IN VIVO SYNTHESIS OF ASCORBIC ACID BY ALLOXAN DIABETIC RATS

by

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A THESIS

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INTRODUCTION

Many plants and animals are capable of synthesizing ascorbic acid. The rat has this capacity to synthesize its ascorbic acid requirement, however, the pathway utilized is still obscure.

During the past 15 years it has been noted that a variety of substances caused increased synthesis of ascorbic acid in the rat. Because of the great variety, chemically and pharmacologically, it was thought that these substances stimulated increased synthesis but did not serve as actual precursors for ascorbic acid. Musulin, Tully, Longenecker, and King[1] pioneered in this study with the observation that the presence of volatile lipid constituents in the unsaponified fraction of Purina dog chow caused increased vitamin C excretion in the rat and the ascorbic acid excretion of the rat decreased from 2 mg to 0.2 mg after three to four days starvation. This low level of ascorbic acid excretion could be maintained if the animals were fed only evaporated milk. They also noted that vacuum-distillable fractions from the unsaponifiable matter of halibut liver oil, oat oil, grass leaf oil, and alfalfa leaf oil caused high excretion. The common fatty acids, sterols, proteins, and sugars did not cause a high vitamin C excretion.

Longenecker, et al[2] utilized the observation that minimal vitamin C excretion follows starvation and maintainance

on evaporated milk as the basis for a method of studying the influence of substances on the synthesis of ascorbic acid. These workers fasted the animals for two days, placed them on an evaporated milk diet until the vitamin C excretion was stabilized, and then added the substance to be tested to the milk. It was subsequently found by Longenecker and others that terpine-like cyclic ketones, carvone and dl piperitone, aliphatic compounds, diisobutyl alcohol and dypropylketone, phenol., barbituric acid derivatives, hypnotics, paraldehyde and Chloretone[2,3], and antihistamines[4] increased the level of ascorbic acid excretion. Definite evidence that these compounds do not serve as actual precursors was obtained by Jackel, Mosback, Burns, and King[5]. They injected methyl-labeled Chloretone into a rat and were unable to show any significant activity in the excreted ascorbic acid. The mechanism of action of these stimulating substances is unknown, but may be the result of direct action on the enzyme systems responsible for the synthesis of ascorbic acid.

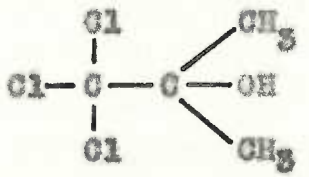
Longenecker's method of accelerated excretion proved to be of value in the search for the actual precursor. Rats were fasted, then placed on an evaporated milk diet as mentioned previously. A stimulating agent was used and then other compounds to be tested were added to the diet.

Because it gives a high level of response, Chloretone[1] has come to be commonly used as a stimulating agent. Twenty mg. of Chloretone daily increases the daily excretion to 20 to 50 mg. In vitro studies were done by using liver slices from Chloretone treated rats which were on the evaporated milk diet.

Early work suggested carbohydrates such as glucose and mannose and sugar derivatives as being possible precursors(6). This was settled by Jackel et al(5), with the injection of uniformly labeled C^{14} glucose into the Chloretone treated rat and the recovery of labeled ascorbic acid. They found approximately 0.5% conversion of the labeled glucose to ascorbic acid. The daily ratio of C^{14} incorporated into ascorbic acid was approximately the same as the ratio of C^{14} labeled glucose to the dietary glucose. That is: $\frac{C^{14}\text{ glucose}}{\text{Dietary glucose}} = \frac{C^{14}\text{ ascorbic acid}}{\text{Total excreted ascorbic acid}}$

This work suggested glucose to be the precursor and the

1. Chlorobutanol, Chloretone (Parke, Davis, & Co.), Trichloro-tert-butyl alcohol



White to colorless crystals; camphor odor and taste; soluble in water, alcohol, Chloroform, ether, acetone, petroleum ether, glacial acetic acid, and oils.

Medical use: anesthetic, antiseptic, and hypnotic.

only major precursor of ascorbic acid synthesis in the rat.

Partial degredation of the excreted ascorbic acid showed that carbon atoms one and two contained about one third of the total activity indicating, in all probability, that the ascorbic acid as well as the administered glucose was uniformly labeled on all carbon atoms. If the glucose had been partially metabolized by anaerobic glycolysis during ascorbic acid synthesis, then there should have been a difference in this activity. Since this was not the case, it was suggested that the entire glucose molecule is utilized for ascorbic acid synthesis.

To gain more conclusive evidence that the whole molecule of glucose is utilized, Horowitz, Doerschuck, and King[7] injected D-glucose-1-C^{14} into the Chloretone stimulated rat. They found approximately 56% of the total activity in carbon six of the excreted ascorbic acid. This strongly suggested that the entire glucose molecule is used for the synthesis and that the glucose one carbon becomes the number six carbon of ascorbic acid.

