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Vitamin C synthesis in rats fed on diets deficient or normal in iron content

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1. Iron deficiency was induced in albino rats by a milk-powder diet. Control groups were given milk powder plus an iron source.

2. The haemoglobin content of the blood was determined once each month. When the haemoglobin level fell to 10 g/100 ml or less in the deficient animals, determinations were made of the vitamin C content of 24 h urine as well as of liver, kidney, adrenals, brain and blood. Additional studies were made of in vitro synthesis of the vitamin by liver homogenate.

3. The urine and tissues of the deficient animals were found to have a higher concentration of the vitamin than those of the control animals. The difference in enzyme synthesis was in the same direction, but was not statistically significant.

4. The results are interpreted as suggesting an increased requirement, by rats, of the vitamin in iron deficiency; this increased requirement is met by increased synthesis.

The effect of vitamin C on the absorption and utilization of iron, and on erythropoiesis, suggests an interrelationship between iron and vitamin C. The administration of vitamin C has been found to increase the absorption of iron (Moore, Arrowsmith, Welch & Minnich, 1939; Bagchi & Chowdhury, 1954).

We investigated the effects of iron deficiency on vitamin C synthesis in rats by comparing the vitamin C content of liver, kidney, adrenals, brain, urine and blood of rats maintained on diets deficient or normal in iron content. We also studied the enzymic synthesis of vitamin C by liver homogenates.

MATERIALS AND METHODS

Two experiments were carried out, the second being essentially a replication of the first.

Animals

Albino rats were used in both experiments. In Expt 1, three groups of five animals, each 7 weeks old and weighing about 65 g at the beginning, were used. In Expt 2, two groups of ten animals, each 8 weeks old and weighing about 70 g at the beginning, were used.

Diet

Iron deficiency was induced by feeding on diets based on milk powder in both experiments. In Expt 1, the animals were given a mixture of equal parts of whole-milk powder (KLIM) and skim-milk powder prepared from buffalo's milk and obtained from Amul Dairy, Anand, with added vitamins (0·6 ml of the vitamin mixture of Schultze (1950) was added to 10 g of the mixture). In Expt 2, skim-milk powder

prepared from buffalo's milk (Amul Dairy, Anand) was used without the addition of either whole-milk powder or vitamin mixture. The change was due to the non-availability of the milk powders used in Expt 1 at the time of Expt 2. The animals in both experiments were given two or three drops of cod-liver oil twice each week. For groups given a diet normal in iron content, 2·85 mg ferric ammonium citrate were added to 10 g of the diet, to provide 0·5 mg iron. In Expt 1 an additional source of iron, namely the ashed powder of fenugreek seeds (*Trigonella foenum graecum*), was used for one of the groups. Fenugreek seeds were ground into flour on a household grinding stone and the flour thus obtained was charred over an open flame until the smoke subsided and then ashed in a muffle furnace at 600°. The ash was found to contain 330·2 mg iron/100 g, and 152·5 mg of the ash were added to 10 g of the diet so as to give 0·5 mg iron.

Period of treatment

The dietary treatment was continued until the haemoglobin content of blood, determined once each month, decreased to 10 g/100 ml or less. This was achieved in 6 months in Expt 1 and in 3 months in Expt 2.

Cages

In both experiments ordinary aluminium-painted wire cages were used. The cages were painted at least once each week to prevent the rats from direct contact with extraneous iron. The animals were housed two or three in a cage in Expt 1 and individually in Expt 2.

Food and water were given *ad lib.* Straight-sided porcelain bowls were used as food containers. Water was glass-distilled and provided in glass bottles with glass nozzles. Food intake was recorded daily and body-weight once each week.

Collection of urine

Urinary excretion of vitamin C was determined when the haemoglobin content of most of the experimental animals was less than 10 g/100 ml. Urine samples were collected twice each day, for 2 consecutive days, in glass bottles containing 6% (w/v) trichloroacetic acid.

In Expt 1, as plastic cages were not then available ordinary round aluminium-painted wire cages and galvanized iron funnels were used. Care was taken, however, to ensure that the urine would drip down immediately after excretion into the glass funnel fitted over the glass bottle so that contamination by contact with metal would be as little as possible. In Expt 2, the animals were kept in plastic cages for urine collection. The cages were kept slightly tilted (15°) over a stand so that the urine would drip down through a hole into a plastic funnel and then into a bottle. The cages were washed with 6% trichloroacetic acid and the washings were added to the sample which was made up to 100 ml and centrifuged. The clear supernatant fluid was used for the determination of ascorbic acid.

