- Freudenberg, K., Carrara, G. & Cohn, E. (1925). *Liebigs Ann.* **446**, 87.
- Freudenberg, K. & Weinges, K. (1958a). *Fortschr. Chem. org. Naturst.* **16**, 1.
- Freudenberg, K. & Weinges, K. (1958b). *Liebigs Ann.* **613**, 61.
- Freudenberg, K. & Weinges, K. (1959). *Chem. & Ind.* p. 486.
- Freudenberg, K. & Weinges, K. (1962). In *The Chemistry of Flavonoid Compounds*, p. 197. Ed. by Geissman, T. A. Oxford: Pergamon Press Ltd.
- Ganguly, A. K., Seshadri, T. R. & Subramanian, P. (1958). *Tetrahedron*, **3**, 228.
- Gierer, J. (1954). *Acta chem. scand.* **8**, 1319.
- Goldstein, J. L., Swain, T. & Tjhi, K. H. (1962). *Arch. Biochem. Biophys.* **98**, 176.
- Harborne, J. B. (1958a). *J. Chromat.* **1**, 473.
- Harborne, J. B. (1958b). *Biochem. J.* **70**, 22.
- Hathway, D. E. (1958). *Biochem. J.* **70**, 34.
- Hathway, D. E. (1962). In *Wood Extractives*, p. 191. Ed. by Hillis, W. E. New York: Academic Press Inc.
- Hayashi, K. (1962). In *The Chemistry of Flavonoid Compounds*, p. 248. Ed. by Geissman, T. A. Oxford: Pergamon Press Ltd.
- Hergert, H. L. (1962). In *The Chemistry of Flavonoid Compounds*, p. 553. Ed. by Geissman, T. A. Oxford: Pergamon Press Ltd.
- Hillis, W. E. (1960). *Nature, Lond.*, **186**, 635.
- Hillis, W. E. & Carle, A. (1958). *Holzforschung*, **12**, 136.
- Hillis, W. E. & Carle, A. (1960). *Biochem. J.* **74**, 607.
- Hillis, W. E. & Carle, A. (1962). *Biochem. J.* **82**, 435.
- Hillis, W. E. & Carle, A. (1963). *Aust. J. Chem.* **16**, 147.
- Hillis, W. E. & Hasegawa, M. (1963). *Phytochemistry*, **2**, 195.
- Hillis, W. E. & Urbach, G. (1959). *J. appl. Chem.* **9**, 665.
- Jurd, L. (1963). *J. org. Chem.* **28**, 987.
- King, H. G. C. & White, T. (1957). *J. chem. Soc.* p. 3901.
- Ley, K. & Mueller, E. (1956). *Chem. Ber.* **89**, 1402.
- Moriconi, E. J., Rakoczy, B. & O'Connor, W. F. (1962). *J. org. Chem.* **27**, 2772.
- Ordin, L. & Skoe, B. P. (1963). *Plant Physiol.* **38**, 416.
- Roberts, E. A. H. (1957). *Chem. & Ind.* p. 1354.
- Roux, D. G. (1959). *J. Amer. Leath. Chem. Ass.* **54**, 614.
- Roux, D. G. (1963). *Biochem. J.* **87**, 435.
- Roux, D. G. & de Bruyn, G. C. (1963). *Biochem. J.* **87**, 439.
- Roux, D. G. & Evelyn, S. R. (1958). *Biochem. J.* **69**, 530.
- Roux, D. G. & Paulus, E. (1961). *Biochem. J.* **80**, 476.
- Roux, D. G. & Paulus, E. (1962). *Biochem. J.* **82**, 320.
- Swain, T. (1952). *Biochem. J.* **53**, 200.
- Swain, T. (1954). *Chem. & Ind.* p. 1144.
- Swain, T. (1962). In *Wood Extractives*, p. 277. Ed. by Hillis, W. E. New York: Academic Press Inc.
- Swain, T. & Hillis, W. E. (1959). *J. Sci. Fd Agric.* **10**, 63.
- Teuber, H. J. & Staiger, G. (1955). *Chem. Ber.* **88**, 802.
- Webb, J. L., Saunders, P. R. & Thienes, C. H. (1949). *Arch. Biochem. Biophys.* **22**, 458.

