The Effect of Vitamin A Deficiency and Dietary α -Tocopherol on the Stability of Rat-Liver Lysosomes

By O. A. ROELS,* MARIAN TROUT AND A. GUHA

Columbia University Institute of Nutrition Sciences, New York, N.Y., U.S.A.

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1. Vitamin A-deficient rats and pair-fed controls were maintained on either normal or raised amounts of dietary α -tocopherol. 2. Their livers were fractionated and 'free' and 'total' lysosomal phosphatase were determined in the various fractions. The rate of release of this enzyme was determined in the mitochondria-lysosome-rich fraction during incubation at pH 5 and 37°. 3. The deficient livers showed increased enzymic activity. 4. Prolonged incubation caused more rapid enzyme release from the mitochondria-lysosome-rich fraction of the vitamin A-deficient rats receiving the normal amount of dietary α -tocopherol than from the equivalent fraction of their pair-fed controls receiving vitamin A. Raised dietary α -tocopherol reversed this phenomenon.

Several workers have shown that relatively high concentrations of retinol lower the stability of biological membranes *in vitro*. Dingle (1963) found that the lysosomes of chick embryos grown in tissue culture release cathepsin more easily when retinol is added to the culture medium, probably owing to the rupture of the lysosomal membrane caused by this addition. de Duve, Wattiaux & Wibo (1962) have demonstrated that the addition of retinol to the mitochondrial fraction of rat-liver homogenates increases the rate of release of lysosomal phosphatase into the incubation medium; they found, however, that the addition of vitamins D₂, E and K had the same effect *in vitro*.

There is evidence (Dingle, Sharman & Moore, 1963) that hypervitaminosis A has an analogous effect *in vivo* on lysosomes. Weissman, Uhr & Thomas (1963) found an increased release *in vitro* of β -glucuronidase from the lysosomes in liver homogenates from guinea pigs suffering from hypervitaminosis A; the unusually high serum β -glucuronidase of these animals indicated that an analogous process might be operating *in vivo*. Fell (1964) and Dingle (1964) have proved that the addition *in vitro* of retinol damages other biological membranes than that of the lysosome: it causes the distension of isolated fibroblasts, the swelling of mitochondria and the haemolysis of pig, ox, rat, rabbit and human erythrocytes (Dingle & Lucy, 1962; Glauert, Daniel, Lucy & Dingle, 1963). However, Lucy & Dingle (1964) discovered that the addition of α -tocopherol to the incubation medium

inhibited the retinol-induced lysis of rabbit erythrocytes, and that vitamin K₁, ubiquinone-30 and squalene had an effect comparable with that of α -tocopherol.

All these observations of the effect of retinol and its derivatives on the stability of biological membranes *in vitro* and *in vivo* were made with large doses of the vitamin. To determine whether the effects of retinol and α -tocopherol on biological membranes observed under these conditions are related to the physiological action of these vitamins, we decided to study the stability and permeability of biological membranes from vitamin A-deficient animals receiving different amounts of dietary α -tocopherol. It has been demonstrated in this Laboratory (Roels, Guha, Trout, Vakil & Joseph, 1964a) that the incorporation *in vitro* of ¹⁴C-labelled amino acids into diaphragm protein from vitamin A-deficient rats was significantly higher than that of pair-fed controls; feeding raised amounts of dietary α -tocopherol to the vitamin A-deficient rats and their controls completely reversed this phenomenon. We explained this observation through the combined effect of both vitamins on membrane permeability and stability.

To study this effect of vitamin A deficiency on membrane stability more specifically, we have determined the release of acid hydrolases from rat-liver lysosomes of vitamin A-deficient rats and pair-fed controls (Roxas, Sessa, Trout, Guha & Roels, 1964; Roels, Trout & Guha, 1964b) and found an increased release of acid ribonuclease, arylsulphatases A and B, cathepsin and β -glycerophosphate phosphatase from the lysosomes of the deficient animals, as well as significantly higher

* Present address: Marine Biology Division, Lamont Observatory, Columbia University, Palisades, N.Y., U.S.A.

total enzymic activity of the lysosomal enzymes in frankly vitamin A-deficient rats. Since analogous observations had been made in vitamin E deficiency in rabbits (Zalkin *et al.* 1962), carbon tetrachloride-induced liver injury in rats (Dianzani, 1963) and various other pathological states (Bitensky, 1963), we were not certain whether our observed lysosomal changes were due to vitamin A deficiency or to non-specific tissue damage.