The purpose of the present study was to ascertain whether or not the synthesis of ascorbic acid by the Chloretone stimulated rat is affected by the diabetic state, in which the primary metabolic deficiency apparently is represented by the absence of or decrease in the hexokinase reaction. In order that glucose may enter the ordinary

metabolic stream for utilization as a source of energy, or the formation of glycogen and fat, it is necessary that it first be converted to glucose-6-phosphate by reaction with adenosinetriphosphate (ATP) under the influence of the enzyme hexokinase. If the diabetic rat forms ascorbic acid at the same levels as the normal animal, then it would appear that phosphorylation of glucose in the hexokinase reaction is unnecessary in the synthesis of ascorbic acid. This would also indicate that the processes of anaerobic glycolysis are not involved in the synthesis of ascorbic acid from glucose and would be in agreement with the tracer findings of King and associates. If the diabetic rat on the other hand has less capacity than the normal animal to synthesize ascorbic acid, then this might indicate involvement of glucose phosphorylation in the process or some other unknown effect.

For our purpose, diabetes was produced in rats by the injection of alloxan which has been shown to destroy the beta cells of the Islets of Langerhans that form insulin.

EXPERIMENTAL WORK

General Plan of Experiments. The rats used in these experiments were twenty-seven adult males and four adult females of the Sprague-Dawley strain, weighing 170 to 250 grams. They were kept in individual metabolism cages.

Four groups of rats were tested. The first group consisted of four rats who were made alloxan diabetic over three months prior to this study. Two had maintained diabetic glucose levels and the other two regressed to within normal limits. They were fasted for two days, then placed on evaporated milk (Darigold). The daily excretion of ascorbic acid was determined several times and then 20 mg. Chloretone added to the diet. Ascorbic acid excretion was determined daily for the first and second days after Chloretone and then every other day through eight days.

The second group, consisting of nine normal animals, was also fasted as the previous group and put on evaporated milk. Ascorbic acid excretion was determined on four separate occasions and then the rats were given alloxan. Each rat received by intramuscular injection, 0.1 ml. of a 10 percent alloxan solution per 100 gm. body weight. The ascorbic acid excretion was followed on the second, fourth, and seventh day after the injection of alloxan. Those animals that didn't show a high fasting blood sugar following

the initial injection of alloxan, were injected again with the same dosage. The ascorbic acid excretion following the second dose of alloxan was determined on the second, fourth, and sixth day. Beginning twenty-one days after the second injection of alloxan, the ascorbic acid excretion was determined on two successive days and then 20 mg. Chloretone was added to the evaporated milk consumed daily by each rat, (35ml.). The excretion was followed on the first, second, fourth, sixth, and eighth day after giving Chloretone. Two of the original nine rats died just prior to the addition of Chloretone, cause of death unknown.

The third group consisting of twelve rats, eight males, and four females, was fasted for two days and put on evaporated milk, as were the other groups. Five had never been given alloxan. The remaining seven, including the four females, were all alloxan diabetic animals. Three determinations of ascorbic acid excretion were made before and four determinations after giving Chloretone as above. The ascorbic acid excretion was determined on the first, second, fourth, and sixth day after giving Chloretone.

The fourth group consisted of six normal male rats which had not been given alloxan. They were fed evaporated milk following a forty-eight hour fast as previously described and then Chloretone was added. Urine ascorbic acid content was determined for two consecutive days preceeding and on the first, second, fourth, and sixth day

after addition of Chloretone to the diet.

Two types of metabolism cages were used. One was made from bottomless jugs with removable floors of galvanized wire screen as shown in figure I. The collecting vessels for this type consisted of 125 ml. Erlenmeyer flasks fitted with three inch funnels covered with a small amount of glass wool to separate feces from urine. The bottomless jugs were kept in a rack of six units which were covered by perforated wooden lids into which water bottle tubes were inserted. Feeding vessels were 100 ml. beakers which were suspended by wire frames close to the floor. The other metabolism cages were made from quarter inch galvanized wire screen and are shown in figure 2. The collecting vessels for the wire cages were eight and ten inch glass funnels with wire gauze in the funnel to separate feces from the urine. The collecting vessels were 250 ml. Erlenmeyer flasks. The urine was preserved by adding 20 ml. of a solution containing 5% metaphosphoric acid and 10% acetic acid to each receiving vessel.

Determination of Ascorbic Acid. Ascorbic acid was determined by the method of Roe and Kuether[8], as modified by Roe and Oesterling[9], Bolomey and Kemmerer[10], and Bolin and Book[11]. Urine was collected as described by Schwartz and Williams[12].

