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 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

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absorption and that only vitamin A alcohol crosses the mucosal-cell membrane to be re-esterified inside the cell. Evidence is also presented here to indicate that the hydrolytic enzyme might be situated on the outer surface of the mucosal-cell membrane, whereas the esterifying enzyme is inside the cell.

MATERIALS AND METHODS

Materials. The sources of the materials used are given in our earlier publications (Ganguly, Krishnamurthy & Mahadevan, 1959; Mahadevan, Murthy, Krishnamurthy & Ganguly, 1961; Mahadevan & Ganguly, 1961).

Methods. Normal male rats of this Institute strain, weighing 120–140 g., were used without prior starving; they were killed by a blow on the head and the intestines were quickly removed and washed twice with 50 ml. of cold physiological saline. From each rat, a segment weighing 1·5 g. (about 20 cm. in length) was cut from the point where the common bile duct enters the intestine. The intestines were everted with thin polyethylene tubing used as a probe and were made into sacs as described by Wilson & Wiseman (1954).

The vitamin A dispersions, prepared according to Seshadri Sastry, Krishnamurthy & Ganguly (1957), usually contained 3 mg. of vitamin A alcohol or an equivalent quantity of the ester and 30 mg. of Tween 20/ml.

The incubation medium, unless otherwise stated, consisted of 14 ml. of calcium-free Krebs–Ringer phosphate solution (Umbreit, Burris & Stauffer, 1947) plus 1 ml. of vitamin A dispersion; the serosal medium was 2·5 ml. of Krebs–Ringer phosphate solution containing 5 mg. of Tween 20. Incubations were carried out in Warburg flasks at 37° with air as the gas phase; the flasks were shaken at 60 cyc./min. for 1 hr. The sacs were then washed with 100 ml. of cold physiological saline directed in a gentle jet from a syringe.

After incubation, the mucosa was scraped off and homogenized and fractionated into particulate and supernatant fractions as described by Mahadevan *et al.* (1963). The procedures adopted for extraction of the medium and the tissue fractions, and for analysis of vitamin A alcohol and its different esters, were those followed in previous investigations (Mahadevan, Krishnamurthy & Ganguly, 1959; Mahadevan & Ganguly, 1961). We have consistently observed that after incubation only traces of vitamin A can be found in the serosal solution. As the aim of this work was to study the events taking place in the mucosal cell we have neglected the serosal solution.

RESULTS

Incubation of the intestinal sacs with vitamin A alcohol or its esters showed that, whereas there was no significant esterification of the vitamin A alcohol, all the esters were hydrolysed to an appreciable extent (Table 1). Since vitamin A is absorbed and stored in the rat as the palmitate (Mahadevan & Ganguly, 1961), the possibility that the palmitate might be absorbed without prior hydrolysis could not be ruled out. But it is most significant in this connexion that, whereas vitamin A acetate was hydrolysed most rapidly, all the higher esters, including the palmitate, were hydrolysed to about the same extent.

Regardless of whether the incubation medium contained the free alcohol, the acetate or the higher esters, the vitamin A alcohol represented the bulk of the total vitamin A content of the particulate fractions. The vitamin A alcohol content of the tissue was greater when the free alcohol or the acetate was in the medium.

Examination of the relative amounts of the free alcohol in the particulate and supernatant fractions shows that the ratio of its distribution was about 10–15:1. The vitamin A alcohol content of the particulate fraction, after incubation with the palmitate, was also essentially similar to that obtained with the other higher esters of the vitamin, suggesting that vitamin A palmitate is treated by the intestine in the same way as the other higher esters.

In contrast, in all cases the vitamin A ester of the mucosa was recovered, after centrifuging, predominantly in the soluble fraction. It should be emphasized here that, irrespective of the nature of the higher ester introduced in the medium, the

Table 1. *Uptake of vitamin A alcohol and its esters by everted intestinal sacs*

Conditions were as described in the Materials and Methods section. Each figure represents the mean of three separate experiments. Values are expressed as μg. of vitamin A alcohol present in the free or esterified form, and are per whole incubation medium (15 ml.) and for whole of the particulate or supernatant fractions of the mucosal-cell homogenate from each sac. ME, Ester in the incubation medium; P, vitamin A palmitate; Al, vitamin A alcohol.