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Studies on Metabolism of Vitamin A

7. LOWERED BIOSYNTHESIS OF ASCORBIC ACID IN VITAMIN A-DEFICIENT RATS*

By P. MALATHI AND J. GANGULY

Department of Biochemistry, Indian Institute of Science, Bangalore 12, India

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Existing information on the effect of vitamin A deficiency on the biosynthesis of ascorbate in animals is contradictory. Thus some workers have produced evidence that vitamin A deficiency decreases the tissue and urinary concentrations (Sure, Theis & Harrelson, 1939; Sangiorgi, 1942; Sutton, Kaeser & Hansard, 1942; Jonsson, Obel & Sjöberg, 1942, 1945) and synthesis *in vitro* (Chatterjee, Kar, Ghosh & Guha, 1961) of ascorbate in rats, and others have claimed that the administration of ascorbate leads to a general improvement in the health and longevity of the vitamin A-deficient animals (Mayer & Krehl, 1948). In contrast, several other investigators claimed that no such relationship exists (Sharman, 1949; Guerrant, 1948; Mapson & Walker, 1948). The most signifi-

cant of these reports seems to be that of Mapson & Walker (1948), who concluded that the decreased tissue and urinary concentrations of ascorbate in vitamin A-deficient rats are caused by inanition and not by the deficiency.

We have previously shown that the tissue concentrations of free phenylalanine and tyrosine are markedly increased in vitamin A-deficient rats (Malathi, Seshadri Sastry & Ganguly, 1961). Ascorbate has been implicated in the metabolism of these two amino acids (La Du & Zannoni, 1961; Knox & Goswami, 1961). It therefore appeared that the increase in these two amino acids might be due to blocks in their metabolic pathway caused by inadequate availability of ascorbate during vitamin A deficiency. In a preliminary communication we have shown that the synthesis of ascorbate is considerably lowered in vitamin A-deficient rats

* Part 6: Subba Rao & Ganguly (1964).

(Seshadri Sastry, Malathi, Subba Rao & Ganguly, 1962), and the present paper describes these results in greater detail.

EXPERIMENTAL

Materials. All chemicals were commercial products. Vitamin A acid was a generous gift from Dr O. Isler of Hoffmann-La Roche, Basle, Switzerland.

Treatment of animals. Male rats of this Institute strain were raised on the vitamin A-deficient diet, as described by Malathi, Subba Rao, Seshadri Sastry & Ganguly (1963). Each deficient rat had a corresponding pair-fed control that received once a week an additional supplement of 250 µg. of vitamin A acetate in 0.1 ml. of groundnut oil. Some of these rats received supplements of ascorbate, as described in individual experiments.

Preparation and assay of enzymes. Rats were killed by heart puncture under light ether anaesthesia and the livers were immediately removed into beakers previously placed in crushed ice. The L-γ-gulonolactone oxidase was prepared and assayed as described by Feinberg & Caputto (1960). The transaminases and *p*-hydroxyphenylpyruvate oxidase were obtained by homogenizing the liver in 66 mM-phosphate buffer, pH 7.0, followed by centrifugation at 100 000g for 30 min. in a Spinco model L ultracentrifuge. The supernatant, which contained about 26–29 mg. of protein/ml., was used as the source of the enzymes, which were assayed by the enol-borate-tautomerase method of Linn, Pitt, Civen & Knox (1958). The keto-enol tautomerase was prepared from pig liver according to the method of Knox (1955).

Analytical methods. The concentrations of free amino acids in blood, liver and kidneys were measured as described by Malathi *et al.* (1961). Urinary ascorbate was determined by the method of Roe & Kuether (1943), and the ascorbate formed in the experiments *in vitro* was measured as described by Mayer, Hassalbach & Boissonnas (1952). Protein was estimated by the biuret reaction (Gornall, Bardawill & David, 1949).