Urine was collected for twenty-four hours for those

FIGURE I

TYPE ONE METABOLISM CAGES

AND THE SUPPORTING RACK

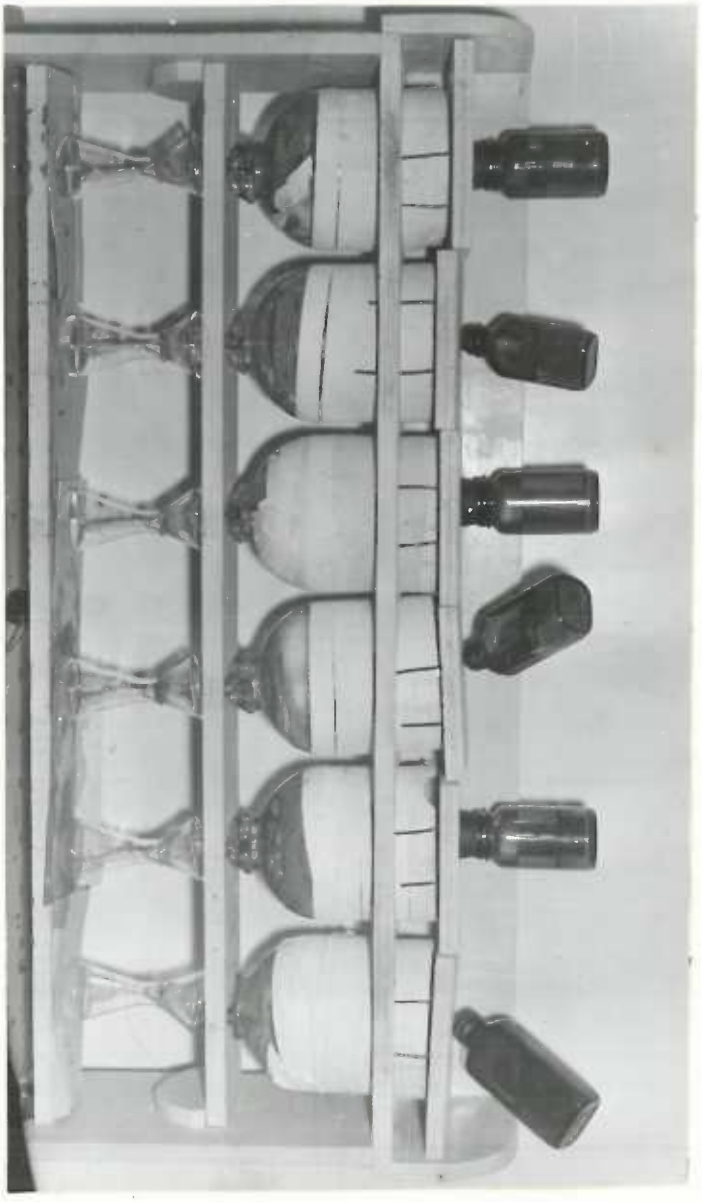
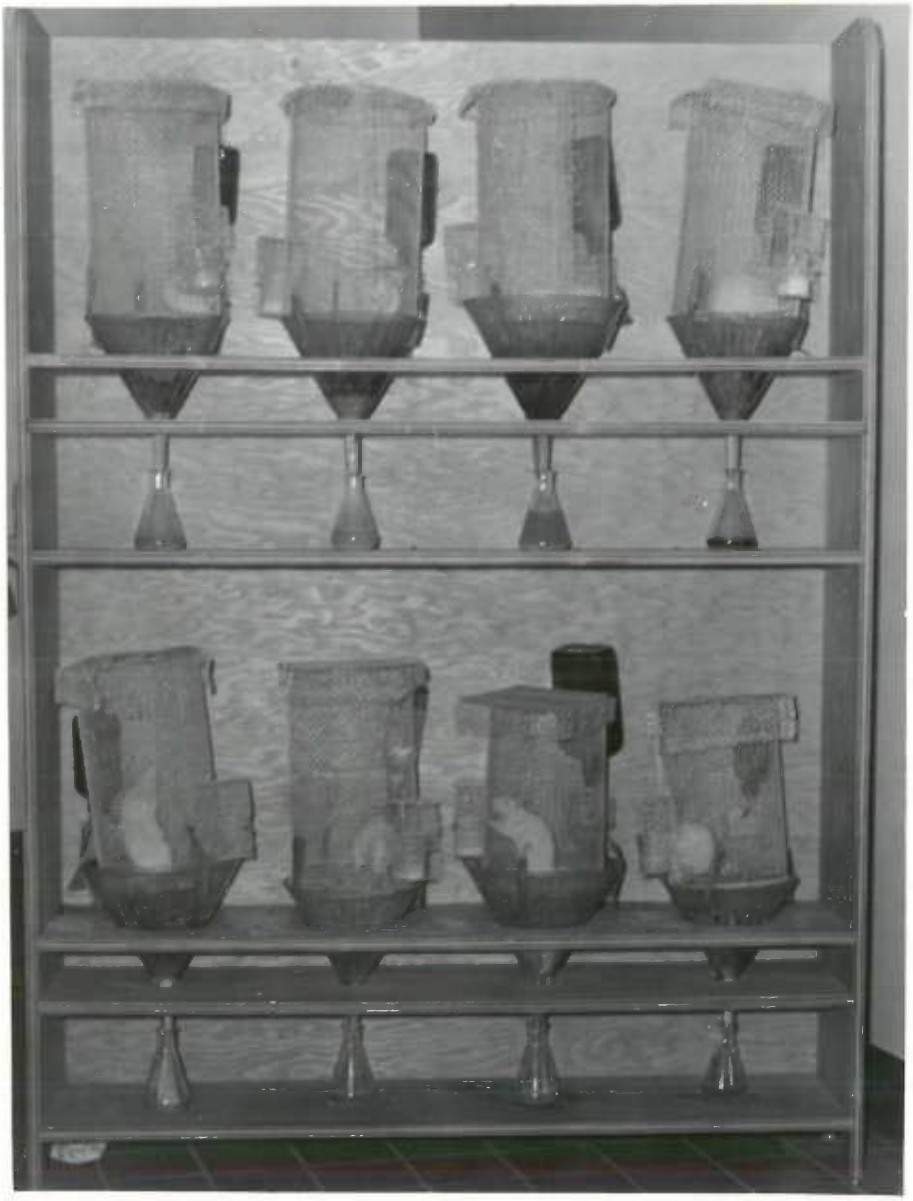