EXPERIMENTAL

Animals and diets. Twenty-one-day-old male albino rats of the Sherman strain, weighing 40–44 g., were housed in individual cages and fed with the experimental diet described by Roels *et al.* (1964a). Male littermates matched for weight formed the pairs; the rat fed with the vitamin A-deficient diet ate *ad lib.* and its littermate receiving the control diet containing vitamin A was pair-fed. One group of vitamin A-deficient rats and their pair-fed controls received a 'normal' dietary amount of α -tocopherol, whereas a second group of pairs was fed with ten times this amount of vitamin E.

The dietary amounts of vitamin A and α -tocopherol are shown in Table 1.

After 5 weeks on this diet, the deficient animals ate less and ceased to grow; they reached a 'plateau stage' in their weight curve (Vakil, Roels & Trout, 1964). We called these rats 'mildly deficient' and all the work reported here was done with animals at this stage of deficiency. After 6–7 weeks on the diet, the deficient rats lost weight rapidly and were called 'frankly deficient'.

Cell fractionation. After 24 hr. starvation, pairs of rats were decapitated and exsanguinated. The livers were quickly removed and immersed in ice-cold 0.25 M-sucrose, pH 7. Fractionation of the livers was done generally according to de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). All the livers were homogenized in the same Potter-Elvehjem homogenizer (diameter 1.8 cm., gap 0.15 mm.) turning at 5000 rev./min. with one up-and-down

stroke for exactly 30 sec. in 0.25 M-sucrose, pH 7, to yield a 1:5 (w/v) homogenate. This homogenate was centrifuged for 10 min. at 500g at 0° in an International refrigerated centrifuge model HR-1 with the no. 856 rotor. The supernatant was decanted and the precipitate was rehomogenized in the same volume of fresh 0.25 M-sucrose, pH 7, with one stroke of the same homogenizer for 30 sec. and again centrifuged for 10 min. at 500g at 0°. This pellet was called the 'nuclear fraction' (N). Both supernatants were combined and formed the 'cytoplasmic extract' (CE). A sample of the CE was centrifuged for 6 min. 42 sec. at 41225g in the Spinco model L centrifuge with rotor no. 40. The supernatant was decanted. The pellet was resuspended in fresh 0.25 M-sucrose, pH 7, and recentrifuged as before. This pellet, containing heavy and light mitochondria, was called the ML fraction. The two combined supernatants were centrifuged at 105000g for 60 min. (Beckman Spinco model L, rotor no. 40). The resulting supernatant is the soluble fraction (S) and the pellet is referred to as the P fraction. The N, ML and P pellets were resuspended in 0.25 M-sucrose, pH 7, so that the total volume of each in millilitres was equal to ten times the weight in grams of the amount of liver from which each was prepared. The N fraction was rehomogenized in the same Potter-Elvehjem homogenizer used to prepare the liver homogenate with six up-and-down strokes of the pestle turning at 5000 rev./min.

Enzymic assay. The technique of Lowry, Rosebrough, Farr & Randall (1951) was used to determine the protein content of the fractions. Crystalline bovine albumin was used as protein standard. 'Free' and 'total' β -glycerophosphate-phosphatase activity was determined in all the fractions generally according to the method described by Gianetto & de Duve (1955) as used by Roels *et al.* (1964b). The fractions were incubated at 37° for 10 min. at pH 5 in a medium containing 0.05 mole of sodium acetate buffer, 0.25 mole of sucrose and 0.05 mole of sodium β -glycerophosphate/l. To assay 'free' activity of the phosphatase. To determine 'total' enzymic activity, alkylphenoxypolyethoxyethanol (Triton X-100, kindly given by Rohm and Haas Co., Philadelphia 5, Pa., U.S.A.) was added to the incubation medium (final concn. 0.1%); this detergent probably breaks the subcellular particles, thereby releasing the enzyme into the medium.