RESULTS

Effect of the administration of chloretone on the urinary excretion of ascorbate in vitamin A-deficient rats. Chloretone markedly stimulates the synthesis, and the consequent urinary excretion, of ascorbate in rats (Longenecker, Fricke & King, 1940). The amount of ascorbate excreted in the urine in response to chloretone can therefore serve as an index for the ascorbate-synthesizing capacity of the animal, and this was used to test the effect of vitamin A deficiency on ascorbate synthesis in rats. For such treatment, three different sets of rats at three stages of deficiency, namely pre-plateau, plateau and deficient, were used. It has been our experience that, with our vitamin A-deficient diet, weanling rats usually stop growing within 4 weeks of receiving it (see Fig. 2). We have chosen to call the fourth week on the depletion diet the pre-plateau stage; the plateau stage represented the

period during which the animals did not show any significant changes in weight; definite loss of weight indicated the deficient stage. For collection of urine, the vitamin A-deficient rats at the various stages of depletion as described above, and the corresponding pair-fed controls, were transferred to separate metabolic cages. The bottoms of these cages and the funnels were previously coated with melted paraffin wax. At first, from each rat 24 hr. urine samples were collected separately into 10 ml. of 10% metaphosphoric acid. After this initial collection, the rats were daily dosed with 5 mg. of chloretone, with the collection of urine samples being continued. Fig. 1 summarizes the patterns of urinary excretion of ascorbate at all the three stages of depletion, as stimulated by chloretone. At the pre-plateau stage there was virtually no difference in the excretion by the two groups. The excretion was slightly less at the plateau stage, and there was a striking decrease at the deficient stage. These results are further assembled in Table 1, where the average food consumption is also given. It is clear from Fig. 1 and Table 1 that the decreased food intake at the plateau stage did not exert any significant influence on the ascorbate excretion, because, when the difference in the food consumption between the pre-plateau and plateau stages was 3.6 g., the peak and average excretions of ascorbate were comparable. That restricted food consumption did not exert any marked influence on ascorbate synthesis is further brought out at the deficient stage. At this stage, where the average food consumption was decreased to 1–2 g. per rat per day, the peak and average ascorbate excretions by the pair-fed normals were not decreased so markedly as in the vitamin A-deficient animals (Fig. 1).

Effect of vitamin A deficiency on the synthesis of ascorbate *in vitro* by the liver. The above experiments showed that under the influence of chloretone vitamin A-deficient rats excrete markedly less ascorbate in their urine, as compared with corresponding pair-fed controls. This could be due either to the lack of the necessary substrates or to the loss of enzyme activities. Table 2 shows that the synthesis of ascorbate from L-γ-gulonolactone *in vitro* by liver microsomes is decreased by about 50% even at the plateau stage, and at the later stages of depletion it was further diminished to about 10% of that of the pair-fed controls. In some rats, where the deficiency had become acute, we have often failed to detect this enzyme activity. The decreased synthesis of ascorbate *in vitro* in vitamin A-deficient rats is in agreement with the results of Chatterjee *et al.* (1961).

A loss of L-γ-gulonolactone-oxidase activity has been noted in the livers of vitamin E-deficient rats also (Chatterjee, Kar, Ghosh & Guha, 1960;

Fig. 1. Chloretone-stimulated urinary excretion of ascorbate by vitamin A-deficient (○) and pair-fed control (●) rats. The conditions of the experiments are described in Table 1. (a) Pre-plateau stage; (b) weight-plateau stage; (c) vitamin A-deficient stage.

Table 1. *Urinary excretion of ascorbate by vitamin A-deficient rats*

Each rat was given 5 mg. of chloretone in 0.1 ml. of groundnut oil every day, and the urine was collected into 10 ml. of 10% metaphosphoric acid every 24 hr. for 7 days. The results are expressed as means $\pm$ S.E.M.

State of deficiency	No. of rats	Average food consumption (g./day)	Average ascorbate excreted (mg./day)
Pre-plateau	6	10.6	7.3 ± 1.3
Pair-fed control	6		7.5 ± 1.8
Weight plateau	6	7.0	6.5 ± 0.8
Pair-fed control	6		6.9 ± 0.2
Vitamin A-deficient	6	1.9	0.89 ± 0.31
Pair-fed control	6		5.8 ± 2.1

Table 2. *Synthesis of ascorbate from L- γ -gulonolactone in vitro by the liver microsomes of vitamin A-deficient and pair-fed normal rats*

The reaction system used was as described in the Experimental section. Vitamin A alcohol, α -tocopherol and menadione were added in 0.1 ml. of an aqueous solution of deoxycholate (25 mg./100 ml.); vitamin A acid was also added thus after being converted into the sodium salt. GSH, EDTA, MnCl_2 and CoCl_2 were added in 0.1 ml. of aqueous solution. The results are given as μ moles of ascorbate synthesized/hr./g. wet wt. of liver, and are expressed as means $\pm$ S.E.M.