FIGURE II

TYPE TWO METABOLISM CAGES

AND THE SUPPORTING RACK



determinations just prior to and just after the addition of Chloretone to the diet. Other collections were made for forty-eight hours. The urine was preserved by adding 20 ml. of a solution containing 5% metaphosphoric acid and 10% acetic acid to each receiving vessel. Extraneous matter was cleared from the urine by centrifugation. The urine was then diluted with distilled water. The amount of dilution depended upon the concentration of ascorbic acid in the urine. While the rats were on the evaporated milk only, 20 ml. of urine was diluted to 100 ml. with water. Maximum dilution of 2 ml. in 200 ml. of water was used while the rats were excreting maximum quantities of vitamin C. During the period when the rats were building up to maximum excretion, dilutions of 5 ml. in 100 ml., and 2 ml. in 100 ml. were used.

Reagents.

1. 2,4-Dinitrophenylhydrazine reagent. Dissolve 2 gms. of 2,4-dinitrophenylhydrazine in 100 ml. of approximately 9 normal H_2SO_4 and filter.

2. Glacial acetic acid.

3. Metaphosphoric-thiourea solution. Prepare a 20 percent solution of metaphosphoric acid in cold distilled water. Filter if necessary and store in a refrigerator. Place 50 ml. of the 20 percent metaphosphoric acid solution and 2 gms. of thiourea in a 100 ml. volumetric flask and shake to dissolve. Dilute to volume and mix.

4. Sodium 2,6-dichlorobenzeneone-indophenol dye solution. Dissolve 100 mg. of dye in 50 ml. of warm water, filter, and store in the refrigerator. Prepare freshly every two weeks.

Procedure for Determination of Ascorbic Acid in Urine.

Transfer 2 ml. of the diluted urine to each of three test tubes (150 by 18 mm.). Add one drop of the dye solution to one of these tubes and shake. Some color should persist; if not, the ascorbic acid is too concentrated and the urine should be further diluted. If some color persists, add a drop of dye to the other tubes. Then add 2 ml. of meta-phosphoric-thiourea solution to all three tubes. This destroys the excess dye. Reserve one tube of the three for the blank; to the others add 1 ml. of the 2,4-dinitro-phenylhydrazine reagent. Mix these well and place them in a water bath at 37° centigrade for exactly three hours. Constant temperature is necessary. Remove the tubes and place them with the blanks in cool tap water for five minutes. Add 5 ml. of glacial acetic acid to each tube and finally to the blank tube add 1 ml. of the 2,4-dinitro-phenylhydrazine reagent. Mix contents of all tubes well and allow to stand thirty minutes. The solution is then trans-fered to pyrex tubes (125 by 15 mm.) which are calibrated and show equal transmission of light in the color range used. Read in a Klett-Summerson photo-electric colorimeter (model 800-3) using a green filter #54 which has a spectral

range of 500-570 millimicrons. The colorimeter is set at zero with each blank.

Method of Determining Blood Glucose. Blood glucose concentration was determined by Somogyi's method[13]. The rats were fasted for approximately twelve hours prior to taking the blood sample.

Reagents.

1. 0.3 N Barium hydroxide

2. 5.0 percent solution of $ZnSO_4 \cdot 7 H_2O$. The $Ba(OH)_2$ must neutralize the $ZnSO_4$ precisely, volume for volume. This is more important than the accuracy of the concentration. Use phenolphthalein as the indicator and titrate to a definite pink which lasts one minute.

3. Sugar reagent (to make one liter)

Anhydrous disodium phosphate	28 gm.
1 N Sodium hydroxide	100 ml.
Rochelle salt	40 gm.
Cupric sulfate (crystalline)	8 gm.
Anhydrous sodium sulfate	180 gm.
Potassium iodide	8 gm.
1 N Potassium iodate	25 ml.

The phosphate and Rochelle salt are dissolved in about 700 ml. of water, the sodium hydroxide is added, and then with stirring, 80 ml. of 10% solution of cupric sulfate is introduced. Sodium sulfate is added and, when dissolved, the solution is diluted to one liter and allowed to stand

for longer than twenty-four hours. Add 8 gm. of potassium iodide and 25 ml. of 1 N potassium iodate to 1 liter of reagent. The top clear part is decanted and the remainder filtered through a good grade of filter paper. This reagent keeps indefinitely.