Only 'total' activity was determined in the S fraction since all the enzyme there is obviously present in the 'free' form.

To determine the rate of release of the enzyme from the lysosomes, portions of the ML fraction were incubated at 37° in the same medium as that used for the enzymic assay, but without substrate. After 10, 20, 40, 60 and 90 min. preincubation, the substrate (β -glycerophosphate) was added so that its final concentration in the medium was 0.05 mole/l. To assay 'free' activity, substrate only was added, whereas substrate containing sufficient Triton X-100 to give 0.1% concentration in the medium was added to assay 'total' enzymic activity. Incubation in the presence of substrate, with or without Triton X-100, was then continued for 10 min. as in the usual enzymic assay. Preincubation at pH 5, 37° (without detergent) damages the lysosomes and enzyme is thereby released into the medium (de Duve *et al.* 1962). The 'free' enzymic activity determined after such preincubation is a measure of the resistance of the lysosomes under these conditions.

Table 1. Vitamin A and α -tocopherol in the diet of the various experimental groups of rats

Results are expressed as mg./kg. of diet.

Diet group ...	Pair-fed		Pair-fed	
	Deficient	Control	Deficient	Control
Retinyl acetate*	0	1.72	0	1.72
α -Tocopherol†	50	50	500	500

* United States Pharmacopeia vitamin A reference solution was used.

† Pure DL- α -tocopherol (Merck, U.S.A.) was used.

RESULTS

The enzyme distribution, recovery and the 'free' and 'total' enzymic activity in the different liver fractions of the four groups of experimental rats are reported in Table 2. 'Percentage recovery' of enzymic activity was obtained by adding the 'total' activity of the N and CE fractions (expressed as μmoles of substrate decomposed/g. of liver/min.) and calculating the 'total' activity of each fraction (N, ML, P and S) as a percentage of this sum.

The total percentage of 'free' enzymic activity was obtained by expressing the sum of 'free' activity in the N and CE fractions (μmoles /g. of liver/min.) as a percentage of the sum of the 'total' activity of (N+CE). In the S fraction, all determinations of the enzymic activity reported

in Table 2 were done in the presence of Triton X-100, preliminary experiments having shown that the 'free' activity in this fraction is equal to its 'total' activity.

The reproducibility of our tissue homogenization and fractionation procedures and of our enzymic assay can best be illustrated as follows: for the 56 rats reported here, the 'free' enzymic activity in the liver ML fraction (see Table 2) (expressed as a percentage of 'total' activity in that fraction) varied from 5 to 9% with only two exceptions: one value of 4% and one value of 14% was found.

Calculation of the specific enzymic activity, on a protein basis, for each of the liver fractions reported in Table 2 gave similar differences between the vitamin A-deficient animals and their respective controls to those shown in Table 2 for enzymic

Table 2. *β -Glycerophosphate-phosphatase activity measured as phosphate liberated in liver fractions from vitamin A-deficient rats and controls receiving different amounts of dietary α -tocopherol*

Incubation was for 10 min. at 37°, pH 5, in 0.25 M-sucrose; 0.05 M- β -glycerophosphate was present for 'free' activity; 0.1% of Triton X-100 was added for 'total' activity.

Fraction	Total activity (%; N+CE taken as 100)	Activity (mμmoles/g. of liver/min.)		Free activity (% of total)
		Free	Total	
A-deficient, normal E intake (−AE); 18 rats				
N	20	280	1659	17
CE	—	1159	6726	17
ML	57	298	4754	6
P	18	391	1572	25
S	8	659	659	100
Total (N+CE)	—	1439	8386	17
A-deficient, high E intake (−AE+); 10 rats				
N	17	313	1225	25
CE	—	1203	5805	21
ML	65	340	4551	7
P	24	514	1701	30
S	9	625	625	100
Total (N+CE)	—	1516	7030	22
Control, normal E intake (AE); 18 rats				
N	15	244	1183	21
CE	—	1121	6720	17
ML	66	336	5187	6
P	19	348	1470	24
S	7	561	561	100
Total (N+CE)	—	1365	7903	17
Control, high E intake (AE+); 10 rats				
N	14	222	892	25
CE	—	969	5407	18
ML	70	412	4440	9
P	25	459	1542	30
S	10	631	631	100
Total (N+CE)	—	1191	6299	19

Table 3. β -Glycerophosphate-phosphatase activity in liver fractions of vitamin A-deficient rats fed on different concentrations of α -tocopherol, compared with the activity in the corresponding liver fraction of their pair-fed controls

N.S., Not significant.