Additions	No. of rats	State of deficiency			
		Weight plateau		Severe deficiency	
		Vitamin A-deficient	Pair-fed control	Vitamin A-deficient	Pair-fed control
None	10	1.16 ± 0.045	2.23 ± 0.111	0.28 ± 0.086	2.15 ± 0.163
GSH (5 μ moles)	10	1.28 ± 0.05	2.32 ± 0.092	0.41 ± 0.03	2.48 ± 0.09
α -Tocopherol (5 μ moles)	10	1.40 ± 0.08	2.62 ± 0.13	0.50 ± 0.02	2.60 ± 0.012
Menadione (5 μ moles)	10	1.31 ± 0.12	2.58 ± 0.12	0.49 ± 0.08	2.60 ± 0.23
EDTA (1 μ mole)	10	1.32 ± 0.24	2.48 ± 0.2	0.50 ± 0.06	2.52 ± 0.41
CoCl_2 (0.5M)	10	1.38 ± 0.2	2.56 ± 0.32	0.52 ± 0.04	2.60 ± 0.32
MnCl_2 (0.5M)	10	1.36 ± 0.3	2.61 ± 0.4	0.50 ± 0.06	2.55 ± 0.40
Vitamin A alcohol (10 μ g.)	10	1.20 ± 0.02	2.21 ± 0.26	0.43 ± 0.04	2.48 ± 0.13
Vitamin A acid (10 μ g.)	10	1.20 ± 0.01	2.26 ± 0.31	0.38 ± 0.10	2.38 ± 0.21

Carpenter, Kitabchi, McCay & Caputto, 1959). This effect of vitamin E deficiency is reversible, because the enzyme activity can be restored *in vitro* by the addition of α -tocopherol, vitamin K₁, GSH, EDTA, Mn²⁺ ions, or Co²⁺ ions. The nature of the inhibition due to vitamin E deficiency, however, is not clear, because the agents mentioned above, as well as several others, markedly stimulate the activity of L- γ -gulonolactone oxidase even in normal rat liver (Chatterjee *et al.* 1960). In contrast, in vitamin A deficiency none of these agents, including vitamin A alcohol or acid, could restore the lost activity, when added *in vitro*. Vitamin A acid or acetate, when given to the vitamin A-deficient rats, however, restored the lost activity completely.

Effect of supplementing the vitamin A-deficient rats with ascorbate. (a) Growth and longevity. Fig. 2 shows that supplementation with ascorbate had a pronounced effect on the growth curves of the vitamin A-deficient rats. Thus, though both groups of vitamin A-deficient rats reached the weight-plateau stage at about the same time, those receiving ascorbate did not lose much weight and lived at least 2-3 weeks longer, as compared with the ascorbate-deprived ones. The vitamin A-deficient rats receiving ascorbate usually showed the characteristic xerophthalmia symptoms, but they were in a healthier state, were not susceptible to infections and did not show the haemorrhagic manifestations generally noticed at the moribund states of vitamin A deficiency. Treatment with chloretone at the pre-plateau and plateau stages of deficiency also increased the longevity of the vitamin A-deficient rats by about 10 days.

(b) Tissue concentrations of phenylalanine, tyrosine and threonine. Table 3 shows that supplementation with vitamin A acetate or acid immediately corrected the increased phenylalanine and tyrosine and decreased threonine concentrations in the plasma of vitamin A-deficient rats. Ascorbate, on the other hand, was able to correct only the increased concentrations of phenylalanine and tyrosine, although at a lower rate. A similar effect

Fig. 2. Effect of the administration of ascorbate on the longevity and body weight of vitamin A-deficient rats. Ascorbate was injected intraperitoneally, as described in Table 3. The arrows indicate where ascorbate supplementations were begun. ○, Vitamin A-deficient; △ and ▲, ascorbate-supplemented vitamin A-deficient; ●, pair-fed control.

Table 3. *Changes in the serum concentrations of phenylalanine, tyrosine and threonine in vitamin A-deficient rats after the oral administration of vitamin A acetate or acid, or intraperitoneal injections of ascorbate*

Where indicated, every alternate day, the rats were supplemented with 50 μ g. of vitamin A acetate in 0.1 ml. of groundnut oil, or 50 μ g. of vitamin A acid as the sodium salt in 0.1 ml. of phosphate buffer, pH 10.5; for ascorbate, each rat received daily intraperitoneally 500 μ g. neutralized with NaHCO₃ in a total volume of 0.1 ml. (The rats were put under light ether anaesthesia and about 0.5 ml. of blood was drawn from each rat by direct heart puncture. The blood samples were first withdrawn at the plateau stage of deficiency, after which the supplementation was started and the blood samples were withdrawn every alternate day.) The results are expressed as μ g./ml. of serum.

