The Influence of Sulfaguanidine on the Biogenesis of Ascorbic Acid in the Rat (33023)

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(Introduced by W. H. Sebrell)
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Previous reports from this laboratory (1,2) have amply demonstrated the adverse influence of sulfaguanidine in low protein diets on the liver levels and urinary excretion of ascorbic acid in the rat. In following up these observations, it was found that, as contrasted with sulfaguanidine, the administration of a broad spectrum antibiotic like chlortetracycline (10 mg/rat per day) did not lower the excretion of the vitamin. Also, in rats fed diets containing sulfaguanidine or sulfasuccidine, there was no correlation between the fecal microfloral counts and ascorbic acid excretion. Therefore, further elucidation of the mode of action of sulfaguanidine was sought in terms of its influence on (a) the liver enzyme system concerned with ascorbic acid biosynthesis, (b) Chloretone stimulated excretion of ascorbic acid, and (c) incorporation of glucose-U-^{14}C into glucuronic and ascorbic acids. The results indicate that the drug may suppress an important metabolic route for the biogenesis of the vitamin other than the well-known glucose–glucuronic acid pathway.

Materials and Methods. As before, observations were made on adult, male albino rats (150–200 gm body wt.). The composition of the diets and details of experimental procedure were the same as described previously (2). The basal diets provided the complete complement of all the vitamins.

Total ascorbic acid in urine and tissues was estimated by the dinitrophenylhydrazine procedure of Roe and Kuether (3). Urinary glucuronic acid was estimated according to Dische (4). The protein content of liver homogenates was estimated by the biuret method of Gornall and associates (5), using crystalline bovine serum albumin as the standard.

The multienzyme system which converts glucuronolactone into ascorbic acid was estimated according to Hassan and Lehninger (6). Rats were decapitated, thoroughly bled and livers were removed. The liver was washed with isotonic KCl (0.15 *M*), wiped with filter paper, and weighed. It was then homogenized in 2.5 volumes of 0.15 *M* KCl. The homogenate was centrifuged at 3000*g* for 20 min in a refrigerated centrifuge at 0–4°C (IEC, PR 2 model). The supernatant was used as the source of enzyme. The assay system consisted of: phosphate buffer (sodium), pH 7.4, 0.02 *M*; NAD, 0.0016 *M*; ATP, 0.0016 *M*; nicotinamide, 0.03 *M*; magnesium chloride, 0.004 *M*; D-glucurono-γ-lactone, 0.004 *M*; rat liver supernatant, 0.5 ml (25–50 mg of protein); and water to 2.5 ml. The reaction mixture held in beakers was incubated in a Dubnoff metabolic shaker for 2 hours at 37°C. At the end of this period, reaction was stopped by adding trichloroacetic acid (5% final concentration) and after centrifugation the supernatant was used for estimating ascorbic acid (7). To the zero hour controls, trichloroacetic acid was added before adding the enzyme. The enzyme activity was expressed in terms of micrograms of ascorbic acid formed in 2 hours per gram of liver or per mg of protein.

The liver UDPG dehydrogenase activity was assayed according to Hollman and Touster (8). Rats were decapitated and after thorough bleeding, livers were removed, washed with ice-cold Tris buffer, pH 7.6–7.7, 0.1 *M*. After the liver was wiped and weighed it was homogenized in 7 volumes of Tris buffer. The homogenate was centrifuged for 5 min at 600*g*. Nine ml of the supernatant was mixed with 1 ml of 1.54 *M* KCl. After swirling for 10 minutes, the solution was centrifuged at 18,000*g* for 90 min in a refrigerated centrifuge at 0–4°C (IEC, HR 1 model). The supernatant from this step was the source of enzyme. The assay system consisted of: glycine–NaOH buffer, pH 8.7, 0.1 *M*; UDPG, 0.6 μmole; NAD, 3.0

μmoles; enzyme, 0.1–0.3 ml (2–6 mg of protein); and water to 3.0 ml. Reaction was started by adding the substrate. The blank cuvette did not have UDPG. Rate of activity was measured by determining the increase in absorbance every minute or more frequently at 340 mμ in a Beckman model DU spectrophotometer with silica cells of 1-cm light path. Enzyme assays were done at two levels and the activity was calculated. A unit of activity is defined as the amount of enzyme required to give an increase in absorbancy of 0.001/min under these conditions.