Normal amount of dietary tocopherol			Increased dietary tocopherol		
Activity/g. of fresh liver (-AE as % of AE)	P (significance of difference between -AE and AE)	Liver fraction	Activity/g. of fresh liver (-AE+ as % of AE+)	P (significance of difference between -AE+ and AE+)	
140	<0.001	N	137	<0.01	
92	<0.001	ML	102	N.S.	
107	<0.02	P	110	N.S.	
117	<0.001	S	99	N.S.	
106	<0.01	Total	112	<0.01	

activity/g. of fresh liver, since there was little difference between the protein content of the liver fractions from the different dietary groups.

There is practically no difference in the 'free' enzymic activity of the various liver fractions between the vitamin A-deficient rats and their respective controls.

'Total' enzyme activity tends to be higher in the liver fractions from the vitamin A-deficient rats than in the controls. The greatest difference is observed in the N fraction. The statistical significances of the differences observed between vitamin A-deficient rats and their respective pair-fed controls are given in Table 3.

In Table 3, the enzymic activity/g. of fresh liver determined in the presence of Triton X-100 for each group of deficient animals has been expressed as a percentage of the corresponding enzyme activity of its control group, taken to be 100%.

Since the deficient rats and their controls were pair-fed, Snedecor's (1959) method for paired observations was used to calculate the significance of the differences reported in Table 2 and Table 3.

The total enzyme activity/g. of liver in the -AE rats (8386 μ moles of substrate decomposed/g. of liver/min., s.d. 400) was significantly higher ($P < 0.001$) than that of the -AE+ rats (7030 μ moles, s.d. 603). Similarly, phosphatase activity/g. of liver of the AE rats (7903 μ moles of substrate decomposed/g. of liver/min., s.d. 220) was significantly higher ($P < 0.001$) than that of the AE+ rats (6299 μ moles, s.d. 296).

Fig. 1 shows the rate of release of β -glycerophosphate phosphatase from the ML fraction of the four different groups of rats.

The enzymic activity shown at zero time in Fig. 1 was obtained without any preincubation, in the same way as the regular enzymic assays shown in Table 2.

Fig. 1. Release of β -glycerophosphate phosphatase from the ML fraction of vitamin A-deficient rats and their controls receiving different amounts of dietary α -tocopherol. Portions were preincubated for 0, 10, 20, 40, 60 and 90 min. at 37°, pH 5, in a medium 0.05M in acetate buffer, 0.25M in sucrose; substrate (0.05M final concentration), without Triton X-100 for 'free' activity and with Triton X-100 (0.1%) for 'total' activity, was added at the end of the preincubation time; incubation was then continued for 10 min. ○—○, Vitamin A-deficient, normal vitamin E intake (-AE; average values for 7 rats); △—△, control, normal vitamin E intake (AE; average values for 7 rats); ●—●, vitamin A-deficient, high vitamin E intake (-AE+; average values for 9 rats); ▲—▲, control, high vitamin E intake (AE+; average values for 9 rats).

There was no release of enzyme from the lysosomes of any experimental group in the first 10 min. of preincubation. Longer preincubation caused a rapid release of enzymic activity in the -AE

group; this release had reached 100% after 40 min. There was little difference in the rate of release of enzymic activity from the lysosomes for both control groups (AE and AE+), whereas the rate of release of the -AE+ group was considerably less than that of the other groups, although all groups had reached 100% release after 90 min. preincubation.