Treatment	Status of rats	Days of treatment						Days of treatment						Days of treatment					
		Concn. of threonine						Concn. of phenylalanine						Concn. of tyrosine					
		1*	3	5	7	9	11	1*	3	5	7	9	11	1*	3	5	7	9	11
None	Vitamin A-deficient	30.5	32	29	26	28	30	88	86	91	84	88	93	81	84	93	90	91	89
	Pair-fed control	46.0	48	50	45	43	48	60	59	63	58	61	63	58	55	52	56	53	58
Vitamin A acetate	Vitamin A-deficient	32	53	48	50	50	48	80	62	63	65	60	58	78	60	58	54	55	59
	Pair-fed control	52	49	50	50	48	46	60	62	65	68	60	58	53	58	50	52	58	56
Vitamin A acid	Vitamin A-deficient	30	48	44	50	52	53	89	68	60	63	58	62	89	68	50	56	54	59
	Pair-fed control	48	50	54	50	49	46	58	64	59	58	62	60	53	53	56	52	58	60
Ascorbate	Vitamin A-deficient	31	28	26	28	28	26	86	70	64	63	66	69	86	76	68	66	65	66
	Pair-fed control	49	53	50	48	49	48	58	66	62	64	68	62	60	62	64	56	58	60

* Values obtained before the supplementation.

Table 4. *Average phenylalanine, tyrosine and threonine contents of the liver and kidneys of the vitamin A-deficient rats receiving supplements of vitamin A acetate or acid or ascorbate*

These values were obtained from the animals described in Table 3 after they had been killed at the end of the experiments. The results are expressed as $\mu\text{g./g.}$ wet wt. of tissue.

Treatment	Status of rats	No. of rats	Threonine content		Phenylalanine content		Tyrosine content	
			Liver	Kidneys	Liver	Kidneys	Liver	Kidneys
None	Vitamin A-deficient	3	1538	2523	1419	1934	1033	1004
	Pair-fed control	3	2108	3729	901	1420	813	731
Vitamin A acetate	Vitamin A-deficient	4	2112	3743	942	1492	784	740
	Pair-fed control	4	2068	3672	926	1513	784	768
Vitamin A acid	Vitamin A-deficient	4	2150	3684	880	1487	794	767
	Pair-fed control	4	2166	3444	888	1529	770	821
Ascorbate	Vitamin A-deficient	7	1569	2369	886	1478	775	798
	Pair-fed control	7	2034	3569	886	1459	747	779

Table 5. *Effect of vitamin A deficiency on phenylalanine-pyruvate-transaminase, tyrosine- α -oxoglutarate-transaminase and p-hydroxyphenylpyruvate-oxidase activities in rats*

The enzymes were assayed as described in the Experimental section. Supplements of vitamin A acetate or acid, or ascorbate, were given as described in Table 3. Ascorbate was neutralized with NaOH to pH 6.5 and added in a volume of 0.1 ml. The results are expressed as means $\pm$ S.E.M.

Treatment	Status of rats	No. of rats	Phenylalanine-pyruvate-transaminase activity ($\mu\text{moles of phenylpyruvate formed/hr./ml. of enzyme}$)	Tyrosine- α -oxoglutarate-transaminase activity ($\mu\text{moles of p-hydroxyphenylpyruvate formed/hr./ml. of enzyme}$)	p-Hydroxyphenylpyruvate-oxidase activity ($\mu\text{moles of p-hydroxyphenylpyruvate disappeared/hr./ml. of enzyme}$)
None	Weight plateau	4	2.2 ± 0.16	2.4 ± 0.13	3.1 ± 0.42
	Pair-fed control	4	2.6 ± 0.23	2.9 ± 0.18	7.2 ± 0.53
None	Vitamin A-deficient	6	2.0 ± 0.26	1.9 ± 0.24	0.88 ± 0.26
	Pair-fed control	6	2.1 ± 0.34	2.0 ± 0.38	6.6 ± 0.31
Ascorbate intraperitoneally	Vitamin A-deficient	6	2.3 ± 0.42	2.6 ± 0.29	5.8 ± 0.38
	Pair-fed control	6	2.8 ± 0.09	2.4 ± 0.30	7.1 ± 0.26
Ascorbate <i>in vitro</i> (5 μmoles)	Vitamin A-deficient	4	2.0 ± 0.18	2.2 ± 0.33	4.7 ± 0.24
	Pair-fed control	4	2.6 ± 0.23	2.9 ± 0.18	7.2 ± 0.53
Vitamin A acid fed	Vitamin A-deficient	4	2.3 ± 0.42	2.4 ± 0.18	6.8 ± 0.48
Vitamin A acetate fed	Vitamin A-deficient	4	2.4 ± 0.41	2.2 ± 0.22	7.0 ± 0.12