4. 2 N H_2SO_4

5. 0.005 N thiosulfate

Procedure. The rat's tail is washed in warm water, dried, and the end of the tail cut with scissors. The blood is collected in an oxalated spot-test dish. 0.1 ml. of whole blood is laked in 3.6 ml. of distilled water. The proteins are precipitated with 0.2 ml. of 0.3 N $Ba(OH)_2$ followed by 0.2 ml. of 5% $ZnSO_4$. The tubes are centrifuged and 2 ml. of the supernatant fluid is placed in large (25 by 200 mm.) tubes. 2 ml. of the sugar reagent is added to the filtrate. The tubes are covered with a large glass marble and heated in boiling water for twelve minutes. Following the heating, they are cooled for five minutes in tap water. 1 ml. of 2 N H_2SO_4 is then added. The tubes are then titrated with thiosulfate to an end point with starch as the indicator. With each series of determinations, a blank with 2 ml. of distilled water and 2 ml. of the sugar reagent is used. A standard sugar concentration is also run with each determination.

RESULTS

The results of the influence of Chloretone on the urinary excretion of ascorbic acid by the rats of group I are shown in Table I. The results will be considered statistically with other groups.

TABLE I

INFLUENCE OF CHLORETONE ON THE EXCRETION OF ASCORBIC ACID BY TWO ALLOXAN TREATED RATS BUT NON-DIABETIC AND TWO ALLOXAN DIABETIC RATS

Values represent mg. per 24 hours

		Rat Number			
		A1	A2	A3	A4
Days on diet*	Days after Chlor#	Fasting blood glucose (mg. %) **			
		139	448	123	83
3		1.20	2.30	1.30	1.70
5		2.40	2.60	1.50	1.50
6		2.60	3.40	1.80	1.90
7		3.00	3.30	2.30	2.50
	Mean:	2.30	2.90	1.85	1.90
	Addition of 20 mg. of Chloretone to the diet				
8	1	7.80	8.30	5.50	3.70
9	2	20.0	14.0	11.0	15.0
10	3	23.0	18.9	16.8	21.9
12	5	26.0	20.0	18.2	24.0

*Darigold® evaporated milk

#Chloretone

**Determined 6 days after discontinuing Chloretone. Still on evaporated milk

TABLE II

EXCRETION OF VITAMIN C, BEFORE AND AFTER ALLOXAN INJECTION, BY RATS ON EVAPORATED MILK

Values represent mg. per 24 hours

		Rat Number								
		B1	B2	B3	B4	B6	B7	B8	B9	B10
Days on Diet	6	0.61	0.48	0.47	0.44	0.49	0.37	0.66	0.90	0.32
	8	0.41	0.46	0.38	0.78	0.55	0.52	0.60	0.80	0.53
	9	0.37	0.68	0.37	1.56	0.74	0.87	0.68	0.73	0.69
	10	0.43	0.51	0.41	1.12	0.69	1.27	0.62	0.87	0.96
	Mean	0.46	0.53	0.41	0.85	0.62	0.76	0.64	0.83	0.63
		Injection of 0.1 ml. per 100 gram body weight of a 10% alloxan solution								
Days after injection	2	0.89	2.13	1.04	2.56	1.31	2.52	1.75	5.36	1.35
	4	0.71	2.20	0.70	2.74	1.99	0.71	0.68	4.37	0.63
	7	1.70	0.52	0.46	4.10	1.74	1.17	0.81	4.95	0.77
		Repeat injection of alloxan to animals no. 1, 2, 3, 6, 7, 8, 10								
	2	1.32	1.42	0.93	4.79	2.00	2.08	1.50	4.20	3.86
	4	2.08	1.40	0.85	5.45	0.82	1.28	2.45	5.20	4.37
	6	2.79	1.79	0.71	5.84	1.69	1.41	2.39	5.87	4.42
Fasting blood Glucose (mg.%)		125	104	151	309	155	107	126	370	250

Table II represents the excretion of ascorbic acid, before and after alloxan injection, by nine normal rats maintained on evaporated milk. Ascorbic acid excretion was increased by all rats two days after the initial injection. Two animals became markedly diabetic and maintained a high level of excretion. Seven did not become diabetic and of these, three maintained a slightly higher than basal level of ascorbic acid excretion. The remaining four were excreting normal quantities by the seventh day. After a second injection of alloxan only one animal of the seven injected maintained an excretion of ascorbic acid in the normal range. Two rats became diabetic following the second injection. The remaining four rats excreted approximately one-half of the ascorbic acid put out by the diabetic animals. The mean ascorbic acid excretion for this group before the alloxan injection was 0.63 mg. per 24 hours.

The influence of Chloretone addition to the diet of the alloxan treated but non-diabetic animals of group II is shown in Table III. Since rats A-3 and A-4 of group I are also of the same type, they are considered statistically with this group. The mean basal excretion before Chloretone while on evaporated milk was 2.03 mg. of ascorbic acid per 24 hours per rat with a standard deviation of 0.56. The mean maximum response to Chloretone was 26.6 mg. per 24 hours. The standard deviation was 5.06.

TABLE XII

THE INFLUENCE OF CHLORETONE ON ASCORBIC ACID EXCRETION BY RATS TREATED WITH ALLOXAN BUT NON-DIABETIC

Values represent mg. per 24 hours

		Rat Numbers			
		B1	B2	B3	B8
Days on Diet*	Diet plus Chlor.#	Fasting blood glucose (mg.%)**			
		125	104	107	126
46		2.28	2.08	0.70	2.49
47		2.66	2.33	1.47	2.85
	Mean:	2.47	2.21	1.09	2.67
	Addition of 20 mg. Chloretone to the diet				
48	1	8.34	6.50	8.15	7.85
49	2	22.5	22.6	32.6	23.1
51	4	26.4	25.3	33.1	25.7
53	6	20.7	25.0	32.3	20.5
55	8	30.0	29.0	29.0	25.3

*Darigold® evaporated milk

#Chloretone

**Determined just before experiment

Table IV represents the influence of Chloretone on the alloxan diabetic rats of group II. They will be considered statistically with the alloxan diabetic rats of group III.