For interpretation of the data illustrated in Fig. 1, it should be stressed that, after the preincubation time indicated on the abscissa, incubation in the presence of substrate was continued for a further 10 min., during which time there may of course have been an additional release of enzyme. The observed interaction of vitamins A and E seems to be rather complex, and the shape of the curves in Fig. 1 indicates at least two distinct effects: all curves have an initial 'induction' period during which the preincubation does not appreciably change the 'release' of enzyme from the lysosomes. This 'induction' period is greatly shortened in the -AE rats and prolonged in the -AE+ group. The rate of release of the enzyme from the lysosomes after the 'induction' period is considerably faster in the -AE than in the -AE+ group. There is very little difference between the two control groups (AE and AE+); their release curves lie in between the two extremes, -AE and -AE+, both for the length of the 'induction' period and for the rate of release during the post-induction period. After 60 min. preincubation, the rate of release decreased for both control groups, whereas this does not occur in either of the deficient groups (-AE and -AE+).

There was no significant difference in 'free' enzyme activity at zero time and after 10 min. preincubation. After 20, 40 and 60 min. preincubation, the release of enzyme from the -AE fraction was significantly higher ($P < 0.001$) than that of the AE fraction. There was no difference in the release of enzyme from the -AE+ fraction and from its control at zero time and after 10 or 20 min. preincubation; after 40 min. of preincubation, however, the enzyme release from the AE+ fraction was significantly higher than that of the -AE+ preparation ($P < 0.01$) and the significance of this difference was even more marked after 60 min. preincubation ($P < 0.001$).

DISCUSSION

It is clear from Table 3 that the total activity of the enzyme was significantly higher in the liver of the vitamin A-deficient animals than in the corresponding controls, for both concentrations of dietary α -tocopherol. However, these differences are relatively small, since we are dealing here with 'mildly' vitamin A-deficient rats. Indeed, we

have previously reported (Roels *et al.* 1964b) that this increase in enzyme activity is a function of the stage of deficiency: as the vitamin A deficiency progresses and the animal starts to lose weight rapidly ('frankly' deficient stage) this increase becomes more marked, mainly in the supernatant fraction. Since an increase of lysosomal hydrolases has also been reported in vitamin E deficiency and in other pathological conditions, it is thought that this phenomenon is non-specific and probably associated with tissue damage from many different causes.

The most important increase in enzymic activity due to vitamin A deficiency in the experiments reported here is in the nuclear fraction, regardless of the amount of dietary α -tocopherol. Since there is little or no difference between the deficient animals and their controls in the protein content of the liver fractions, it seems unlikely that this increase of enzymic activity in the N fraction of the livers of the deficient animals is due to a greater percentage of unbroken cells in this fraction. β -Glycerophosphate phosphatase is specifically a lysosomal enzyme (Neil & Horner, 1964); it appears therefore that the N fraction from vitamin A-deficient rat liver is more highly contaminated with lysosomes than that from control animals. Since there is little difference in 'free' activity in the N fraction between deficient animals and controls, the most likely explanation of this observation seems to be an increase in the density of the lysosomes from the deficient animals, under our experimental conditions, regardless of the amount of dietary α -tocopherol. However, the possible adsorption of lysosomal enzyme on the nuclei or cell debris cannot be excluded, although it would be hard to explain why this adsorption would be greater in the deficient animals than in pair-fed controls. Another conclusion to be drawn from Table 3 is the lower activity/g. of liver in the ML fraction of the -AE group compared with the AE group; this is compensated for by an increase of enzyme in the S fraction, indicating that more -AE lysosomes were ruptured under our experimental conditions and had released their enzyme into the soluble phase. This is in contrast with the -AE+ and AE+ groups where there is no difference in enzymic activity/g. of liver in the ML and S fractions resulting from vitamin A deficiency. The total enzyme/g. of liver was 20-25% higher in the liver of the rats on the lower α -tocopherol intake regardless of their vitamin A status (Table 2). This seems to be parallel with the observation (Zalkin *et al.* 1962) that there is a considerably increased activity of muscle lysosomal enzymes in vitamin E deficiency in several species.

As is shown in Table 2, there was practically no difference in 'free' activity between vitamin A-

deficient rats and controls for all particulate fractions, indicating that intact lysosomes present in the fractions were equally resistant to incubation at pH 5 for 10 min. in the medium used, whether they came from vitamin A-deficient rat livers or from controls. This is of particular importance for the ML fractions, which contain 57–70% of all the enzyme present in the tissue; only 5–9% of the enzyme in this fraction was determined as 'free' activity under our experimental conditions in the different groups of animals.

