Effects of Fasting on Gulonolactone Hydrolase, Gulonate NADP Oxidoreductase, and Hepatic Ascorbate in Male and Female Rats (37555)

D. W. STUBBS AND J. F. GRIFFIN
(Introduced by M. M. Guest)
Department of Physiology, University of Texas Medical Branch, Galveston, Texas 77550

That dietary factors can influence metabolism of L-ascorbic acid in rats was first elucidated by Roberts and Spiegl in 1947 (1). In 1963 Stirpe *et al.* (2) reported diminution of L-ascorbic acid synthesis from D-glucuronolactone *in vitro* by liver extracts from starved rats. This effect of starvation on biosynthesis of L-ascorbic acid in rats has been confirmed by Chandrasekhara, Rao and Srinivasan (3) and by Sasmal *et al* (4). However, apart from concurrence that the activity of gulonolactone O_2 oxidoreductase (EC1.1.3b) is decreased, agreement about other changes in enzymic activities acting between D-glucuronic acid and L-ascorbic acid in the biosynthetic pathway of starved rats is lacking. Sasmal *et al.* (4) have reported a decrease in glucuronolactone hydrolase (EC3.1.1.19) after fasting, in contrast to the finding of Stirpe and Comporti (5) that starvation produces no change in the activity of this enzyme. The results reported by Stirpe and Comporti for starved rats appear comparable to a similar lack of change in this enzyme in hypophysectomized rats despite a diminished biosynthesis of L-ascorbic acid in both cases, and is consistent with the view that glucuronolactone hydrolase is not part of the principal biosynthetic pathway *in vivo* (6). Sasmal *et al.* (4) have also reported a striking decrease in the activity of gulonolactone NA DP oxidoreductase (EC1.1.1.20) following starvation, although they did not use a direct or specific assay for this enzyme (changes in activity were estimated by inference from L-ascorbic acid synthesis from D-glucuronolactone in a multienzyme system). On the other hand, Stirpe and Comporti (5) using a more direct enzymic assay found no change in gulonate NADP oxidoreductase (EC 1.1.1.19)[1] in fasted animals.

None of the published studies on effects of starvation on biosynthesis of L-ascorbic acid from D-glucuronic acid have reported data on the activity of gulonolactone hydrolase (EC3.1.1.18), an important enzyme in this part of the biosynthetic pathway whose activity appears to be the rate-determining step in hypophysectomized rats (6, 8, 9). Because of similarities in diminished polysomal protein synthesis in livers of hypophysectomized rats and of starved rats (10–13), and the possible significance of such changes in the production of specific hepatic enzyme proteins, it was felt to be necessary that further study of the relationship of fasting to L-ascorbic acid metabolism include investigation of the effects of inanition on gulonolactone hydrolase, an enzyme whose activity has been shown to be specifically decreased by hypophysectomy (9). Furthermore, since there is some uncertainty about the influence of fasting on the activity of gulonate NADP oxidoreductase, additional study of the effects of starvation on this enzyme was also deemed of interest.

Materials and Methods. Animals and experimental procedure. The 62 animals used

[1] In rat liver, gulonolactone NADP oxidoreductase (named for the reduction of D-glucuronolactone to L-gulonolactone) and gulonate NADP oxidoreductase (named for the reduction of D-glucuronate to L-gulonate) are apparently synonyms for the same enzymic activity, since partially purified preparations (with a 350- to 500-fold increase in specific enzymic activity) are essentially equally effective with either D-glucuronate or D-glucuronolactone as substrates (7).

in this experiment were male and female rats of the Charles River CD strain. Initially rats received were 28 days of age and were placed on a complete diet (normal protein test diet) for at least 2 wk. The normal protein test diet, which was obtained from Nutritional Biochemicals Corp., contained 27% protein, 59% sucrose, 10% vegetable oil, and 4% salt mixture and ascorbic acid-free vitamin diet fortification mixture. After 2 wk no food was provided for 15 males and 15 females for a period of 9 days of fasting. Sixteen males and 16 females, serving as fed controls, were maintained on the complete diet over the same period of time. All of the animals received tap water *ad libitum*. Enzyme assays and liver ascorbate determinations were performed on animals sacrificed after the first, third, sixth, and ninth days of the experiment.

Enzyme assays. Animals were decapitated, exsanguinated, and livers were rapidly removed. Part of the liver was utilized for analysis of L-ascorbate acid content and part for enzymic assays. The liver sample for enzymic assay was weighed to the nearest milligram and homogenized in cold isotonic KCl. The homogenate was centrifuged for 1 hr at an average centrifugal force of 54,000*g* to remove nuclei, mitochondria, microsomes, and other particulate elements from the cytoplasm. The cytoplasmic supernatant fraction was drawn off and assayed for the activities of gulonolactone hydrolase and gulonate NADP oxidoreductase. Gulonolactone hydrolase activity was measured manometrically by a modification of the method of Winkelman and Lehninger (14). Gulonate NADP oxidoreductase activity was determined spectrophotometrically by a modification of the method of Ul-Hassan and Lehninger (15). The specific modifications of these methods were essentially as described elsewhere (16).