The daily excretion of ascorbic acid by the normal rats of group III while on evaporated milk only is shown

in Table V. These results are considered with those of all the normal rats on evaporated milk only (from Tables II, V, IX). The mean ascorbic acid excretion by rats on evaporated milk only was 0.71 mg. per 24 hours with a standard deviation of ± 0.38.

TABLE IV

THE INFLUENCE OF CHLORETONE ON ASCORBIC ACID EXCRETION BY ALLOXAN DIABETIC RATS

Values represent mg. per 24 hours

		Rat Number		
		B6	B9	B10
Days on Diet*	Diet plus Chlor.#	Fasting blood glucose **		
		155	370	250
46		1.29	5.16	3.35
47		1.62	3.27	4.70
	Mean:	1.46	4.22	4.03
	Addition of 20 mg. Chloretone to the diet			
48	1	8.70	5.67	3.95
49	2	22.9	22.4	16.8
51	4	31.5	30.9	19.3
53	6	33.4	34.7	14.8
55	8	26.0	35.3	16.5

*Darigold® evaporated milk

#Chloretone

**Determined just before this experiment

TABLE V

EXCRETION OF ASCORBIC ACID BY NORMAL RATS ON AN EVAPORATED MILK DIET

Values represent mg. per 24 hours

	Rat Number				
	C1	C2	C8	C9	C10
Days on Diet*	Fasting Blood Glucose (mg.%)**				
	68	78	87	100	81
5	0.32	1.36	0.68	1.75	1.56
7	0.43	1.18	0.48	1.66	1.82
8	0.37	0.80	0.52	1.80	1.48
Mean:	0.37	1.10	0.59	1.74	1.62

Group mean: 1.08 mg. per 24 hours

Standard deviation: ± 0.543

*Darigold ® evaporated

**Determined 15 days after Chloretone was discontinued. Animals still on evaporated milk diet.

The excretion of ascorbic acid by the normal rats of group III in response to Chloretone is shown in Table VI. The results will be considered with group IV.

The ascorbic acid excretion of the alloxan diabetic rats of group III is shown in Table VII. The total mean (from Tables I, IV, and VII) excretion while on evaporated milk only was 3.4 mg. per 24 hours per rat and the standard deviation was ± 1.14.

TABLE VI

EXCRETION OF ASCORBIC ACID BY NORMAL RATS

WITH CHLORETONE ADDED TO DIET

Values represent mg. per 24 hours

	Rat Number				
	C1	C2	C8	C9	C10
	Fasting Blood Glucose (mg.%)**				
	68	78	87	100	81
Days on Diet*	Mean basal excretion#				
	0.37	1.10	0.59	1.74	1.62
1	4.54	7.39	10.2	13.3	10.3
2	17.9	20.0	19.8	31.6	17.4
4	21.1	23.5	26.7	42.5	27.5
6	23.9	24.0	32.8	42.8	32.2

#When fed evaporated milk only (From Table V)

*Darigold® evaporated milk plus 20 mg. Chloretone

Group mean of highest level: 31.1 mg. per 24 hours

Standard deviation: ± 6.98

**Determined 15 days after Chloretone was discontinued. Animals still on evaporated milk diet.

Table VIII shows the excretion of ascorbic acid by alloxan diabetic rats when stimulated by Chloretone. The total mean maximum response to Chloretone (from Tables I, IV, and VII) was 26.5 mg. per 24 hours per rat. The standard deviation was±6.3.

TABLE VII

EXCRETION OF ASCORBIC ACID BY ALLOXAN DIABETIC RATS ON AN EVAPORATED MILK DIET

Values represent mg. per 24 hours

	Rat Number						
	C3	C4	C5	C6	C7	C10	C11
Days on Diet*	Fasting Blood Glucose (mg.%)**						
	174	153	372	473	412	183	275
6	2.96	4.90	4.23	2.29	5.00	2.83	3.56
7	3.32	4.15	4.29	1.63	5.25	2.83	3.13
8	2.43	5.00	4.90	1.39	6.17	3.08	4.12
Mean:	2.92	4.68	4.49	1.77	5.47	2.91	3.62

Group mean: 3.69 mg. per 24 hours

Standard deviation ± 1.21

*Darigold® Evaporated milk

**Determined just before experiment

The influence of Chloretone on the ascorbic acid excretion of the six normal rats of group IV is shown in Table IX. These results are considered with the results from Table VI. The mean maximum level of ascorbic acid excretion by normal rats was 22.2mg. per 24 hours per rat. The standard deviation was ± 10.25

Table X represents the statistical comparison of the normal, alloxan diabetic, and the alloxan treated but non-diabetic rats while on evaporated milk only and evaporated

milk plus 20 mg. Chloretone daily. For the sake of convenience the alloxan treated but non-diabetic group will be designated as the alloxan resistant group.

