Studies on Metabolism of Vitamin A

3. THE MODE OF ABSORPTION OF VITAMIN A ESTERS IN THE LIVING RAT*

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Since the work of Gray, Margareidge & Cawley (1940) with rats, followed by that of Eden & Sellers (1950) with other animals, it has been generally believed that during absorption extensive hydrolysis followed by resynthesis of vitamin A esters takes place in the intestine. In previous communications from this Laboratory it was shown that, when vitamin A alcohol or acetate is given orally, the vitamin is ultimately stored in the liver as the palmitate (Mahadevan, Krishnamurthy & Ganguly, 1959; Mahadevan & Ganguly, 1961). Vitamin A acetate was not detected in the intestinal muscles, blood and liver of rats (Mahadevan *et al.* 1959), showing that the acetate had undergone hydrolysis and re-esterification at the time of absorption.

However, some workers (Sobel, 1952; Popper, Steigmann & Dyniewicz, 1950), who measured the blood concentration or liver storage of vitamin A after oral administration of aqueous dispersions of vitamin A esters, were led to suggest that the esters need not be hydrolysed at the time of absorption. Haubold, Heuer, Lowe & Rohuschinsky (1960) support this concept of particulate absorption of vitamin A esters. Two schools of thought thus seem to exist on the question of the necessity of hydrolysis and resynthesis of vitamin A esters during absorption.

In this paper it is shown that hydrolysis of vitamin A esters is essential for absorption in the rat. In the next paper (Mahadevan, Seshadri Sastry & Ganguly, 1963) it is demonstrated that the same mechanism also obtains in the system *in vitro*. These results were presented at the symposium on Vitamin Metabolism at Calcutta (Mahadevan & Ganguly, 1962).

MATERIALS AND METHODS

Materials. The sources of the materials used have previously been described (Ganguly, Krishnamurthy & Mahadevan, 1959; Mahadevan & Ganguly, 1961). The

various esters of vitamin A were prepared enzymically according to Mahadevan & Ganguly (1961).

Methods. Male rats of this Institute strain, weighing 120–140 g., were starved for 24 hr. and dosed orally with 3 mg. of vitamin A alcohol or an equivalent quantity of the ester, dissolved in 0.5 ml. of groundnut oil or in 0.5 ml. of Tween 20 (polyoxyethylene sorbitan monolaurate). The rats were killed 2–3 hr. later under light ether anaesthesia by drawing as much blood as possible from the heart. Extraction of the tissues and subsequent analysis for vitamin A alcohol and its different esters were carried out as detailed by Ganguly *et al.* (1959), Mahadevan *et al.* (1959) and Mahadevan & Ganguly (1961).

RESULTS

It is shown in Table 1 that, when vitamin A alcohol was fed in groundnut oil, considerable amounts of the palmitate were present in the lumen, whereas, with Tween 20 as the carrier, in addition to the palmitate there was a significant quantity of the laurate. The palmitate was the major ester in the mucosa in both cases and it was almost the only ester in the rest of the tissues. These findings are in agreement with those reported earlier (Mahadevan & Ganguly, 1961).

More striking results were obtained on feeding with the esters. Whatever ester was fed, considerable hydrolysis always occurred in the lumen, and more alcohol was formed when the esters were administered as Tween dispersions than with the oily solutions. In all cases the lumen contained substantial amounts of the palmitate, and with the Tween 20 some vitamin A laurate also was found. In the mucosa, the relative proportion of vitamin A alcohol was always more than that of the total esters. However, the nature of the esters in this tissue was of particular interest in that, irrespective of the type of ester ingested, the relative concentration of the palmitate (as compared with the other esters) was lower in the contents and was markedly higher in the mucosa. There was practically no difference in this respect between the animals receiving the oily solution and the Tween dispersion. It seems significant that small but definite amounts of the fed ester were consistently recovered from the mucosa.

* Part 2: Subba Rao, Seshadri Sastry & Ganguly (1963).

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Table 1. *Vitamin A ester composition of the contents, mucosa and muscles of the small intestine, blood and liver of rats 2-3 hr. after feeding with vitamin A alcohol or different vitamin A esters*

Form of vitamin A fed	Intestine											
	Contents				Mucosa				Muscles			
	Ester (μg.)				Ester (μg.)				Ester (μg.)			
	A	S	P	M	A	S	P	M	L	A	P	OE
	Alcohol	(a)	(b)	(c)	Alcohol	(a)	(b)	(c)	(d)	Alcohol	(a)	(b)
Alcohol	0	12	69	30	871	0	10	120	20	395	140	22
Acetate	225	11	120	32	770	27	3	105	8	330	127	15
Laurate	153	8	73	13	873	35	4	157	15	390	185	22
Stearate	0	13	152	28	534	0	2	108	7	184	107	7
Palmitate	0	727	127	12	439	0	17	97	6	194	98	5
Linoleate	0	535	79	6	642	0	29	147	7	278	138	11
	0	9	830	15	417	0	2	93	4	205	107	6
	0	6	675	10	678	0	3	156	7	290	140	6
	0	6	95	550	8	305	0	2	96	181	118	4