The animals were killed at the end of the experimental period, and determinations were made of the vitamin C content of liver, adrenals, kidney, brain and blood.

Additional results were obtained on the haemoglobin content and red blood cell count of blood and the iron and protein contents of plasma and liver.

Collection of tissue and blood

The animal was anaesthetized with ether, the abdomen was cut open and 0·15 ml of heparin solution containing 1·5 mg heparin was injected into the inferior vena cava. After 1–2 min, a blood sample was removed by heart puncture. The animal was also bled through the inferior vena cava, and the blood collected in a measuring cylinder kept in ice. When all the blood was drained, the liver was quickly excised, wiped between filter papers and weighed, and 1 g of the tissue was kept in a test tube containing 10 ml 6% trichloroacetic acid. The adrenals, kidney and brain were quickly removed, weighed and immediately placed in tubes containing 10 ml 6% trichloroacetic acid. All the tissues were homogenized with 6% trichloroacetic acid with a porcelain pestle and mortar in the cold room. The homogenates were centrifuged in the cold room, and the supernatant fluid was diluted to 50 ml with 6% trichloroacetic acid.

One ml of heparinized blood was added to 3 ml of ice-cold 6% trichloroacetic acid and used for vitamin C determination by the dinitrophenylhydrazine method of Roe & Kuether (1943).

Determination of haemoglobin, red blood cells and white blood cells

The haemoglobin content of the blood was determined by the acid haematin method in a Sohli-Adem's haemoglobinometer (Wintrobe, 1958). Red blood cells were counted as 10^6/mm^3 and white blood cells as 10^3/mm^3 in a counting chamber by the procedure given by Wintrobe (1958).

Protein

Nitrogen contents of the liver and plasma were determined by the micro-Kjeldahl method. Protein content was taken as $N \times 6{\cdot}25$.

Iron

A portion of the liver and plasma was ashed separately and the iron content of the ash was determined by the thiocyanate method (Hawk, Oser & Summerson, 1954).

In vitro synthesis of vitamin C

The in vitro synthesis of vitamin C from D-glucuronolactone by liver homogenate was studied, the method being essentially that of Chatterjee, Ghosh, Ghosh, Roy & Guha (1957). The assay system contained: phosphate buffer (pH 7·0), 350 μM; D-glucuronolactone, 125 μM; potassium cyanide, 225 μM; tissue homogenate, 1 ml containing 250 mg liver. The total volume was 5 ml. The incubation was carried out at 37° for 2 h and the reaction was stopped with 1 ml 20% trichloroacetic acid. The precipitated protein was removed by centrifugation and the supernatant liquid assayed for vitamin C content by titration with 2,6-dichlorophenol indophenol dye.

Table 1. *Composition of liver and blood in rats fed on a diet deficient or normal in iron content*

(Mean values with their standard errors)

	Expt 1*			Expt 2*	
	Group 1	Group 2	Group 3	Group 4	Group 5
Iron content of diet	Deficient	Normal	Normal	Deficient	Normal
Source of iron	None	Fenugreek ash†	Ferric ammonium citrate†	None	Ferric ammonium citrate†
No. of rats	5	5	5	7	10
Body-weight (g)	140 ± 6·3	177 ± 18·2	191 ± 16·6	139 ± 8·9	173 ± 7·1
Liver protein (g/100 g)‡	17·6 ± 0·38	18·3 ± 0·14	19·6 ± 0·47	15·9 ± 0·77	18·9 ± 0·67
Liver iron (mg/100 g)	3·7 ± 0·16	4·4 ± 0·20	4·9 ± 0·30	3·4 ± 0·17	4·5 ± 0·22
Plasma protein (g/100 ml)‡	5·4 ± 0·28	7·4 ± 0·32	7·7 ± 0·37	4·9 ± 0·26	7·3 ± 0·35
Plasma iron (mg/100 ml)	0·47 ± 0·02	0·54 ± 0·02	0·63 ± 0·04	0·47 ± 0·03	0·63 ± 0·02
Haemoglobin (g/100 ml)	10·3 ± 0·66	16·1 ± 1·66	17·1 ± 0·66	9·8 ± 0·21	16·1 ± 0·22
Red blood cells (10^{-6}/mm^3)	5·9 ± 0·28	7·4 ± 0·32	11·2 ± 1·06	4·6 ± 0·15	10·3 ± 0·18
Blood volume (ml)	5·9 ± 0·21	6·8 ± 0·21	7·6 ± 0·62	4·5 ± 0·37	5·7 ± 0·32

* Period of treatment, 6 months in Expt 1 and 3 months in Expt 2.
† Added in an amount providing 0·5 mg iron per rat per day.
‡ N × 6·25.