Hepatic ascorbate. The sample of liver removed for ascorbate analysis was weighed to the nearest milligram and homogenized in cold 10% trichloroacetic acid. Precipitated protein and debris were removed by centrifugation and aliquots of the supernatant were analyzed for "total ascorbic acid" by a modification of the method of Lowry, Bessey and Burch (17). Differences in means for enzymic activities and hepatic ascorbate concentrations were tested for significance by Student's t test.

Results and Discussion. The effects of fasting on gulonolactone hydrolase activity in both male and female rats are summarized in Table I. The data not only show a rapid and significant decrease ($p < 0.001$) in enzymic activity from the first day of starvation, but also a diminution to the same low level in both sexes after 9 days of fasting, thereby completely obliterating the previously present sexual differences in gulonolactone hydrolase activity.

The data for gulonate NADP oxidoreductase activity in fed and fasted rats of both sexes are summarized in Table II. No diminution in the activity of this enzyme was found in either sex as a result of starvation. Indeed, the only significant difference found between fed and fasted animals was an increase in gulonate NADP oxidoreductase activity in females after the first day of fasting ($p < 0.05$). Since no subsequent differ-

TABLE I. Gulonolactone Hydrolase Activities in Fed and Fasted Rats of Both Sexes.

Day of expt	Males		Females	
	Fed	Fasted	Fed	Fasted
1	143.5 ± 3.6[a,c]	119.7 ± 3.1[b,c]	110.5 ± 2.5[c]	78.0 ± 2.5[b,c]
3	155.3 ± 5.0[c]	102.8 ± 4.3[b,c]	108.5 ± 2.9[c]	74.7 ± 3.9[b,c]
6	144.7 ± 2.7[c]	92.2 ± 7.7[b,c]	118.2 ± 3.5[c]	70.2 ± 3.8[b,c]
9	152.0 ± 1.8[c]	80.0 ± 4.4[b]	121.5 ± 2.7[c]	81.3 ± 3.2[b]

[a] Each entry is average of 4 animals (except fasted males and females at 9 days are 3 each) as microliters CO_2/15 min of assay ± standard error of the mean.

[b] Significantly different from fed control of same sex ($p < 0.001$).

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

TABLE II. Gulonate NADP Oxidoreductase Activities in Fed and Fasted Rats of Both Sexes.

Day of expt	Males		Females	
	Fed	Fasted	Fed	Fasted
1	0.353 ± 0.013[a,c]	0.337 ± 0.004[c]	0.278 ± 0.008[c]	0.305 ± 0.006[b,c]
3	0.340 ± 0.011[c]	0.360 ± 0.010[c]	0.296 ± 0.011[c]	0.294 ± 0.015[c]
6	0.332 ± 0.010[c]	0.321 ± 0.018[c]	0.301 ± 0.006[c]	0.292 ± 0.019[c]
9	0.335 ± 0.015[c]	0.334 ± 0.005[c]	0.267 ± 0.016[c]	0.300 ± 0.010[c]

[a] Each entry is average of 4 animals (except fasted males and females at 9 days are 3 each) as change in absorbance of NADPH (340 nm) over 20 min period ± standard error of the mean.

[b] Significantly different from fed control of same sex ($p < 0.05$).

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

ences were found to be significant in females for the remainder of the experiment, and none at any time in males, the difference in gulonate NADP oxidoreductase may be the result of chance (expected to occur by chance between 5 and 2.5 out of 100).

The concentrations of total ascorbic acid found in the livers of fed and fasted rats of both sexes are given in Table III. Although the hepatic ascorbate concentrations in fasted males or females are not statistically different from those in fed controls of the same sex on the first day of starvation, they are significantly lower ($p < 0.05$) for the remainder of the experiment. Not only do the decreases in concentration of hepatic ascorbate parallel the decrease in gulonolactone hydrolase activity after the first day, but also sexual differences in hepatic ascorbate concentrations between males and females after 9 days of starvation are obliterated.

Since a decrease in the protein synthesizing function of hepatic polysomes is a defect common to both hypophysectomized and starved animals (10–13), and since the protein of hepatic enzymes is dependent on polysomal translation, as are other proteins of liver, similarities in the pattern of diminished enzymic activities in conditions associated with decreased protein anabolism, such as hypophysectomy or starvation, might be expected. Thus the decreased gulonolactone hydrolase activity found in fasted animals is similar to the specific decrease in this enzymic activity found in hypophysectomized rats of both sexes and in puromycin-treated rats of both sexes (16). A partial explanation of the rapidity and specificity of the diminution in gulonolactone hydrolase activity may lie in the relatively short half-life of only 7 to 8 hr estimated for this enzyme (16).