TABLE VIII

EXCRETION OF ASCORBIC ACID BY ALLOXAN DIABETIC RATS WITH CHLORETONE ADDED TO DIET

Values represent mg. per 24 hours

	Rat Number						
	C3	C4	C5	C6	C7	C10	C11
	Fasting Blood Glucose (mg.%)**						
	174	153	372	473	412	133	275
Days on Diet*	Mean Basal Excretion#						
	2.92	4.63	4.49	1.77	5.47	2.91	3.62
1	9.06	10.4	17.6	10.6	18.9	8.50	11.9
2	21.4	15.8	24.5	20.3	35.2	15.6	17.5
4	19.8	24.0	30.3	20.0	27.5	18.5	24.1
6	23.1	28.4	26.9	24.7	30.7	19.9	21.5

#When fed evaporated milk only (from Table VII)

*Darigold® Evaporated milk plus 20 mg. Chloretone

Group mean of highest level: 27.0 mg. per 24 hours

Standard deviation ± 4.7

**Determined just before start of experiment

TABLE IX

THE INFLUENCE OF CHLORETONE ON ASCORBIC ACID EXCRETION BY NORMAL RATS NOT GIVEN ALLOXAN

Values represent mg. per 24 hours

		Rat Number					
		D1	D2	D3	D4	D5	D6
Days on Diet*	Days after Chlor.#	Fasting Blood Glucose (mg.%)**					
		59	69	74	90	96	104
6		0.46	0.33	0.71	0.69	0.53	0.47
7		0.40	0.38	0.56	0.49	0.51	0.39
	Mean:	0.43	0.36	0.64	0.59	0.52	0.43
		Addition of 20 mg. Chloretone to diet					
8	1		0.38	2.38	1.56	3.60	4.30
9	2	2.30	2.45	6.18	6.78	9.96	8.38
11	4	8.28	7.25	13.0	9.30	14.4	11.7
13	6	10.4	12.7	11.6	14.0	17.6	20.3

Group mean before Chloretone: 0.50 mg. per 24 hours

Standard deviation: ± 0.11

Group mean of highest level after Chloretone: 14.7 mg. per 24 hours

Standard deviation: ± 3.61

*Darigold® Evaporated milk

#Chloretone

**Determined just after Chloretone administration

TABLE X

STATISTICAL COMPARISON OF THE EXCRETION OF ASCORBIC ACID BY NORMAL, ALLOXAN DIABETIC, AND ALLOXAN RESISTANT ANIMALS ON AN EVAPORATED MILK DIET AND EVAPORATED MILK PLUS CHLORETONE

	Mean mg./24 hours	Standard Deviation	Diff. of Means[1]	Std. error of means[2]	t.[3]	P[4]
Evaporated Milk Only						
Alloxan Diabetic	3.4 mg.	± 1.14	2.69	0.285	9.44	≪0.005
vs						
Normal rats	0.71	± 0.38				
Alloxan Resistant	2.03	± 0.56	1.22	0.198	6.18	≪0.005
vs						
Normal rats	0.71	± 0.33				
Alloxan Resistant	2.03	± 0.56	1.37	0.515	2.66	<2.5
vs						
Alloxan diabetic	3.40	± 1.14				
Evaporated Milk plus Chloretone						
Alloxan Diabetic	26.5	± 6.3	4.3	3.68	1.17	>10
vs						
Normal rats	22.2	± 10.25				
Alloxan Resistant	26.5	± 5.06	4.3	4.49	0.96	>10
vs						
Normal rats	22.2	± 10.25				

1 Difference between means of the two groups

2 Standard error of means $= \sqrt{\frac{55_1 + 55_2}{N_1 + N_2 - 2}} \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}$

3 $t. = \frac{\text{Difference of means}}{\text{Std. error of means}}$

4 Level of significance

DISCUSSION

The purpose of following the ascorbic acid excretory response to alloxan injection was to ascertain the effect of the drug itself on the excretion. The rise in excretion followed by a return to normal indicates that the toxic action of the drug, other than diabetogenic action, is not long lasting. The rise in excretion could be explained by an alarm reaction[14]. Govian[15] followed the ascorbic acid response in the adrenal cortex following alloxan injection. He attributed a fall in adrenal cortical ascorbic acid to the alarm reaction.

It is apparent from the t. values shown in Table X that the excretion of ascorbic acid by diabetic and normal rats while on evaporated milk, is different. The diabetic rat excretes far greater quantities of ascorbic acid than the normal while on evaporated milk only. Among the possible explanations for this observation are the following: (1) Alloxan has a toxic action on the kidney causing increased excretion. (2) There is competition in tubular reabsorption mechanisms between glucose and ascorbic acid with preference for reabsorption of glucose, thus causing increased ascorbic acid excretion and in turn, increased basal synthesis. Competition between glucose and ascorbic acid reabsorption has been demonstrated in the dog by Selkurt[16]. (3) Increased blood sugar by mass action

effect stimulates increased synthesis. There is some evidence to show that added glucose feeding doesn't increase synthesis of ascorbic acid. Perhaps the sustained high level of blood sugar in the diabetic, however, does increase the rate of synthesis.

