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Studies on Metabolism of Vitamin A

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

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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-^{14}C]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-^{14}C]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-^{14}C]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-^{14}C]mevalonate *in vitro*, the enzymes were obtained as described by Bucher & McGarrahan (1956). Liver was homogenized for not more

than 60 sec. with a loose homogenizer of the Potter–Elvehjem type (0·5 mm. clearance) in 2 vol. of 0·1 M-phosphate buffer, pH 7·4, containing nicotinamide (30 mM) and $MgCl_2$ (4 mM). The supernatant, obtained after centrifugation of the homogenate for 15 min. at 10 000 *g*, was used as the source of the enzymes.

The incubation mixtures consisted of 10 000 *g* supernatant (2 ml.), [2-^{14}C]mevalonolactone (500 μg.; 5×10^4 counts/min.), NAD (3 μM), NADP (1·5 μM), GSH (30 μM), ATP (3 μM), nicotinamide (30 mM) and $MgCl_2$ (15 μM). Vitamin A alcohol was added in 0·01 ml. of 10% (v/v) ethanol, vitamin A acid as the sodium salt in 0·1 ml. of 0·1 M-phosphate buffer, pH 10·5, and ascorbate in 0·1 ml. of aqueous solution after neutralization with NaOH. The total volume was made up to 3·0 ml. with 0·1 M-potassium phosphate buffer, pH 7·4. Incubations were for 3 hr. at 37°, when the reactions were stopped with 10 ml. of ethanol containing 5 mg. of crystalline cholesterol as carrier.

Analytical methods. Portions of the liver in the experiments *in vivo* were minced and suspended in 10 vol. of ethanol. The suspension was then saponified with 40% (w/v) NaOH (1 ml./g. of tissue) in the presence of pyrogallol (0·1 g./g. of tissue). The whole reaction mixture in the experiments *in vitro* was mixed with 10 ml. of ethanol and saponified as described above. The unsaponifiable materials were extracted three times with light petroleum, and the pooled extracts were washed free of alkali, dried *in vacuo* and dissolved in a small volume of light petroleum. The samples were then chromatographed on columns of 10 g. of deactivated (4%) alumina, as described by Joshi, Jayaraman & Ramasarma (1963), by using the following successive eluents: 50 ml. of light petroleum, 50 ml. of 5% (v/v) diethyl ether in light petroleum, 50 ml. of 10% (v/v) diethyl ether in light petroleum, 60 ml. of 20% (v/v) diethyl ether in light petroleum and, finally, 50 ml. of diethyl ether. Successive 10 ml. fractions were collected. Light petroleum alone eluted the hydrocarbons including squalene, 5% diethyl ether in light petroleum eluted the ubiquinones, and 20% diethyl ether in light petroleum eluted the bulk of the sterols. All fractions or suitable samples were plated on aluminium planchets as uniformly and with as thin a layer as possible. The radioactivity was measured with a Geiger–Müller tube (Tracerlab, type TGC2, no. 27998, with a window thickness of 1·9 mg./cm.^{2} and an operating voltage of 1400 v) attached to a scaler (Nuclear–Chicago, model 151 A). Corrections were made for self-absorption and background. Each sample was counted in triplicate and sufficient numbers of counts were taken to decrease the error of counting to less than 5%.

Ubiquinones were estimated from the change in extinction at 275 mμ on the addition of borohydride to ethanolic solutions (Crane, Lester, Widmer & Hatefi, 1959). Cholesterol was estimated by the Liebermann–Burchard reaction, and protein by the biuret method (Gornall, Bardawill & David, 1949).