of these agents on the liver and kidney concentrations of these amino acids, at the end of the experiments, is shown in Table 4.

Effect of vitamin A deficiency on the transamination reactions of phenylalanine and tyrosine, and on the oxidation of p-hydroxyphenylpyruvate, by rat-liver enzymes. Table 5 shows that, though the transaminases remained unaffected at all the stages of vitamin A deficiency, p-hydroxyphenylpyruvate-oxidase activity was decreased to less than 50 % at the weight-plateau stage and to about 10 % at the acute stages of deficiency. The intraperitoneal administration or the addition *in vitro* of ascorbate was partially effective, and the feeding of vitamin A acetate or acid was fully effective, in restoring the lost activity.

DISCUSSION

The present results support the contention that the biosynthesis of ascorbate is diminished in vitamin A-deficient rats. This conclusion seems to

disagree with that of Mapson & Walker (1948), who showed that, on treatment with chloretone, the vitamin A-deficient rats and the pair-fed controls excrete in their urine comparable amounts of ascorbate, and that in normal rats restriction of food intake together with the administration of chloretone leads to a decrease in the urinary excretion of ascorbate from 12.4 mg./24 hr. to 6.9 mg./24 hr. at the peak period. Our results show that restriction of food intake of the chloretone-treated normal rats lowers the urinary excretion of ascorbate from 9.0 mg./24 hr. to 7.6 mg./24 hr. at the peak period, but we have also shown that the chloretone-treated vitamin A-deficient rats excrete decreasing amounts of ascorbate with the progress of vitamin A deficiency, when compared with the corresponding pair-fed controls. Towards the end of the experiment the chloretone-treated vitamin A-deficient rats excreted very little ascorbate, whereas the corresponding pair-fed animals excreted significantly higher amounts.

There seem to be several explanations for this disagreement. The rats used in the two Laboratories were of different strains. The rats used by Mapson & Walker (1948) weighed as much as 245 g., whereas our animals seldom weighed more than 100 g.; moreover, a few of their depleted animals had traces of vitamin A in their livers. Also, their deficient rats seem to have survived longer than ours. It is therefore possible that their rats were not sufficiently depleted of vitamin A, and this might readily explain the discrepancy. However, the same report contains some evidence in favour of the lowering of the synthesis of ascorbate in vitamin A-deficient rats. Thus the blood ascorbate concentrations of the vitamin A-deficient rats were lower than those of the normal animals, and the difference could not be eliminated by pair-feeding, and at the terminal stages of deficiency the urinary response to chloretone was decreased.

That starvation alone is not responsible for such marked decrease in the synthesis of ascorbate is further shown by Table 2. At the stage of acute vitamin A deficiency, when the average food intake falls to only 1-2 g./day, there was practically no change in the synthesis of ascorbate from L- γ -glulonolactone *in vitro* by the liver enzyme of the pair-fed controls, whereas the deficient rats had suffered a marked loss.