The alloxan treated but non-diabetic animals excreted statistically greater quantities of ascorbic acid than the normal while on evaporated milk only. This alloxan resistant group may also be different than the alloxan diabetic rats in regard to their basal excretion. The confidence coefficient (P. value) is slightly less than 2.5%. This greater excretion by the alloxan resistant rats may indicate that alloxan has a toxic action on the kidney or that the rats are mildly metabolically diabetic which possibly modifies the ascorbic acid excretion. Perhaps both factors may be operating. It is obvious that this work only poses the question as to the mechanisms involved and that more investigation is necessary.

As shown in Table X there is no statistical difference in ascorbic acid excretion between the normal, diabetic, and alloxan treated but non-diabetic animals in response to Chloretone. This supports the thesis that the hexokinase reaction is not necessary for ascorbic acid synthesis, and in turn, that the glucose molecule is not fragmentated in the anaerobic glycolytic pathway before the synthesis

of ascorbic acid. This is in agreement with the findings of Horowitz and associates[7].

SUMMARY

1. The synthesis of ascorbic acid as stimulated by Chloretone is not impaired in the alloxan diabetic rat, indicating that the hexokinase reaction is not necessary for this synthesis.

2. Ascorbic acid excretion by the alloxan diabetic rat and the alloxan treated but non-diabetic rat is greater than for the normal rat while on evaporated milk only. Explanations for this are discussed.

BIBLIOGRAPHY

1. Musulin, R. R., Tully, R. H., 3rd., Longenecker, H. E., and King, C. G. Vitamin C Synthesis and Excretion by the Rat. J. Biol. Chem., vol. 129, pp. 437-444, 1939.

2. Longenecker, H. E., Musulin, R. R., Tully, R. H., 3rd., and King, C. G. An Acceleration of Vitamin C Synthesis and Excretion by Feeding Known Organic Compounds to Rats. J. Biol. Chem., vol. 129, pp. 445-453, 1939.

3. Longenecker, H. E., Fricke, H. H., and King, C. G. The Effect of Organic Compounds upon Vitamin C Synthesis in the Rat. J. Biol. Chem., vol. 135, pp. 497-510, 1940.

4. West, E. S. and Peterson, R. D. The Effects of Antihistaminic Drugs Upon the Excretion of Ascorbic Acid by Rats. J. Allergy, vol. 20, pp. 344-349, 1949.

5. Jackel, S. S., Mosbach, E. H., Burns, J. J., and King, C. G. The Synthesis of L-Ascorbic Acid by the Albino Rat. J. Biol. Chem., vol. 186, pp. 569-579, 1950.

6. Smythe, C. V. and King, C. G. A Study of Ascorbic Acid Synthesis by Animal Tissues in Vitro. J. Biol. Chem., vol. 142, pp. 529-541, 1942.

   Guha, B. C. and Ghosh, B. Biosynthesis of Ascorbic Acid. Nature, vol. 138, p. 844, 1936.

7. Horowitz, H. H., Doerschuk, A. P., and King, C. G. The Origin of L-Ascorbic Acid in the Albino Rat. J. Biol. Chem., vol. 199, pp. 193-198, 1952.

8. Roe, J. H. and Kuether, C. A. The Determination of Ascorbic Acid in Whole Blood and Urine Through the 2,4-Dinitrophenylhydrazine Derivative of Dehydroascorbic Acid. J. Biol. Chem., vol. 147, pp. 399-407, 1943.

9. Roe, H. H. and Oesterling, M. J. The Determination of Dehydroascorbic Acid and Ascorbic Acid in Plant Tissues by the 2,4-Dinitrophenylhydrazine Method. J. Biol. Chem., vol. 152, pp. 511-517, 1944.

10. Bolomey, R. A. and Kemmerer, A. R. The Determination of Ascorbic Acid; A Simplification of the Roe Method. J. Biol. Chem., vol. 165, pp. 377-378, 1946.

11. Bolin, D. W. and Book, L. Oxidation of Ascorbic Acid to Dehydroascorbic Acid. Science, vol. 106, p. 451, 1947.

12. Schwartz, M. A. and Williams, J. M., Jr. Further Studies on Folic Acid-Ascorbic Acid Interrelationships in the Rat. J. Biol. Chem., vol. 197, pp. 481-484, 1952.

13. Somogyi, M. A New Reagent For the Determination of Sugars. J. Biol. Chem., vol. 160, pp. 61-68, 1945.

Somogyi, M. Determination of Blood Sugar, J. Biol. Chem., vol. 160, pp. 69-73, 1945.

14. Selye, H. The Physiology and Pathology of Exposure to Stress, ed. 1, Acta, Inc., Montreal, Canada, 1950.

15. Covian, M. R. L'acide ascorbique surrenal a pres l'action de l'alloxane. Compt. rend. Soc. de biol., vol. 143, p. 1257, 1949.

16. Selkurt, E. E. The Influence of Glucose Renal Tubular Reabsorption and P-Aminohippuric Acid Tubular Excretion on the Simultaneous Clearance of Ascorbic Acid. Am. J. Physiol., vol. 142, pp. 182-190, 1944.