Three rats were used in each group. Each rat was given orally 3 mg. of vitamin A alcohol or an equivalent quantity of the ester in 0.5 ml. of groundnut oil (a) or 0.5 ml. of Tween 20 (b). Results are averages of three separate experiments with individual rats. Values are expressed as μg. of vitamin A alcohol in the free or esterified form per whole organ for intestinal contents, for mucosa and muscles, and for liver, and per 10 ml. of blood. A, Acetate; S, stearate; P, 'palmitate'; M, 'myristate'; L, 'laurate'; OE, other esters. The 'other esters' in intestinal muscles, blood and liver represent esters other than the palmitate; the acetate was totally absent from these three tissues in all the cases. The reason for denoting the higher esters as 'palmitate' etc. can be found in Mahadevan & Ganguly (1961).

Distribution of vitamin A alcohol and its different esters between the particulate and supernatant fractions of the mucosal-cell homogenate

The above-mentioned experiments indicated that extensive hydrolysis of vitamin A esters in the lumen is followed by resynthesis, probably inside the cell. Also, the fact that the fed ester did not appear in any appreciable quantity in the mucosa suggested that vitamin A alcohol might be the molecular species that crosses the cell membrane during absorption. Further experiments were therefore planned to obtain additional evidence in support of such a possibility.

In one set of experiments, where rats were dosed with vitamin A acetate in groundnut oil, the intestinal mucosa was collected after 3 hr. and homogenized in 0.25 M-sucrose. The nuclear and mitochondrial fractions were separated as described by Ganguly *et al.* (1959) and the microsomal fraction was then sedimented by centrifuging the resultant supernatant at 100,000g for 90 min. in the Spinco model L ultracentrifuge. All the fractions were analysed for vitamin A alcohol and total vitamin A esters. As shown in Table 2, about 77% of the total free alcohol of the homogenate was present in the combined particulate materials, of which nearly 73% occurred in the microsomal fraction alone. In contrast, the greater part of the vitamin A esters (70%) of the homogenate was present in the supernatant fraction. More than half of the esters of the particulate materials was recovered from the microsomal fraction.

Since the above trials showed that the bulk of both free and esterified vitamin A associated with the particulate materials can be located in the microsomal fraction alone, in later experiments the mucosal-cell homogenates were centrifuged only once at 100,000g for 90 min. in the ultracentrifuge to separate them into two fractions, the particulate and supernatant. Rats were dosed with vitamin A alcohol, acetate, stearate, linoleate, laurate or palmitate, and the two fractions of the mucosal cells were analysed for vitamin A alcohol and the different esters. In every instance the particulate fraction had higher concentrations of vitamin A alcohol. The supernatant fraction, on the other hand, contained the vitamin almost exclusively in the esterified form. However, this ester consisted mostly of the palmitate, with traces of the administered ester. Again the fed ester was associated more with the particles than with the soluble fraction of the cell (Table 3).

DISCUSSION

Although it has been generally assumed that vitamin A esters are hydrolysed and re-esterified

Table 2. *Intracellular distribution of vitamin A alcohol and ester in the intestinal mucosa of rats after feeding with vitamin A acetate in groundnut oil*

Each rat was given orally 3 mg. of vitamin A acetate in 0.5 ml. of groundnut oil and killed 2–3 hr. later. The mucosal homogenates were fractionated as described in the text. Results are the average of three separate experiments with individual rats; values are expressed as $\mu\text{g.}$ of vitamin A alcohol in the free or esterified form and are for the whole of the intestinal mucosa from each rat.

Tissue fraction	Vitamin A alcohol		Vitamin A ester	
	($\mu\text{g.}$)	(% of homogenate)	($\mu\text{g.}$)	(% of homogenate)
Homogenate	157	100	163	100
Nuclear	22	12.7	13	7.9
Mitochondrial	10	6.4	7	4.3
Microsomal	90	57.3	28	17.2
Cell sap	37	23.6	115	70.0

Table 3. *Distribution of vitamin A alcohol and its esters between the particulate and supernatant fractions of the homogenates of the intestinal mucosa of rats 2–3 hr. after the feeding with vitamin A alcohol or its esters in groundnut oil*

Feeding procedure was the same as described in Table 2. Only two fractions, i.e. the particulate and supernatant, were obtained by centrifuging the homogenates at 100000g for 90 min. For definitions of abbreviations, see Table 1.

