and hepatic gulonolactone hydrolase activity were persistence of sexual differences for both kinds of data from animals on the 8% protein diet, and abolition of significant sexual differences in both tissue ascorbate and hepatic gulonolactone hydrolase in animals on the 0% protein diet.

The significant decrease in gulonolactone hydrolase previously demonstrated in hypophysectomized rats along with a similar diminution in ascorbate synthesis *in vivo* and a decline in tissue ascorbate concentrations

TABLE III. Tissue Ascorbate Concentration with Diets of Differing Protein Content.

Tissue and diet (% protein)	Males		Females	
Days:	9	16	9	16
Liver				
27 (controls)	37.1 ± 2.3[a,c]	36.6 ± 0.6[c]	23.2 ± 0.5[c]	25.0 ± 0.9[c]
8	26.3 ± 1.1[b,c]	26.1 ± 1.3[b,c]	19.9 ± 0.9[b,c]	17.7 ± 1.0[b,c]
0	20.4 ± 0.8[b]	13.5 ± 1.1[b]	18.8 ± 1.1[b]	12.9 ± 1.0[b]
Lung				
27 (controls)	38.1 ± 1.1[c]	37.0 ± 1.2[c]	30.8 ± 0.8[c]	30.5 ± 1.0[c]
8	25.8 ± 1.6[b,c]	30.7 ± 1.0[b,c]	20.9 ± 0.8[b,c]	24.1 ± 0.9[b,c]
0	18.9 ± 1.6[b]	14.9 ± 1.2[b]	18.9 ± 1.4[b]	15.0 ± 0.5[b]
Kidney				
27 (controls)	18.0 ± 0.7[c]	17.9 ± 0.8[c]	12.7 ± 0.5[c]	13.3 ± 0.7[c]
8	11.8 ± 0.8[b,c]	11.8 ± 0.6[b,c]	9.3 ± 0.4[b,c]	9.3 ± 0.5[b,c]
0	8.0 ± 0.3[b]	9.2 ± 0.4[b]	6.5 ± 0.6[b]	8.7 ± 0.2[b]
Heart				
27 (controls)	9.0 ± 0.4[c]	8.6 ± 0.3[c]	6.6 ± 0.3[c]	6.9 ± 0.2[c]
8	7.1 ± 0.7	5.9 ± 0.3[b]	5.9 ± 0.7	5.2 ± 0.4[b]
0	6.5 ± 0.6[b]	4.3 ± 0.2[b]	6.7 ± 0.6	4.3 ± 0.4[b]

[a] Each entry is the average of four animals as mg total ascorbate/100 g tissue ± standard error of the mean.

[b] Significantly different from controls of same sex on same day of experiment ($p < 0.02$).

[c] Significant sexual difference from corresponding animals of opposite sex ($p < 0.05$).

have drawn attention to the possible importance of this enzyme in regulating the biosynthesis of L-ascorbic acid, and have led to the suggestion that this enzyme acts at the rate-limiting step in these animals (6). Decreases in tissue ascorbate which parallel decreases in gulonolactone hydrolase activity as a result of low protein or protein-free diets, as shown in this study, are consistent with a rate-determining role of this enzyme in protein-depleted animals as well. The considerable diminutions in enzymic activity following only 9 days of the low protein diet and the profound decreases following 16 days on the protein-free diet are more than sufficient in magnitude to account for the decreased levels of ascorbate in the several tissues analyzed (assuming that tissue accumulation of ascorbate is dependent on hepatic supply). That these changes in gulonolactone hydrolase are not artifacts due to possible nonspecific changes is indicated by the persistence of normal activity of gulonate NADP oxidoreductase which is maintained in all animals despite the feeding of low protein or protein-free diets to some. Furthermore the maintenance of normal gulonate NADP oxidoreductase activities which include the usual differences between sexually maturing male and female rats is suggestive that the effects of protein depletion on sex differences in gulonolactone hydrolase are not mediated by alterations in sex hormone secretion.

In a previous study with fasted animals and a carbohydrate-free diet, Stirpe, Comporti and Caprino (20) concluded that the principal dietary component determining L-ascorbic acid biosynthesis was carbohydrate. However, since the feeding of the carbohydrate-free diet for the same length of time as fasting in another group of animals did not have nearly as much effect as starvation, it seems likely that a different or additional influence may also be important. The results obtained in the present investigation with high carbohydrate, low protein or protein-free diets suggest that protein is the principal dietary constituent affecting biosynthesis of L-ascorbic acid. Other conditions accompanied by diminished translational function of hepatic ribosomes such as hypophysectomy (1, 2), puromycin administration (21) and starvation (8) are also associated with specific decreases in gulonolactone hydrolase activity and/or decreased tissue accumulation of ascorbate. These observations support the concept normal biosynthesis of L-ascorbic acid in rats is dependent upon normal protein anabolism to maintain adequate activities or amounts of enzymes such as gulonolactone hydrolase.

1. Korner, A., *in* "Recent Progress in Hormone Research" (G. Pincus, ed.), Vol. 21, p. 205. Academic Press, New York (1965).

2. Staehelin, M., Biochem. Z. **342**, 459 (1965).

3. Salomon, L. L., and Stubbs, D. W., Ann. N. Y. Acad. Sci. **92**, 128 (1961).

4. Korner, A., Biochim. Biophys. Acta **174**, 351 (1969).

5. Salomon, L. L., and Stubbs, D. W., Biochem. Biophys. Res. Commun. **5**, 349 (1961).

6. Stubbs, D. W., and Salomon, L. L., Fed. Proc. Fed. Amer. Soc. Exp. Biol. **21**, 472 (1962).

7. Stubbs, D. W., and Haufrect, D. B., Arch. Biochem. Biophys. **124**, 365 (1968).

8. Sox, H. C., and Hoagland, M. B., J. Mol. Biol. **20**, 113 (1966).

9. Gaetani, S., Massotti, D., and Spandoni, A., J. Nutr. **99**, 307 (1969).

10. Jefferson, L. S., and Korner, A., Biochem. J. **111**, 703 (1969).

11. Enwonwu, C. O., and Sreebny, L. M., J. Nutr. **101**, 501 (1971).

12. Stubbs, D. W., and Griffin, J. F., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **30**, 522 (1971).

13. Stirpe, F., and Comporti, M., Biochem. J. **95**, 354 (1965).

14. Mukherjee, D., Kar, N. C., Sasmal, N., and Chatterjee, G. C., Biochem. J. **106**, 627 (1968).

15. Sasmal, N., Kar, N. C., Mukherjee, D., and Chatterjee, G. C., Indian J. Biochem. **6**, 96 (1969).

16. Chatterjee, G. C., Sasmal, D., Kar, N. C., Sasmal, N., Majumder, P. K., Roy, R. K., and Banerjee, S. K., Indian J. Biochem. Biophys. **8**, 163 (1971).

17. Winkelman, J., and Lehninger, A. L., J. Biol. Chem. **233**, 794 (1958).

18. Ul Hassan, M., and Lehninger, A. L., J. Biol. Chem. **233**, 123 (1956).

19. Lowry, O. H., Bessey, O. A., and Burch, H. B., Proc. Soc. Exp. Biol. Med. **80**, 361 (1952).

20. Stirpe, F., Comporti, M., and Caprino, G., Biochem. J. **86**, 232 (1963).

21. Nemath, A. M., and De La Haba, G., J. Biol. Chem. **237**, 1190 (1962).

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