Form of vitamin A in the medium	Incubation-medium vitamin A (μg.)			Intestinal-mucosa vitamin A (μg.)					
				Particulate fraction			Supernatant fraction		
	ME	P	Al	ME	P	Al	ME	P	Al
Alcohol	—	10	2200	—	38	200	—	85	23
Acetate	1120	16	630	27	26	225	1·5	55	18
Laurate	3000	9	293	10	4	57	6·0	69	3
Stearate	3000	7	245	6	6	66	7·5	46	5
Linoleate	3100	4	277	5	6	45	7·0	52	4
Palmitate	—	2900	255	—	8	62	—	50	6

palmitate was the major component (about 80% of the total esters) in the soluble fraction. In all the trials the soluble fraction contained comparable quantities of the palmitate even when the palmitate itself was in the medium. This supports the argument that the mechanism of absorption of vitamin A palmitate is the same as that of the other higher esters.

Vitamin A acetate could be found both in the particles and in the supernatant fraction only when the acetate was present in the incubation medium. In all other instances we have consistently failed to detect any trace of it and in this do not agree with Loran & Althausen (1959).

Rate of uptake of vitamin A alcohol and stearate

Table 2 gives the results of experiments in which the rate of uptake of vitamin A alcohol or the stearate was studied. The results give a picture similar to those presented in Table 1. The bulk of the vitamin A alcohol was always in the particles, whereas the ester, which was mostly the palmitate, was concentrated in the soluble fraction, regardless of whether the alcohol or the stearate was used. With the alcohol, again there was practically no palmitate or any other ester in the medium. Here also the particles took up the alcohol. The amount increased from 20 to 190 μg. within the interval of 15–60 min. of incubation. The vitamin A palmitate content of the particles increased only from 5 to 42 μg. within the same period of time. At each time studied, the quantity of palmitate in the soluble fraction was greater than the amount of free vitamin A, and the quantity of palmitate was always greater than that present in the corresponding particulate fraction.

When vitamin A stearate was added (Table 2) initially, vitamin A alcohol was liberated progressively into the medium, but virtually no palmitate appeared. Again the particles contained relatively more alcohol and the soluble fraction more palmitate. Stearate was present only in small amounts that showed little change with time.

Effect of activators and inhibitors on the uptake of vitamin A alcohol and esters

The above experiments, together with those conducted with the living animal (Mahadevan *et al.* 1963), have shown that the process of absorption of vitamin A esters consists of their hydrolysis followed by resynthesis. The mucosal cells contain enzymes that carry out both the reactions and several compounds have shown preferential activating or inhibitory effects on these enzymes (Mahadevan *et al.* 1961). Experiments were therefore designed where such activators or inhibitors were used with a view to obtaining more precise information about the role played by these enzymes in the absorption process.

Taurocholate. Sodium taurocholate activates the hydrolysis but inhibits the synthesis of vitamin A esters by homogenates, as well as by soluble enzymes prepared from the intestinal mucosa of rats (Mahadevan *et al.* 1961). Table 3 shows that inclusion of the taurocholate invariably increased the hydrolysis of both the acetate and the stearate, with the result that more vitamin A alcohol appeared in the medium, as well as in the particles. Contrary to expectation, the amount of vitamin A palmitate increased in quantity in both soluble and particulate fractions when taurocholate was present.