Form of vitamin A fed	Particulate fraction						Supernatant fraction					
	Ester ($\mu\text{g.}$)					Alcohol ($\mu\text{g.}$)	Ester ($\mu\text{g.}$)					Alcohol ($\mu\text{g.}$)
	A	S	P	M	L		A	S	P	M	L	
Alcohol	0	6	25	15	3	284	0	4	90	5	3	96
Acetate	24	2	22	5	1	252	14	1	73	2	0	87
Laurate	0	1	26	4	10	145	0	0	71	1	3	39
Stearate	0	9	17	2	3	135	0	4	62	2	1	39
Palmitate	0	3	19	1	2	142	0	3	74	2	1	41
Linoleate	0	2	28	8	2	129	0	2	68	3	0	59

during absorption, a critical examination of the existing literature reveals that no conclusive proof has ever been produced in support of this assumption. Thus Gray *et al.* (1940) reported that, after feeding vitamin A alcohol to rats in the form of fish-liver-oil distillate, nearly all the vitamin of the intestinal wall was present as the free alcohol at the height of absorption. Apparently contradictory results were, however, produced by Eden & Sellers (1950), who showed that the vitamin was largely in the esterified form in the intestinal wall of calves and sheep fed either with the ester or the free alcohol. Nevertheless, complete hydrolysis of the fed ester had occurred in the intestinal lumen of some of the animals. The work of Glover, Goodwin & Morton (1948) and of Thompson, Ganguly & Kon (1949) also showed that at the time of absorption vitamin A is found predominantly esterified in the intestinal wall. Though these observations indicated that during absorption extensive hydrolysis and resynthesis of the esters take place in the intestine, Sobel (1952) and Popper *et al.* (1950) were of the opinion that if the ester is administered in a finely emulsified state it might be absorbed without hydrolysis.

As stated earlier (Mahadevan *et al.* 1959), in rats vitamin A acetate is absorbed only after hydrolysis, whereas the present results (Table 1) demonstrate that the higher esters also are hydrolysed in the lumen of the intestine. Further, in all cases except where the palmitate was fed it was not possible for us to recover more than traces of the fed ester from the intestinal muscles and blood. Irrespective of the ester administered, the predominant ester in all the tissues was the palmitate and the same was true even when the free alcohol was fed. These observations thus strongly support the hypothesis that the esters are hydrolysed and are then absorbed as the free alcohol. Since at the time of active absorption the palmitate predominates in the intestinal tissues, it might be argued that vitamin A palmitate may be absorbed without hydrolysis. However, the data presented here (Tables 1 and 3) show that it is also hydrolysed to a similar extent.

The hydrolysis of the fed esters seems to be a common feature for both oily solutions and Tween dispersions (Table 1). Moreover, with both types of carriers and with all types of esters, the palmitate was the major ester recovered from the mucosal

cells, which makes it abundantly clear that the Tween dispersions also are not absorbed without prior hydrolysis.

We have shown here (Tables 2 and 3) that the major portion of the vitamin A alcohol of the mucosal cell is invariably found associated with the particulate materials. Similar observations were made with the intestinal mucosa of the rat (Ganguly *et al.* 1959) and the guinea pig (Glover, Green & Stainer, 1959). The presence of small amounts of the fed ester in these particles might mean that it is actually adsorbed on the outer surface of the mucosal-cell membrane and is in the process of being hydrolysed. At the same time, in every case, there was some palmitate also in the particulate fraction. It is possible that it represents the ester that is being resynthesized on the particles after the free alcohol has crossed the cell membrane; the larger concentration of the palmitate in the supernatant fraction would suggest that once resynthesized it is automatically released into the cytoplasm.

SUMMARY

1. After starvation for 24 hr. rats were fed with vitamin A alcohol or its various esters in groundnut oil or as aqueous dispersions in Tween 20, and the free alcohol and the esters were analysed in the tissues of the intestinal tract, blood and liver 3 hr. after the dose.

2. Regardless of the ester fed, there was considerable vitamin A alcohol in all tissues analysed, but the palmitate predominated in the intestinal mucosa and was almost the only ester in the other tissues.

3. The particulate materials of the mucosal-cell homogenate contained the bulk of the vitamin A alcohol of the cells, whereas the supernatant had the

major portion of the ester, which was mostly as the palmitate, with traces of the fed ester.

4. It is suggested that all esters of vitamin A are hydrolysed, whereupon the vitamin A alcohol crosses the cell membrane and is re-esterified, preferentially with palmitic acid, inside the cell.

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Studies on Metabolism of Vitamin A

4. STUDIES ON THE MODE OF ABSORPTION OF VITAMIN A BY RAT INTESTINE *IN VITRO*

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In the preceding paper (Mahadevan, Seshadri Sastry & Ganguly, 1963) experiments with living animals have shown that vitamin A esters are

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hydrolysed and resynthesized during absorption. In this paper, where experiments conducted *in vitro* with everted intestinal sacs of rats are described, further evidence is produced to show that prior hydrolysis of vitamin A esters is essential for the