RESULTS AND DISCUSSION

The results of the two experiments are summarized in Tables 1–4. As was expected, the haemoglobin and RBC content of blood, and the protein and iron contents of plasma and liver were decreased in animals fed on the basal diet without iron supplementation. On the other hand, quite unexpectedly, the vitamin C content of the tissues was found to be greater in these animals than in those fed on a normal diet. This was also true of urine. The increase in both tissue concentration and urinary excretion would suggest a greater synthesis of vitamin C under conditions of iron deficiency in the rat.

Table 2. *Weight of different organs in rats fed on a diet deficient or normal in iron content*

(Mean values with their standard errors)

	Expt. 1			Expt 2	
	Group 1	Group 2	Group 3	Group 4	Group 5
Iron content of diet	Deficient	Normal	Normal	Deficient	Normal
Source of iron	None	Fenugreek ash*	Ferric ammonium citrate*	None	Ferric ammonium citrate*
No. of rats	5	5	5	7	10
Liver wt (g)	4·3 ± 0·3	5·3 ± 0·5	5·6 ± 0·2	4·08 ± 0·2	4·87 ± 0·2
Kidneys wt (g)	1·49 ± 0·12	1·59 ± 0·14	1·46 ± 0·12	1·10 ± 0·037	1·39 ± 0·063
Adrenals wt (g)	0·036 ± 0·005	0·034 ± 0·002	0·033 ± 0·003	0·036 ± 0·004	0·035 ± 0·002
Brain wt (g)	1·58 ± 0·08	1·58 ± 0·09	1·55 ± 0·07	1·46 ± 0·035	1·49 ± 0·036

* Added in an amount providing 0·5 mg iron per rat per day.

The in vitro synthesis of vitamin C by liver homogenate was 1·68 ± 0·207 μmoles/h g liver with the deficient diet and 1·20 ± 0·174 μmoles/h g liver with the normal diet. Although the difference in synthesis between the two groups was not statistically significant, it was in the direction expected. The lack of significance might have been due to the small number of animals used.

The blood levels of the vitamin were not found to differ significantly between the different groups. This confirms the general impression that blood level is not always a reliable criterion of vitamin C nutrition.

In general, the values in Expt 1 were higher than those in Expt 2. We believe this to have been due to the differences in the diet, period of treatment and environmental temperature at the time of death. It will be recalled that the animals in Expt 1 were fed on a mixture of skim-milk and whole-milk powders so that the fat content of the food was higher. Further, the whole-milk powder was reinforced with vitamin D and the animals also received a vitamin supplement. The period of treatment was 6 months in Expt 1 and 3 months in Expt 2. The animals were killed in February in Expt 1, a much colder month than November in which they were killed in Expt 2.

In the rat an increase in tissue concentration or in urinary excretion of vitamin C or in both has been reported in exposure to cold (Monier, Byer & Weiss, 1953) and in treatment with drugs (Burns, Conney, Dayton, Evans, Martin & Taller, 1960). Rats

Table 3. *Vitamin C content of organs, blood and urine of rats fed on a diet deficient or normal in iron content*

(Mean values with their standard errors)