RESULTS

Effect of supplementation with ascorbate on the incorporation of [2-^{14}C]*mevalonate into the unsaponifiable materials of the liver of vitamin A-deficient rats.* After being on the vitamin A-deficient diet for 2 weeks, the rats were divided into two groups; the animals of one group were given daily by intraperitoneal injection 0·5 mg. of ascorbate (neutralized to pH 6·5–7·0 with solid sodium hydrogen carbonate) in a total volume of 0·2 ml., and those in the other group remained unsupplemented. Rats of both groups had corresponding normal pair-fed controls. One week after the weight plateau was reached, one rat from each group was given intraperitoneally 10 μc of [2-^{14}C]mevalonate in 0·3 ml. of water (pH 7·0) and killed 4 hr. later, when the livers were excised and analysed as described in the Experimental section. These experiments were repeated with three individual rats from each group; the results obtained from one such set of experiments are given in Fig. 1, the patterns of distribution in the other two being similar. Table 1

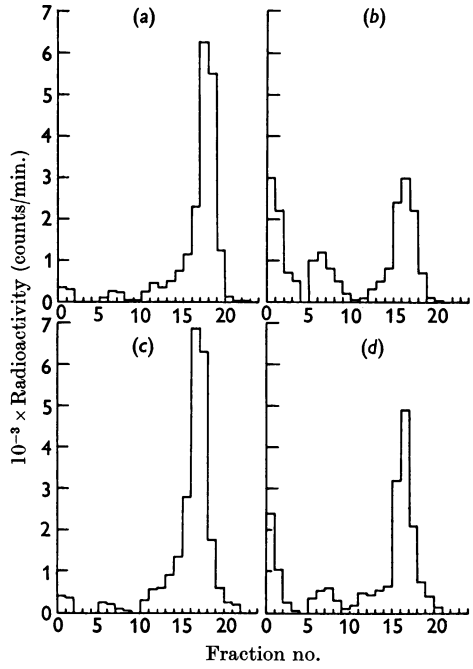

Fig. 1. Distribution of radioactivity among the squalene, ubiquinone and sterol fractions in the livers of vitamin A-deficient and pair-fed control rats. The conditions of the experiments are described in the Results section. Each Figure represents the analysis carried out on a portion of the whole liver obtained from one rat of each group. Fractions 1–5 represent the squalene portion, fractions 5–10 the ubiquinones, and fractions 15–20 the sterols; a small portion of the sterols was eluted in fractions 10–15. (*a*) Pair-fed control (total liver, 5·32 g.; portion analysed, 2·8 g.); (*b*) vitamin A-deficient (total liver, 3·8 g.; portion analysed, 2·2 g.); (*c*) ascorbate-supplemented pair-fed control (total liver, 4·9 g.; portion analysed, 2·9 g.); (*d*) ascorbate-supplemented vitamin A-deficient (total liver, 5·0 g.; portion analysed, 2·5 g.).

Table 1. *Incorporation of* [2-^{14}C]*mevalonate into the liver unsaponifiable materials of vitamin A-deficient rats*

The conditions of the experiments are described in the Results section. The results are expressed per whole liver and are averages of three separate experiments in each group. The values in parentheses represent average amounts of the respective compounds.

		Total radioactivity (counts/min.)				Sp. radioactivity	
Treatment	Status of rats	Total unsaponifiable materials	Squalene fraction	Ubiquinone fraction	Sterol fraction	Ubiquinone (counts/min./μmole)	Sterol (counts/min./mg.)
None	Vitamin A-deficient	38020	11760	6460 (2·3 μmoles)	20300 (9·2 mg.)	2808	2206
	Pair-fed control	40200	1480	1020 (0·64 μmole)	37480 (11·44 mg.)	1593	3276
Ascorbate administered	Vitamin A-deficient	39700	7490	4200 (1·33 μmoles)	27700 (9·8 mg.)	3158	2826
	Pair-fed control	39900	1400	1090 (0·68 μmole)	37200 (11·0 mg.)	1603	3381

Table 2. *Incorporation of* [2-^{14}C]*mevalonate* in vitro *into the unsaponifiable materials of the liver of vitamin A-deficient rats*

The reactions were carried out as described in the Experimental section. The results are averages of four separate experiments in each group and are expressed per reaction mixture.