However, on prolonged preincubation of the ML fraction at pH 5, very important differences between the stability of the lysosomes from the different groups of animals became apparent, as is shown in Fig. 1.

The release curves of the enzyme in the ML fraction of both control groups (AE and AE+) are remarkably close and compare quite well with a similar release curve of β -glycerolphosphate phosphatase from the ML fraction of 'normal' rat liver published by de Duve *et al.* (1962). The amount of dietary α -tocopherol therefore did not change the stability of the liver lysosomal membrane in either of our control groups. In contrast, for the vitamin A-deficient groups, the amount of dietary α -tocopherol was an important factor in lysosome stability: at the lower tocopherol intake, the liver lysosomes from the vitamin A-deficient rats were considerably less stable than controls, whereas the liver lysosomes of the -AE+ group showed much greater resistance to lysis caused by incubation at 37°, pH 5, than controls. Dietary vitamin A therefore appears to regulate the stability of the lysosomal membrane: when dietary α -tocopherol is normal, retinol stabilizes the membrane (compare the release curves in Fig. 1 of the -AE and AE groups), whereas vitamin A labilizes the membrane at abnormally high α -tocopherol intakes (compare the release curves of -AE+ and AE+).

Lucy & Dingle (1964) observed that the hypotonic release of haemoglobin from rabbit erythrocytes during incubation at 20° is retarded by adding 1 μ g. of retinol/ml. to the incubation medium. They concluded that retinol, at this concentration, has a membrane-stabilizing effect, whereas larger doses (15 μ g. of retinol/ml. in the medium) considerably increased the rate of erythrocyte lysis during incubation at 37°. They found that the addition of 50 μ g. of α -tocopherol/ml. of incubation medium slowed down the retinol (15 μ g./ml.)-induced lysis of rabbit erythrocytes at 37°.

Roels *et al.* (1964a) have demonstrated that -AE+ rat liver contains considerably more α -tocopherol (273 μ g./g. of dry tissue) than AE+ liver (189 μ g./g. of dry tissue). Since tissue α -tocopherol accumulates mainly in the mitochondrial and microsomal fractions (Draper & Alaupovic, 1959),

it could be argued that the ML fraction of the -AE+ rats probably contained more α -tocopherol than that of the AE+ rats, and that lysosome stability may thus have been governed by the amounts of α -tocopherol present during the preincubation period. The results obtained with the liver ML fractions from the -AE and AE groups militate against this since we found before that the -AE group tends to store somewhat more α -tocopherol (135 μ g./g. of dry liver) than the AE group (107 μ g./g. of dry liver), and the -AE lysosomes were considerably less stable than those of the AE group. Moreover, de Duve *et al.* (1962) reported that the addition of α -tocopherol (125 μ g./ml. of incubation medium) *in vitro* increased the rate of release of lysosomal enzyme from the ML fraction of normal rat liver. We believe therefore that the action *in vitro* of the α -tocopherol naturally present in the ML fractions of our rat livers cannot explain the difference in the rate of enzyme release we observed.

It is unlikely that the observed changes are due to the differences in the amount of retinyl ester and retinol stored in the livers of our different groups of rats, since this ester appears mainly in the supernatant fraction (Krishnamurthy, Seshadri Sastry & Ganguly, 1958). Moreover, when control rats were fed retinoic acid (which is not stored in the liver, but promotes normal growth), we found the same pattern of lysosomal enzyme distribution as when retinyl acetate was used as dietary supplement for the controls (Roels *et al.* 1964b).

Two possible mechanisms can be envisaged to explain the observed differences in lysosome stability resulting from the different dietary amounts of vitamins A and E. First, the vitamins or their metabolites may influence the activity of an enzyme or enzymes capable of attacking the lysosomal membrane. Secondly, the vitamins or their metabolites may directly or indirectly influence the structure of the lysosomal membrane; we believe that our data are more compatible with the second hypothesis.

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