	Expt 1			Expt 2		Significance of difference between means—value of t		
	Group 1	Group 2	Group 3	Group 4	Group 5	1~2	1~3	4~5
Iron content of diet	Deficient	Normal	Normal	Deficient	Normal	—	—	—
Source of iron	None	Fenugreek ash†	Ferric ammonium citrate†	None	Ferric ammonium citrate†	—	—	—
No. of rats	5	5	5	7	10	—	—	—
Vitamin C content (mg) of:								
Liver	—	—	—	1·25 ± 0·08	1·18 ± 0·04	—	—	0·83
Kidneys	0·42 ± 0·08	0·35 ± 0·01	0·33 ± 0·07	0·27 ± 0·022	0·22 ± 0·17	0·79	0·83	1·79
Adrenals	0·24 ± 0·21	0·17 ± 0·004	0·11 ± 0·01	0·14 ± 0·015	0·10 ± 0·004	3·25*	5·63**	2·58*
Brain	0·81 ± 0·01	0·61 ± 0·02	0·59 ± 0·01	0·57 ± 0·011	0·48 ± 0·025	8·73**	15·21**	3·83**
Blood (per 100 ml)	2·33 ± 0·12	3·56 ± 0·13	2·56 ± 0·30	1·02 ± 0·07	0·89 ± 0·08	6·95**	0·91	0·17
Urine (per 24 h)	1·73 ± 0·17	1·34 ± 0·11	1·41 ± 0·15	1·21 ± 0·044	1·00 ± 0·37	3·10*	1·99	3·68**

* Significant at 5 % level. ** Significant at 1 % level.
† Added in an amount providing 0·5 mg iron per rat per day.

Table 4. *Vitamin C content of tissues per* 100 *g body-weight of rats fed on a diet deficient or normal in iron content*

(Mean values with their standard errors)

	Expt 1			Expt 2		Significance of difference between means—value of t		
	Group 1	Group 2	Group 3	Group 4	Group 5	1~2	1~3	4~5
Iron content of diet	Deficient	Normal	Normal	Deficient	Normal	—	—	—
Source of iron	None	Fenugreek ash†	Ferric ammonium citrate†	None	Ferric ammonium citrate†	—	—	—
No. of rats	5	5	5	7	10	—	—	—
Vitamin C content (mg/100 g body-weight) of:								
Liver	—	—	—	1·01 ± 0·07	0·69 ± 0·03	—	—	4·10**
Kidneys	0·29 ± 0·03	0·21 ± 0·02	0·16 ± 0·01	0·22 ± 0·017	0·13 ± 0·008	2·22	4·06**	4·74**
Adrenals	0·17 ± 0·01	0·05 ± 0·001	0·08 ± 0·005	0·13 ± 0·016	0·06 ± 0·003	9·23**	6·43**	3·54**
Brain	0·58 ± 0·02	0·36 ± 0·04	0·31 ± 0·02	0·46 ± 0·022	0·28 ± 0·019	4·89**	9·64**	6·14**

** Significant at 1 % level.
† Added in an amount providing 0·5 mg iron per rat per day.

fed on a high-fat diet show an increased concentration of the vitamin in the interscapular adipose tissue (Page & Babineau, 1950). Exposure to cold and treatment with drugs are believed to increase vitamin C requirement in species susceptible to scurvy such as guinea-pigs, monkeys and man, and the administration of large doses of the vitamin is believed to help acclimatization to cold by monkeys (Dugal & Frontier, 1952). It would appear then that certain metabolic and physiological stress conditions increase the requirement for vitamin C which is met by increased synthesis in species with the capacity for such synthesis. In species susceptible to scurvy, subjected to similar stress, we should expect the situation to be met by a decrease in urinary excretion, which appears to be so in guinea-pigs exposed to cold (Dugal, 1961). It is interesting to note that vitamin C has been found to have a remarkable capacity to prevent or retard symptoms resulting from a deficiency of other vitamins (Terroine, 1960).

Other stress conditions such as a deficiency of B vitamins (Terroine, 1960), vitamin A (Sure, Theis & Harrelson, 1939) and protein (Yanovskaya, 1952; Stirpe, Comporti & Caprino, 1963; Chandrasekhara, Rao & Srinivasan, 1963), amino acid imbalance (Chandrasekhara, Rao & Srinivasan, 1965), and x-irradiation (Stirpe *et al.* 1963; Minuto & Luzzatto, 1957) are found to depress vitamin C synthesis in rats. It might be that, in these circumstances, the nutrients needed for the synthesis of the vitamin are not available in adequate amounts. Some of these conditions are also found to depress food intake, which would result in decreased availability of nutrients. With vitamin A deficiency and with x-irradiation, no differences in synthesis were found when food intake was matched between the experimental and control groups (Stirpe *et al.* 1963; Chatterjee & McKee, 1965).

It seems plausible that iron deficiency constitutes a stress condition in rats and that vitamin C plays a compensatory role in this condition.

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