			Percentage distribution of the total radioactivity of the unsaponifiable materials	
Additions	Status of rats	Radioactivity of total unsaponifiable materials (counts/min.)	Squalene fraction	Sterol fraction
None	Vitamin A-deficient	3860	52·3	46·3
	Pair-fed control	6980	13·2	87·0
Vitamin A alcohol (50 μg.)	Vitamin A-deficient	3600	55·0	44·2
	Pair-fed control	7020	12·1	86·0
Vitamin A acid (50 μg.)	Vitamin A-deficient	4020	52·2	48·7
	Pair-fed control	7100	11·7	86·7
Ascorbate (10 μmoles)	Vitamin A-deficient	3790	50·1	48·8
	Pair-fed control	6940	12·8	86·3
None	Ascorbate-supplemented vitamin A-deficient	5470	30·1	71·2
	Ascorbate-supplemented pair-fed control	7200	12·0	88·3
Vitamin A alcohol (50 μg.)	Ascorbate-supplemented vitamin A-deficient	4980	29·7	72·2
	Ascorbate-supplemented pair-fed control	6900	14·3	85·5
Vitamin A acid (50 μg.)	Ascorbate-supplemented vitamin A-deficient	5080	30·1	71·9
	Ascorbate-supplemented pair-fed control	6880	11·6	87·3

shows the average values from each group: the total incorporation of radioactivity into the unsaponifiable materials was the same in all the groups. Both Table 1 and Fig. 1 show that in the vitamin A-deficient rats the incorporation was markedly higher in the squalene and ubiquinone fractions, with a correspondingly lower radioactivity in the sterol fraction, as compared with the pair-fed controls. This is in agreement with previous reports (Gloor & Wiss, 1959*b*). However, the administration of ascorbate led to a significant change in the pattern of distribution of radioactivity in the three fractions of the deficient liver, whereas it had no effect on the normal animals. Thus, in the vitamin A-deficient rats, the percentage distribution of radioactivity in the sterol, ubiquinone and squalene fractions was 53, 17 and 30; the corresponding values in the pair-fed normal animals were 94, 2·5 and 3·5, and in the ascorbate-supplemented vitamin A-deficient rats were 70, 10·5 and 19 respectively.

The results of the experiments on the incorporation of [2-^{14}C]mevalonate *in vitro* are summarized in Table 2. Whereas in the studies *in vivo* the total

radioactivities in the liver unsaponifiable materials were similar in all the groups, they were less in the studies *in vitro* in the vitamin A-deficient rats than in the pair-fed controls. However, in the vitamin A-deficient rats, both *in vivo* and *in vitro*, the incorporation of radioactivity was less in the sterol fraction and higher in the squalene fraction. This agrees with the results of Wiss & Gloor (1960) and Weber, Gloor & Wiss (1960). In contrast, the prior administration of ascorbate to the vitamin A-deficient rats led to a considerable increase in the total incorporation of the labelled mevalonate into the liver unsaponifiable materials *in vitro*; the radioactivities were higher in the sterols and lower in the squalene fraction, as compared with the ascorbate-deprived vitamin A-deficient rats. Additions of vitamin A alcohol or acid or of ascorbate *in vitro* had no effect on the incorporation of mevalonate.

DISCUSSION

We have shown that vitamin A deficiency causes a marked decrease in the synthesis of ascorbate and in the *p*-hydroxyphenylpyruvate-oxidase activity in rats; the latter could only partially be corrected by the administration or addition *in vitro* of ascorbate, although injection of ascorbate normalizes the increased concentrations of phenolic amino acids in the tissues of the vitamin A-deficient rats (Malathi & Ganguly, 1964).

In the present experiments with vitamin A-deficient rats, treatment with ascorbate led to a decrease in the concentration of ubiquinones in the liver. It also lowered the incorporation of labelled mevalonate into squalene and ubiquinones, and increased that into the sterols. Since ascorbate corrects the elevated concentrations of free phenylalanine and tyrosine in the tissues of vitamin A-deficient rats, and since phenylalanine is the precursor of the benzenoid ring of ubiquinones, the administration of ascorbate should lead to a decrease in the availability of the ring precursors for ubiquinone synthesis, and this may therefore partly explain these observations.