In scorbutic guinea pigs the *p*-hydroxyphenylpyruvate-oxidase activity is normal (Knox, 1958), but the feeding of tyrosine to such animals leads to an inhibition of the enzyme, which is caused by an excess of the substrate (*p*-hydroxyphenylpyruvate) produced (Zannoni & La Du, 1959). In normal animals ascorbate protects this enzyme from the inhibition (La Du & Zannoni, 1961; Knox & Goswami, 1961). Our results show that, of the enzymes concerned in the metabolism of phenylalanine and tyrosine, tested in the vitamin A-deficient rats, the activity of *p*-hydroxyphenylpyruvate oxidase is appreciably decreased. Throughout the vitamin A deficiency, the transamination of tyrosine with α -oxoglutarate is maintained at the normal level, indicating a steady rate of formation of *p*-hydroxyphenylpyruvate. Extra tyrosine was not given to the vitamin A-deficient rats, but the *p*-hydroxyphenylpyruvate oxidase was depressed, and the feeding of ascorbate or its addition *in vitro* was partially effective in restoring the lost activity. This suggests that vitamin A deficiency lowers the concentration of this enzyme itself and that the decreased enzyme is further inhibited by the *p*-hydroxyphenylpyruvate because of the insufficient supply of ascorbate. The loss of *p*-hydroxyphenylpyruvate oxidase therefore seems to be caused by a combined effect of vitamin A and ascorbate deficiencies. Meunier, Zwingelstein & Jouanneteau (1953) have reported an increased excretion of

tyrosyl derivatives in tyrosine-fed vitamin A-deficient rats, and that this could be reversed by dietary ascorbate.

Our results have clearly shown that, with the progress of depletion of vitamin A, the animal loses its ability to synthesize ascorbate, leading to practically no synthesis at the acute vitamin A-deficiency stage. Ascorbate deficiency is thus superimposed on vitamin A deficiency, so that the resulting effect is a combination of the two. Since the rat can synthesize ascorbate, it is never included in the vitamin A-free diet. The question therefore naturally arises whether ascorbate should be included in such diets in order to obtain vitamin A deficiency free of ascorbate deficiency.

SUMMARY

1. The effect of vitamin A deficiency on the biosynthesis of ascorbate in rats was studied.

2. The chloretone-stimulated urinary excretion of ascorbate and its synthesis *in vitro* from L- γ -glulonolactone by liver microsomes were markedly diminished in vitamin A-deficient rats, as compared with those in corresponding pair-fed normals; at the severe stages of deficiency both urinary excretion and synthesis *in vitro* of ascorbate were lowered to negligible values.

3. The administration of ascorbate to vitamin A-deficient rats resulted in less pronounced loss in weight, increased longevity and better health, as compared with ascorbate-deprived vitamin A-deficient ones.

4. The liver *p*-hydroxyphenylpyruvate oxidase of rats was considerably depressed during vitamin A deficiency. The administration of ascorbate partially restored the lowered enzyme activity, and brought to normal the increased concentrations of phenylalanine and tyrosine in the plasma of vitamin A-deficient rats.

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REFERENCES

- Carpenter, M. P., Kitabchi, A. E., McCay, P. B. & Caputto, R. (1959). *J. biol. Chem.* **235**, 2814.
Chatterjee, I. B., Kar, N. C., Ghosh, N. C. & Guha, B. C. (1960). *Arch. Biochem. Biophys.* **86**, 154.
Chatterjee, I. B., Kar, N. C., Ghosh, N. C. & Guha, B. C. (1961). *Ann. N.Y. Acad. Sci.* **92**, 36.
Feinberg, R. H. & Caputto, R. (1960). *Biochem. biophys. Res. Commun.* **3**, 110.
Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
Guerrant, N. B. (1948). *Science*, **108**, 724.
Jonsson, G., Obel, A. L. & Sjöberg, K. (1942). *Z. Vitaminforsch.* **12**, 300.