Tween 20. Tween 20 inhibits the synthesis, but not the hydrolysis, of vitamin A esters by the soluble mucosal enzyme (Mahadevan *et al.* 1961). It is shown in Table 4 that an increase of the con-

Table 2. *Uptake of vitamin A alcohol or stearate by everted intestinal sacs with increasing time of incubation*

Incubations were carried out as described in the Materials and Methods section. For each time-interval two intestinal sacs were used and the results are the averages of the pair. Values are for the whole incubation medium (15 ml.) and for the particulate or supernatant fractions of the mucosal-cell homogenate obtained from each sac. Values are expressed as μg. of vitamin A alcohol present as the free alcohol or in the esterified form. For definitions of abbreviations, see Table 1.

Form of vitamin A in the medium	Time (min.)	Incubation-medium vitamin A (μg.)			Intestinal-mucosa vitamin A (μg.)					
					Particulate fraction			Supernatant fraction		
		ME	P	Al	ME	P	Al	ME	P	Al
Alcohol	15	—	0	2500	—	5	20	—	9	3
	30	—	1	2400	—	13	63	—	31	8
	45	—	3·5	2500	—	24	85	—	44	14
	60	—	6·5	2100	—	42	190	—	78	27
Stearate	15	2800	0	80	10	2	30	2	15	3
	30	2800	0	140	5	3	47	4	29	5
	45	2700	2	180	6	4	60	4	37	6
	60	2400	5	225	6	6	67	4	48	7

centration of the Tween from 2 to 20 mg./ml. led to slightly increased hydrolysis of the vitamin A esters in the medium. It is more significant that, instead of any inhibition, there was actually an increased synthesis of vitamin A palmitate.

Tetraethyl pyrophosphate and di-isopropyl fluorophosphate. According to our experience (Mahadevan *et al.* 1961), tetraethyl pyrophosphate, even at low concentrations, inhibits the hydrolysis of vitamin A acetate by the mucosal enzyme, whereas di-isopropyl fluorophosphate inhibits that of both the acetate and the higher ester, but neither agent has any effect on the esterifying enzyme. Table 5 shows that, when vitamin A alcohol was used, the presence or absence of tetraethyl pyrophosphate had no effect on the alcohol or palmitate contents of the particulate and supernatant fractions, whereas, in contrast, it inhibited the hydrolysis of the acetate of the medium with a concomitant lowering of the alcohol and palmitate concentrations in both cell fractions. The presence or absence of tetraethyl pyrophosphate had no effect on the uptake of the stearate, but di-isopropyl fluorophosphate had the same effect on the stearate as tetraethyl pyrophosphate on the acetate. It is striking that, whenever the hydrolysis of an ester was inhibited, it appeared in considerable quantities in the particulate materials in the unhydrolysed state. There were also small amounts of the unhydrolysed ester in the supernatant fraction. It is possible that it actually originated from the particulate materials and was mechanically removed into the supernatant fraction during the process of homogenization and centrifuging.

Table 3. *Effect of sodium taurocholate on the uptake of vitamin A alcohol, acetate or stearate by everted intestinal sacs*

Each experiment represents the average of at least two determinations. Conditions were as described in the Materials and Methods section, except that, when the sodium taurocholate was used, 1 ml. of an aqueous solution containing 100 mg. of the bile salt was added at the expense of the Ringer phosphate buffer. Values are expressed as in Table 1, in which the abbreviations are defined.

Form of vitamin A in the medium	Incubation-medium vitamin A (μg.)		Intestinal-mucosa vitamin A (μg.)					
			Particulate fraction			Supernatant fraction		
	ME	Al	ME	P	Al	ME	P	Al
Vitamin A alcohol								
(*a*) Without taurocholate	30	2400	0	43	232	0	83	28
(*b*) With taurocholate	10	2500	0	49	330	0	95	58
Vitamin A acetate								
(*a*) Without taurocholate	2400	630	25	27	296	10	75	16
(*b*) With taurocholate	1900	1120	37	32	343	3	90	27
Vitamin A stearate								
(*a*) Without taurocholate	2800	231	4	8	54	6	42	5
(*b*) With taurocholate	2000	995	16	20	120	12	50	9

Table 4. *Effect of Tween* 20 *on the uptake of vitamin A alcohol, acetate or stearate by the everted intestinal sac*

Conditions were as described in Table 3. The 2 mg. of Tween/ml. represents the amount of detergent usually present in the whole medium as used for preparing the vitamin A dispersion. When the extra Tween was used, 1 ml. of an aqueous solution containing 270 mg. of Tween 20 was added at the expense of the buffer. Other conditions and procedures were the same as described in previous Tables. Values are expressed as in Table 1, in which the abbreviations are defined.