Ascorbate takes part in microsomal electron transport (Staudinger, Krisch & Leonhauser, 1961), and it has been suggested that this may be connected with hydroxylation reactions involving the transfer of electrons from $NADH_2$ or $NADPH_2$ to molecular oxygen (Staudinger *et al.* 1961). There are several cases where ascorbate has been implicated in hydroxylation reactions. Thus the hydroxylating processes required in the biosynthesis of corticosteroids are ascorbate-dependent (Hofmann & Staudinger, 1951; Kahnt & Wettstein, 1951; Staudinger, 1958; Kernsten, Leonhauser & Staudinger, 1958). Ascorbate is also known to participate in the formation of 5-hydroxytryptophan from tryptophan (Cooper, 1961), and of hydroxyproline from proline (Stone & Meister, 1962). Axelrod, Udenfriend & Brodie (1954) showed that the hydroxylation of acetanilide, aniline and antipyrine is depressed in scurvy. The normal metabolism of phenylalanine involves the introduction of several hydroxyl groups, and some of these are dependent on the availability of ascorbate (Levin, Levenberg & Kaufman, 1960; Knox & Goswami, 1961).

Gloor & Wiss (1959*b*) and Wiss & Gloor (1960) have suggested that in vitamin A-deficient rats the conversion of squalene into cholesterol is affected. This conversion requires, among other reactions, the introduction of a hydroxyl group. If the decreased conversion of squalene into cholesterol in vitamin A deficiency were unchanged by supplementation with ascorbate, then the squalene fraction would have shown higher radioactivity, but it was actually decreased, with the sterol fraction showing an increase. Ascorbate, therefore, partially reverses the effect of vitamin A deficiency, and this might well be at the step of hydroxylation.

Besides ascorbate, several other factors, e.g. NAD or NADP and the optimum activities of the enzymes, would be required for the conversion of squalene into cholesterol. Johnson & Wolf (1960) have shown that the systems that regenerate $NADPH_2$ are not affected by vitamin A deficiency in the rat, but there is no information as to whether or not the concentrations of the nucleotides (NAD and NADP) themselves are lowered. Further, it is possible that the concentrations of the enzymes are also decreased. In the above experiments *in vivo* ascorbate could correct only partially the altered pattern of biosynthesis of ubiquinones and sterols, as caused by the vitamin A deficiency. Therefore, in addition to the indirect effect due to the lowered ascorbate synthesis, vitamin A deficiency seems to be directly responsible for some of these defects in isoprenoid metabolism.

Of the several species of animals studied, this effect of vitamin A deficiency on isoprenoid metabolism seems to be characteristic of the rat only. Thus in vitamin A-deficient guinea pigs the incorporation of [2-^{14}C]mevalonate is lower into the hydrocarbon and ubiquinone fractions and higher into the sterol fraction (Phillips, 1961). Also, in both vitamin A-deficient guinea pigs and chicks the amounts of ubiquinones in liver remain unaffected (Phillips, 1961; Lowe, Morton, Cunningham & Vernon, 1957).

SUMMARY

1. The effect of the administration of ascorbate on isoprenoid metabolism in vitamin A-deficient rats was studied.

2. The administration of ascorbate to vitamin A-deficient rats led to a lowered synthesis of ubiquinones in the livers compared with that in the livers of ascorbate-deprived vitamin A-deficient animals. The incorporation of [2-^{14}C]mevalonate into the sterols was higher and that into the squalene and ubiquinone fractions lower in the ascorbate-supplemented vitamin A-deficient animals, when compared with those in the ascorbate-deprived vitamin A-deficient rats.

3. Liver preparations *in vitro* from ascorbate-supplemented vitamin A-deficient rats showed increased incorporation of [2-^{14}C]mevalonate into the unsaponifiable materials, with greater radioactivity in the sterol fraction than in the squalene fraction, as compared with the ascorbate-deprived vitamin A-deficient rats. The addition of vitamin A alcohol or acid or ascorbate had no effect.

P.M. is a Lady Tata memorial scholar.

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The Distribution of ^{59}Fe in Albino Rats after Intravenous Administration in the Ionic or Chelated Form

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Our previous investigations have shown that the simultaneous administration of chelating agents can profoundly alter the tissue distribution of ^{60}Co in albino rats (Sivaramakrishnan, Sreenivasan, Sekhara Varma & Brahmanandam, 1962*a*). In an attempt to explain the changes produced we have studied the exact state in which the cobalt chelate exists in the blood after intravenous administra-

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