- Jonsson, G., Obel, A. L. & Sjöberg, K. (1945). *Z. Vitaminforsch.* **15**, 115.
- Knox, W. E. (1955). In *Methods in Enzymology*, vol. 2, p. 289. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Knox, W. E. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, vol. 11, p. 307. London: Pergamon Press Ltd.
- Knox, W. E. & Goswami, M. N. D. (1961). *Ann. N.Y. Acad. Sci.* **92**, 192.
- La Du, B. N. & Zannoni, V. G. (1961). *Ann. N.Y. Acad. Sci.* **92**, 175.
- Linn, E. C. C., Pitt, B. M., Civen, M. & Knox, W. E. (1958). *J. biol. Chem.* **233**, 668.
- Longenecker, H. E., Fricke, H. H. & King, C. G. (1940). *J. biol. Chem.* **135**, 497.
- Malathi, P., Seshadri Sastry, P. & Ganguly, J. (1961). *Nature, Lond.*, **189**, 660.
- Malathi, P., Subba Rao, K., Seshadri Sastry, P. & Ganguly, J. (1963). *Biochem. J.* **87**, 305.
- Mapson, L. W. & Walker, S. E. (1948). *Brit. J. Nutr.* **2**, 1.
- Mayer, J. & Krehl, W. A. (1948). *J. Nutr.* **35**, 523.
- Mayer, K. H., Hassalbach, C. H. & Boissonnas, R. A. (1952). *Helv. chim. acta*, **35**, 1781.
- Meunier, J., Zwingelstein, G. & Jouanneteau, J. (1953). *Bull. Soc. Chim. biol., Paris*, **35**, 1189.
- Roe, J. H. & Kuether, C. A. (1943). *J. biol. Chem.* **147**, 399.
- Sangiorgi, M. (1942). *Boll. Soc. ital. Biol. sper.* **17**, 369.
- Seshadri Sastry, P., Malathi, P., Subba Rao, K. & Ganguly, J. (1962). *Nature, Lond.*, **193**, 1080.
- Sharman, I. M. (1949). *Brit. J. Nutr.* **3**, x.
- Subba Rao, K. & Ganguly, J. (1964). *Biochem. J.* **90**, 104.
- Sure, B., Theis, R. M. & Harrelson, R. T. (1939). *J. biol. Chem.* **129**, 245.
- Sutton, T. S., Kaeser, H. E. & Hansard, S. L. (1942). *J. biol. Chem.* **144**, 183.
- Zannoni, V. G. & La Du, B. N. (1959). *J. biol. Chem.* **234**, 2925.

Biochem. J. (1964), **92**, 527

Studies on Metabolism of Vitamin A

8. EFFECT OF ASCORBATE ON ISOPRENOID METABOLISM IN VITAMIN A-DEFICIENT RATS

By P. MALATHI AND J. GANGULY

Department of Biochemistry, Indian Institute of Science, Bangalore 12, India

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The biosynthesis of ubiquinones probably takes place by a condensation of the appropriate polyisoprenoid chain with the benzoquinone nucleus (Lynen, Eggerer, Henning & Kessel, 1958; Stoffel & Martius, 1960; Lynen, 1961). Mevalonate is rapidly incorporated into the side chain of the ubiquinones (Gloor & Wiss, 1958), and not into the ring (Gloor, Schindler & Wiss, 1960). The ring, however, can be derived from phenylalanine (Olson, Dialameh & Bentley, 1961; Bentley, Ramsey, Springer, Dialameh & Olson, 1961; Ramasarma, 1961).

The ubiquinone concentration in the liver of vitamin A-deficient rats is markedly increased (Lowe, Morton & Harrison, 1953; Morton & Phillips, 1959), and in the liver of such rats the incorporation of [2-¹⁴C]mevalonate is higher in the squalene and ubiquinone fractions and lower in the sterol fraction (Gloor & Wiss, 1959*a, b*). The concentrations of free phenylalanine and tyrosine in the tissues of vitamin A-deficient rats are higher (Malathi, Seshadri Sastry & Ganguly, 1961) and can be corrected by the administration of ascorbate (Malathi & Ganguly, 1964). Possibly, therefore, one of the reasons for the increased concentration of

ubiquinones in the liver of the vitamin A-deficient rats is the availability of larger amounts of the phenolic amino acids. If this were so, the injection of ascorbate into vitamin A-deficient rats should lower the increased ubiquinone concentrations in the liver. Our results show that such treatment is partially effective in decreasing the raised incorporation of [2-¹⁴C]mevalonate into the squalene and ubiquinone fractions, with a concomitant rise in the radioactivity of the sterol fraction of the vitamin A-deficient liver.

EXPERIMENTAL

Materials. [2-¹⁴C]Mevalonolactone was generously given by Dr O. Isler of Hoffmann-La Roche, Basle, Switzerland. Diethyl ether and light petroleum (b.p. 40–60°) were distilled and stored over anhydrous sodium sulphate. Alumina used for chromatography was supplied by E. Merck. The rest of the materials were as described by Malathi & Ganguly (1964).

Animals. Animals (all male) were treated as described by Malathi, Subba Rao, Seshadri Sastry & Ganguly (1963).

Enzymes. For the incorporation of [2-¹⁴C]mevalonate *in vitro*, the enzymes were obtained as described by Bucher & McGarrah (1956). Liver was homogenized for not more