Form of vitamin A in the medium	Incubation-medium vitamin A (μg.)			Intestinal-mucosa vitamin A (μg.)					
				Particulate fraction			Supernatant fraction		
	ME	P	Al	ME	P	Al	ME	P	Al
Vitamin A alcohol									
(*a*) 2 mg. of Tween/ml.	4	37	2500	0	45	240	0	83	32
(*b*) 20 mg. of Tween/ml.	2	14	2600	0	52	290	0	97	53
Vitamin A acetate									
(*a*) 2 mg. of Tween/ml.	2400	10	675	28	24	258	6	75	22
(*b*) 20 mg. of Tween/ml.	2300	6	740	32	32	290	7	87	30
Vitamin A stearate									
(*a*) 2 mg. of Tween/ml.	2800	7	205	4	8	57	3	48	7
(*b*) 20 mg. of Tween/ml.	2800	3	257	5	10	63	3	62	12

Table 5. *Effect of tetraethyl pyrophosphate and di-isopropyl fluorophosphate on the uptake of vitamin A alcohol, acetate or stearate by everted intestinal sacs*

Conditions for incubation in the absence of the inhibitors were as described in the Materials and Methods section. In the other cases the everted sac was preincubated, without shaking, at 37° for 15 min. in 13 ml. of Ringer phosphate solution and 1 ml. of a dispersion of the inhibitor in water (effected by a small quantity of ethanol). Then 1 ml. of the vitamin A dispersion was added and the incubation was continued for 60 min. Values are averages of three separate experiments, where at least two sacs were used in each. Values are expressed as in Table 1, in which the abbreviations are defined. The final concentration of inhibitor in all cases was 0·1 mM. TEPP, Tetraethyl pyrophosphate; DFP, di-isopropyl fluorophosphate.

Form of vitamin A in the medium	Incubation-medium vitamin A (μg.)		Intestinal-mucosa vitamin A (μg.)					
			Particulate fraction			Supernatant fraction		
	ME	Al	ME	P	Al	ME	P	Al
Vitamin A alcohol								
(*a*) Without TEPP	32	3300	0	40	214	0	78	37
(*b*) With TEPP	20	3300	0	38	222	0	66	39
Vitamin A acetate								
(*a*) Without TEPP	2300	785	28	41	256	13	94	41
(*b*) With TEPP	2100	30	150	2	18	53	10	13
Vitamin A stearate								
(*a*) Without TEPP	2800	253	0	9	63	7	48	4
(*b*) With TEPP	3000	240	10	8	58	6	53	7
(*c*) Without DFP	2900	260	3	10	65	7	57	7
(*d*) With DFP	3100	25	93	2	6	13	6	2

DISCUSSION

In experiments with intact animals it is rather difficult to evaluate the exact role of the enzymes of the intestinal mucosa in the absorption. The everted intestinal sac lends itself to a more controlled investigation of this problem at the mucosal-cell level. This technique has been used successfully to study the mechanism of absorption of sugars (Crane, 1960), amino acids (Spencer, Bow & Markulis, 1962), lipids (Johnston, 1959; Smith & Treadwell, 1958) and vitamins (Cooper & Castle, 1960).