For studying the influence of the drug on Chloretone stimulated excretion of ascorbic acid in the rat, two groups of rats were fed a 10% casein diet and a 10% casein diet containing 4% sulfaguanidine, respectively. After the animals were on the diets for 2 weeks, the urinary excretion of glucuronic and ascorbic acids was determined. Then all the animals were given a single dose of Chloretone in peanut oil (50 mg/rat in 0.1–0.2 ml). Urine samples were collected daily during the subsequent 5 days for the estimation of glucuronic and ascorbic acids.

To determine the incorporation of ^{14}C-glucose into glucuronic and ascorbic acids, each rat was injected with 50 μC (0.25 mg of glucose, 6.6×10^7 cpm) intraperitoneally and immediately transferred to a metabolism cage. Collection of urine and separation of ascorbic acid were done in a manner similar to that described by Jackel *et al.* (9). For separation and radioassay of the urinary glucuronic acid, 10 ml of the HCl eluate from the anion exchanger (Amberlite IR-4B) used in the separation of ascorbic acid was evaporated to dryness at 35–40°C *in vacuo* in a flash evaporator and then dissolved in 0.25 ml of water. A 0.1–0.2-ml sample of this concentrated solution was subjected to paper chromatography on Whatman no. 1 filter paper with butanol:acetic acid:water (4:1:1 v/v) as the solvent, along with an authentic sample of D-glucuronic acid. The region corresponding to glucuronic acid was cut out with the help of guide strips and eluted with water. An aliquot of the eluate was taken for estimating glucuronic acid and another aliquot was dried in a planchet. Radioactivity was measured with a Tracerlab SC-16 windowless gas-flow counter in conjunction with a Tracerlab 1000 Scaler.

Results and Discussion. The observations recorded here further confirm the adverse influence of sulfaguanidine on the liver concentration and urinary excretion of vitamin C in rats fed a 10% casein diet. As pointed out earlier (1,2), the parallel decrease in the liver levels and urinary excretion is a definite indication that the drug affects the biosynthesis of the vitamin. This is substantiated by the comparatively low response evoked by Chloretone in animals receiving the drug (Fig. 1). Chloretone is known to stimulate markedly the biogenesis of the vitamin through enhancing UDPG dehydrogenase activity in the liver(10).

In elucidating the manner in which sulfaguanidine influences the biogenesis of the vitamin, one of the possibilities to be investigated first was its influence on the multienzyme system responsible for converting glucuronolactone to ascorbic acid. The results in Table I show very clearly that the drug had no influence on the concentration of this enzyme system in the liver. Also, the addition of sulfaguanidine as well as sulfasuccidine at 10^{-4} *M* to 10^{-5} *M* concentrations to the assay system did not influence the enzyme activity. Obviously if the sulfa drug were influencing any of the enzymatic steps concerned with the biogenesis of the vitamin, it should be at or before the stage of formation of glucuronic acid (or the lactone) and not beyond it.

That the administration of sulfaguanidine lowers the UDPG dehydrogenase activity in the liver is evident from the data presented in Table II. However, this decrease in the dehydrogenase activity does not seem to affect the biosynthesis of glucuronic acid as judged by the incorporation of glucose-U-^{14}C into glucuronic acid (Table III).

The data in Table III also show that the drug does not lower the incorporation of radioactivity from the labeled glucose into ascorbic acid; in fact, in three out of five rats, the incorporation was nearly double and the excretion of the vitamin being only a third of that in the controls the specific activity was

FIG. 1. Effect of sulfaguanidine on the Chloretone stimulated excretion of glucuronic and ascorbic acids. Six adult, male rats in each group; 50 mg of chloretone per rat per day.

Urinary glucuronic acid (mg/day)			
before Chloretone	○	7.88 ± 0.52	$(0.001 < p < 0.01)$
	●	6.30 ± 0.11	
First day after Chloretone	○	20.29 ± 1.40	$(0.01 < p < 0.05)$
	●	14.31 ± 1.08	
Urinary ascorbic acid (mg/day)			
before Chloretone	○	1.04 ± 0.18	$(p < 0.001)$
	●	0.49 ± 0.03	
Second day after Chloretone	○	16.10 ± 1.30	$(p < 0.001)$
	●	7.65 ± 0.81	

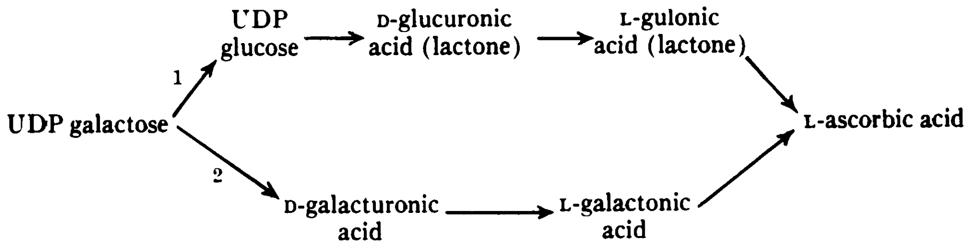

FIG. 2.