The close correspondence between changes in biosynthetic enzymic activity and changes in tissue or blood ascorbate concentration in a variety of circumstances including hypo-

TABLE III. Hepatic Total Ascorbic Acid Content in Fed and Fasted Rats of Both Sexes.

Day of expt	Males		Females	
	Fed	Fasted	Fed	Fasted
1	35.1 ± 0.7[a,c]	31.1 ± 1.7[c]	26.5 ± 1.5[c]	24.4 ± 1.1[c]
3	37.2 ± 0.8[c]	28.2 ± 0.7[b,c]	27.4 ± 0.5[c]	23.0 ± 0.8[b,c]
6	35.8 ± 1.4[c]	25.3 ± 0.4[b,c]	26.0 ± 0.9[c]	22.7 ± 0.9[b,c]
9	36.6 ± 1.6[c]	23.1 ± 0.6[b]	25.5 ± 0.7[c]	23.2 ± 0.5[b]

[a] Each entry is average of 4 animals (except fasted males and females at 9 days are 3 each) as mg/100 g of liver ± standard error of the mean.

[b] Significantly different from fed control of same sex ($p < 0.05$).

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

physectomy (18), injection of growth hormone (6), emetine toxicity (19), castration (18), and changes in normal male and female rats with sexual maturity (21, 22) gives considerable indirect evidence that the tissue levels of total ascorbic acid are greatly influenced by the rate of hepatic biosynthesis. It has been suggested that the sexual differences in the supply of ascorbate due to the higher enzymic activities in males are responsible for the differences in tissue ascorbate between males and females (22). The similar close correspondence between changes in gulonolactone hydrolase activity and changes in hepatic ascorbate in this study lends further support to this concept.

Although the feeding of a carbohydrate-free diet did not have nearly as much influence on ascorbate synthesis as did fasting for the same period of time, Stirpe, Comporti and Caprino (2) suggested that effects of starvation are primarily those of carbohydrate deprivation. However, preliminary results from feeding of high carbohydrate, low protein diets have indicated that low protein content produces effects on gulonolactone hydrolase activity and tissue ascorbate content comparable to those of starvation over the same period of time (23). Thus, several factors affecting the activity of gulonolactone hydrolase such as the specific diminution in activity caused by the metabolic inhibitor of protein synthesis, puromycin; the specific induction of increased gulonolactone hydrolase by the protein-anabolic hormone, growth hormone; and the association of decreased gulonolactone hydrolase activity with diminished ability of hepatic polysomes to synthesize protein in starvation, all suggest an important influence of protein metabolism on the biosynthesis of L-ascorbic acid in rats. It would appear that in relation to ascorbate biosynthesis, one of the principal deficiencies in fasting is a protein deficiency having a significant effect on the activity of gulonolactone hydrolase.

Summary. Starvation results in a rapid and significant decrease in gulonolactone hydrolase activity in both males and females while gulonate NADP oxidoreductase activity is not diminished in either sex. The fall in gulonolactone hydrolase activity is followed by a significant fall in hepatic ascorbate content. Differences in both gulonolactone hydrolase and hepatic ascorbate, dependent on sex, are obliterated after 9 days of fasting.

1. Roberts, E., and Spiegl, C. J., J. Biol. Chem. **171**, 9 (1947).

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

3. Chandrasekhara, N., Rao, M. V. L., and Srinivasan, M., Indian J. Biochem. **5**, 22 (1968).

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

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

6. Salomon, L. L., Stubbs, D. W., and Daginawala, H. F., Proc. Soc. Exp. Biol. Med. **115**, 800 (1964).

7. Shimazono, N., and Mano, Y., Ann. N. Y. Acad. Sci. **92**, 91 (1961).

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

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

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

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

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

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

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

15. Ul Hassan, M., and Lehninger, A. L., J. Biol. Chem. **223**, 123 (1956).

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

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

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

19. Chatterjee, A. K., Datta, S. C., and Ghosh, B. B., Brit. J. Pharmacol. **44**, 810 (1972).

20. Stubbs, D. W., McKernan, J. B., and Haufrect, D. B., Proc. Soc. Exp. Biol. Med. **126**, 464 (1967).

21. Stubbs, D. W., and Swann, H. G., Fed. Proc., Fed. Amer. Soc. Exp. Biol. **28**, 371 (1969).

22. Stubbs, D. W., Tex. Rep. Biol. Med., in press.

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

Received March 22, 1973. P.S.E.B.M., 1973, Vol. 144.