After feeding rats with vitamin A alcohol, some vitamin A ester was always found in the lumen of the small intestine (Mahadevan & Ganguly, 1961). In the present experiments *in vitro* the esters were almost completely absent from the incubation medium when the free alcohol was used. The enzyme of the intestinal contents therefore must carry out the esterification of vitamin A alcohol in the intestinal lumen of the living animal, and this enzyme has been removed by the washing procedure, as might be expected from the work of Pelot & Grossman (1962). The enzymic reactions (esterification and hydrolysis) observed with the washed everted intestinal sac should therefore be due to the mucosal enzymes.

In the intact rat, regardless of the carrier used or the form given, vitamin A is ultimately absorbed and stored in the liver as the palmitate (Mahadevan & Ganguly, 1961). Fractionation of the homogenate of the intestinal-mucosal cells of such rats has shown that the particulate materials contain the bulk of the vitamin A alcohol, whereas the supernatant fraction, which presumably represents the soluble contents of the cell, had the major portion of the ester, mostly as the palmitate (Mahadevan *et al.* 1963). In the present experiments *in vitro* also, irrespective of the form of vitamin A used in the incubation medium, the particulate materials of the mucosal cells had the bulk of the alcohol, whereas the supernatant fraction contained most of the ester, which was again the palmitate. When incubated with the alcohol in the medium the particulate materials contained significant quantities of the palmitate also, which increased progressively with time (Table 2). We have already discussed and suggested that in the living animal vitamin A alcohol crosses the mucosal-cell membrane and is re-esterified inside the cell on the particulate materials, preferably as the palmitate, which in turn is released into the cytoplasm (Mahadevan *et al.* 1963). A similar mechanism seems to be operative in the experiments *in vitro* also. Thus, although the everted sac system is an artificial one, it seems to simulate closely the behaviour in the living animal, thereby supporting the views of Hogben (1960) and Smyth (1961) that the everted-sac technique is not altogether unphysiological. However, the soluble enzyme preparation from the mucosal cells displays no particular preference for palmitic acid for the esterification of vitamin A (Murthy, Mahadevan, Seshadri Sastry & Ganguly, 1961). One explanation for the preponderance of the palmitate in the mucosal cells, even in the system

where this fatty acid was not included, may be that the palmitic acid is preferentially donated by some endogenous lipids.

The rapid appearance of vitamin A palmitate in the mucosal cells, but not in the medium, after incubation of the sac with the vitamin A alcohol, strongly suggests that the re-esterification takes place inside the cell. The inability of Tween 20 and sodium taurocholate, which have been shown to inhibit the solubilized esterifying enzyme of the same tissue (Mahadevan *et al.* 1961), to inhibit re-esterification in the sac would provide additional evidence for the intracellular esterification. In contrast, the everted sac rapidly hydrolysed the medium ester, and its hydrolytic enzyme was readily inhibited by the organophosphorus compounds. This marked difference in the response of the hydrolytic and esterifying enzymes to the respective inhibitors indicates that they are probably differently located in the intact cell. However, the easy access of vitamin A ester and the inhibitor to the hydrolytic enzyme would suggest that it is in more intimate contact with the medium, and therefore probably situated on the outer surface of the cell membrane. Nevertheless, the hydrolytic enzyme may be of little significance in the absorption of vitamin A in the living animal, because the luminal enzyme, being in a soluble state and of markedly higher specific activity (Mahadevan *et al.* 1961), would probably hydrolyse all of the dietary vitamin A esters.

SUMMARY

1. After incubation of everted intestinal sacs prepared from normal male rats, in media containing aqueous dispersions of vitamin A alcohol or its various esters, the particulate and supernatant fractions of the mucosal-cell homogenates were analysed.

2. Although there was no significant esterification of vitamin A alcohol, considerable hydrolysis of all the esters occurred in the medium.

3. In all cases the particulate materials contained the bulk of the vitamin A as alcohol, whereas the ester predominated in the supernatant, where it was mostly in the form of vitamin A palmitate.

4. The effect of inhibitors and activators of the enzymes hydrolysing and synthesizing vitamin A esters has been studied. The results are discussed in relation to the possible situation of the enzymes in the cell.

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