TABLE I. Urinary Glucuronic and Ascorbic Acids and Activity of Enzyme System Forming Ascorbic Acid from Glucuronolactone in Rats Fed Diets Containing Sulfaguanidine.[a]

Diet	Urinary excretion (mg/day)		Liver ascorbic acid		Ascorbic acid[b] (μg) synthesized in 2 hours	
	Glucuronic acid	Ascorbic acid	(mg/gm of fresh tissue)	Total (mg)	per gm of liver	per mg of protein
1. 10% Casein	10.21 ± 0.34	1.60 ± 0.17	0.42 ± 0.007	3.57 ± 0.17	482.8 ± 50.8	3.08 ± 0.36
2. 10% Casein + 4% sulfaguanidine	7.68 ± 0.28	0.51 ± 0.02	0.35 ± 0.013	2.66 ± 0.15	604.8 ± 62.7	3.98 ± 0.44
3. 30% Casein	11.51 ± 0.40	2.02 ± 0.24	0.42 ± 0.013	3.78 ± 0.18	537.6 ± 40.8	3.09 ± 0.19
4. 30% Casein + 4% sulfaguanidine	9.88 ± 0.17	0.81 ± 0.14	0.36 ± 0.015	2.94 ± 0.14	630.9 ± 56.8	4.01 ± 0.34
Statistical analysis[c]						
1–2	***	***	***	***	NS	NS
1–3	*	NS	NS	NS	NS	NS
3–4	**	***	**	**	NS	*

[a] Six adult, male rats in each group distributed by simple randomization. Period of feeding: 3 weeks.
[b] Assayed according to Hassan and Lehninger (6), for details see text.
[c] * = Significant at 5% level; ** = significant at 1% level; *** = significant at 0.1% level; NS = not significant.

increased about fivefold. In respect of glucuronic acid, there was no increase in either incorporation of total radioactivity or specific activity because the decrease in urinary glucuronic acid was not of that magnitude as in ascorbic acid, and individual variations annulled, on the average, the effect of even this decrease. The only postulate that would best fit all these findings is the possible existence of a major metabolic route for the biogenesis of the vitamin in the rat which does not involve the well-known glucose–glucuronic acid sequence but is inhibited by sulfaguanidine. When this happens, there is presumably a tendency for greater diversion of glucose and glucuronic acid for the biosynthesis of the vitamin though this does not compensate to any large extent the decrease due to the inhibitory action of the drug.

The question would then arise as to what this alternate pathway is. Some light has been thrown on this question in certain preliminary experiments. In continuation of our studies with lactose (2), galactose has also been found to counteract effectively the depressant action of sulfaguanidine (unpublished results), a finding which suggests that the drug may be inhibiting the formation of the vitamin from galactose. Two alternative routes proposed for the biogenesis of the vitamin from galactose are illustrated in Figure 2. The trend of the results presented here as also some of the data reported by Conney and associates (10) on the incorporation of galactose-1-^{14}C into glucuronic and ascorbic acids in 3-methylcholanthrene-treated rats favor the second alternative. That galactose is a better precursor, for ascorbic acid than glucose has been reported by Evans and collaborators (11) who found a relatively large incorporation of galactose-1-^{14}C into ascorbic acid as compared with glucose-1-^{14}C. Also, D-galacturonate is reported to be a better substrate for L-hexonate dehydrogenase than D-glucuronate (12). Moreover, the same microsomal enzyme preparation which converts D-glucuronolactone into ascorbic acid has also been shown to convert L-galactonolactone to ascorbic acid (13). If it is assumed that conversion of galactose into ascorbic acid makes a larger contribution to the overall

TABLE II. Urinary Excretion of Glucuronic Acid and Liver UDPG Dehydrogenase Activity in Rats Fed Diets Containing Sulfaguanidine.[a]

Diet	Urinary glucuronic acid (mg/day)		UDPG dehydrogenase units[b] per gm liver		UDPG dehydrogenase units[b] per mg protein	
A. 1. 10% Casein	6.56 ± 0.29	[c]	1103 ± 47	[d]	6.80 ± 0.37	[d]
2. 10% Casein + 4% sulfaguanidine	5.29 ± 0.41		769 ± 94		4.50 ± 0.50	
B. 3. 10% Casein	9.97 ± 0.78	[d]	1193 ± 69	[d]	9.03 ± 0.57	[e]
4. 10% Casein + 4% sulfaguanidine	6.49 ± 0.44		853 ± 48		5.95 ± 0.03	

[a] Six adult, male rats in each group distributed by simple randomization. Period of feeding: 3 weeks.
[b] Assayed according to Hollman and Touster (8), for details see text.
[c] Significant at 5% level.
[d] Significant at 1% level.
[e] Significant at 0.1% level.

biogenesis of the vitamin in the rat and that this pathway is inhibited by sulfaguanidine, it would satisfactorily account for all the observed experimental facts. Explorative investigations with ^{14}C labeled galactose seem particularly indicated as there is little information on the relative contributions of glucose and galactose to ascorbic acid biosynthesis in the rat nor are the sequence of reactions in the galactose pathway well delineated. It would appear from the present studies that sulfaguanidine may prove a handy tool in such studies.

Summary. In animals fed diets containing sulfaguanidine, the response to Chloretone stimulation of ascorbic acid excretion was considerably suppressed. The enzymatic synthesis of the vitamin from glucuronolactone by liver homogenates of such animals was not decreased nor was the enzyme activity affected by addition of the sulfa drug to the system *in vitro*. A significant lowering was observed in the liver UDPG dehydrogenase activity in animals receiving sulfaguanidine but the incorporation of glucose-U-^{14}C into either glucuronic or ascorbic acid was unaltered. These results indicate that the sulfa drug lowers the liver level and excretion of the vitamin by

TABLE III. Incorporation of Glucose-U-^{14}C into Urinary Glucuronic and Ascorbic Acids in Rats Fed Diets Containing Sulfaguanidine.[a]

Diet	Urinary excretion (mg/day)		Radioactivity (cpm × 10^{-3}) Total (48 hour)		Radioactivity (cpm × 10^{-3}) (per mg)	
	Glucuronic acid					
1. 10% Casein	7.92 ± 0.42	[b]	57.04 ± 9.46	NS[d]	3.56 ± 0.45	NS
2. 10% Casein + 4% sulfaguanidine	5.92 ± 0.58		54.60 ± 14.57		4.23 ± 0.76	
	Ascorbic acid					
1. 10% Casein	1.38 ± 0.19	[c]	13.20 ± 0.89	NS	5.29 ± 0.91	[b]
2. 10% Casein + 4% sulfaguanidine	0.42 ± 0.13		21.80 ± 4.30		32.01 ± 8.17	

[a] Five adult, male rats in each group distributed by simple randomization. Period of feeding: 4 weeks.
[b] Significant at 5% level.
[c] Significant at 1% level.
[d] NS = not significant.

decreasing its biosynthesis via a pathway that may not directly involve glucose as the precursor.

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Collection of Rosette-Forming Spleen Cells by BPA Density Gradient Centrifugation* (33024)

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Studies on cellular or biochemical aspects of antibody formation are often difficult to interpret because of the low percentage of antibody producing cells among the cells of active lymph nodes or spleens. An attempt to enrich the population of antibody producing cells based on the physical properties of these cells has met with some success (1,2).

It has recently been shown that when spleen cells or lymph node cells (LNC) obtained from animals immunized with sheep red blood cells (SRBC) are mixed with SRBC they form rosettes (3,4) consisting of antibody producing cells and SRBC attached to them. These rosettes could be assumed to have a density intermediate between that of antibody producing cells and that of SRBC. Since bovine plasma albumin (BPA) density gradient centrifugation has been used successfully for separation of cells where differences in density might be involved (2,5,6) this approach was applied to the separation of antibody producing cells from suspensions of cells of active lymph nodes or spleens.

Materials and Methods. $C_{57}Bl/6$ male mice and Balb female mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. The mice were immunized intraperitoneally with 0.2 ml of 50% SRBC washed in PBS. Rabbits were immunized with 0.5 ml of 50% SRBC injected into each hind footpad, with an interval of 5–6 weeks between first and second injections.

Rosette test. Cells from spleens or popliteal lymph nodes of immunized mice or rabbits were washed once with Eagle's medium supplemented with 10% fetal calf serum (Microbiological Associates). The rosette test was performed in a final volume of 1.0 ml of Eagle's medium containing 20–30 million mouse spleen cells and 2% SRBC, or rabbit LNC with 0.5% SRBC. The cell mixture was incubated for 2 hours at 37°C without shaking

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