Studies on Metabolism of Vitamin A

ABSORPTION OF RETINAL (VITAMIN A ALDEHYDE) IN RATS

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1. Young rats with low reserves of vitamin A were dosed with retinal in groundnut oil, and the stomach, the contents, mucosa and muscles of small intestine, the blood and the liver were analysed at periods up to 24 hr. after dosing. 2. Up to 6 hr. after the dose, retinal was present in high concentrations in the contents, mucosa and muscles of the intestine. Small but significant amounts were present in blood and liver at all times. 3. The intestinal mucosa and muscles always contained small amounts of retinol and its esters. 4. A study of the distribution of the three forms of the vitamin within the mucosal cell showed that most of the mucosal retinal enters the cell unchanged. 5. When protein-depleted rats were similarly given retinal, the rate of reduction of the aldehyde, and the consequent deposition in the liver of retinol and its esters, progressively decreased with reduced protein intake.

It has been widely accepted that retinal is one of the intermediates in the conversion of β -carotene into retinol. However, even after massive doses of β -carotene no significant amounts of retinal have yet been isolated from the contents or wall of the small intestine of rats, but considerable quantities of retinol and its esters have been detected in the intestinal wall. This had led to the general belief that the reduction of the aldehyde in the intestine is a rapid process and that it takes place in the intestinal wall.

A survey of the existing literature on the fate of retinal in the gastrointestinal tract of rats revealed that the most noteworthy work in this regard was reported by Glover, Goodwin & Morton (1948), who detected the alcohol and the esters, but not the aldehyde, in the intestinal wall of rats, 4–24 hr. after an oral dose of the aldehyde. This observation also appeared to strengthen the view that the reduction of retinal to the alcohol in the intestine is an extremely rapid process.

However, these workers analysed the whole wall of the small intestine, and thus gained no information about the different phases of absorption, as might be obtained from a separate analysis of the mucosa and muscles of the small intestine. By separating the mucosa and muscles of the small intestine and fractionating the mucosal cells, we have, in recent years, investigated, with rats, the

mechanism of absorption of retinol and its esters (Mahadevan, Seshadri Sastry & Ganguly, 1963*a,b*) and also of cholesterol (Ganguly, Krishnamurthy & Mahadevan, 1959; Murthy, David & Ganguly, 1963). Utilizing these techniques we have re-investigated the absorption of retinal in rats and now show that during the early intervals after a dose of the aldehyde the bulk of it can be recovered unchanged from the mucosal cells, whereas only relatively minor amounts of the alcohol and esters can be found.

MATERIALS AND METHODS

Sources of materials. Retinal was prepared according to Ball, Goodwin & Morton (1948). The sources of the other materials have been described by Mahadevan, Murthy & Ganguly (1962) and Deshmukh, Malathi & Ganguly (1964).

Animals. Male rats were used as described by Mahadevan, Deshmukh & Ganguly (1964); they had very low initial liver vitamin A reserves when used for the experiments.

Dosing and subsequent analysis. Retinal was stored at -20° as a solution in light petroleum ($40-60^\circ$) and was purified before use by chromatography on deactivated alumina columns by the method of Olson (1961). After deprivation of food for 24 hr., rats were dosed with 5 mg. of retinal dissolved in 0.2 ml. of groundnut oil, and killed at given time-intervals by heart puncture while under light ether anaesthesia. Blood, intestine and liver were immediately transferred into beakers chilled in crushed ice. Further treatment of the intestine for the separation of contents, mucosa and muscles, and fractionation of the mucosal cells, was according to Murthy *et al.* (1963). Carotenoids were extracted by the procedure of Ganguly *et al.* (1959). Retinal,

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retinol and its esters were separated chromatographically by a modification of the method of Olson (1961). The retinyl esters were eluted by 100 ml. of light petroleum, which was followed by 40–50 ml. of 1% (v/v) acetone in light petroleum to remove the aldehyde, and 40 ml. of 6% (v/v) acetone in light petroleum to remove the alcohol. The three retinol derivatives were estimated as described by Mahadevan *et al.* (1962).

RESULTS

It is shown in Table 1 that within $\frac{1}{2}$ hr. of dosing rats with retinal it appeared in large amounts in the contents of the small intestine. It was present in considerable quantities for up to 6 hr., but after 12 and 24 hr. intervals only traces could be found. Almost simultaneously with its appearance in the contents, appreciable amounts of the aldehyde could be found in the mucosa, where its concentration was significant so long as that in the intestinal contents remained high. Some unchanged aldehyde was present in the intestinal muscles, blood and

liver at all time-intervals. While its concentrations in the mucosa and muscles seemed to depend on its amount in the intestinal contents, those in blood tended to show a slight peak of absorption at 3 hr., and those in the liver reached a maximum after 3 hr. which was maintained till 24 hr. The alcohol and its esters also appeared in the mucosa within $\frac{1}{2}$ hr. of the dose. They were still present in considerable quantities at 6 hr., at which time significant amounts of the aldehyde could also be detected in this tissue. As expected, the alcohol and its esters appeared in both intestinal muscles and blood even at $\frac{1}{2}$ hr. Both the ester and the alcohol could be found in the liver within $\frac{1}{2}$ hr. of the dose and the ester continued to be deposited there, so that after 24 hr. a total of 975 μ g. of the ester and alcohol was present.

Our previous work with retinol and its esters showed that the ester must be hydrolysed before it can enter the mucosal cell (Mahadevan *et al.* 1963a,b). Similar attempts have been made here to

Table 1. *Distribution of retinal, retinol and its esters in the contents, mucosa and muscles of the small intestine and in blood and liver of rats after a dose of retinal*

Rats with low vitamin A reserves were given 5 mg. of retinal in 0.2 ml. of groundnut oil, and the distribution of retinal, retinol and its esters was determined in the various tissues at the given time-intervals, as described in the Materials and Methods section. All values are expressed as μ g./whole tissue, except for intestinal contents and blood, where they are expressed as μ g./whole contents and μ g./100 ml. of blood. Values are averages of four. (The 0 hr. values were those given for the rats receiving 20% protein diet in Table 3.) Average liver weight was 5.6 g.

Time-interval (hr.)		Intestine				Blood	Liver
		Stomach	Contents	Mucosa	Muscles		
$\frac{1}{2}$	Retinyl ester	—	5.0	21.5	8.0	25.0	45.0
	Retinol	—	3.0	10.0	1.5	22.0	9.0
	Retinal	2625	270	147	17.7	11.0	1.5
1	Retinyl ester	—	5.0	42.5	10.0	37.0	105
	Retinol	—	4.0	6.2	2.5	27.0	4.0
	Retinal	2563	345	179	17.0	21.0	3.0
2	Retinyl ester	—	2.5	49.5	15.0	48.0	195
	Retinol	—	2.5	7.0	1.0	25.0	6.0
	Retinal	2075	257	182	18.0	25.0	4.0
3	Retinyl ester	—	4.0	40.0	20.0	51.0	250
	Retinol	—	2.5	6.5	1.5	28.0	14.0
	Retinal	1495	235	126	10.0	26.0	5.0
4	Retinyl ester	—	3.0	24.0	27.5	37.5	618
	Retinol	—	3.0	6.8	4.0	30.5	15.0
	Retinal	1291	226	112	17.5	19.0	4.0
6	Retinyl ester	—	2.0	18.0	20.0	35.0	711
	Retinol	—	2.5	6.0	1.0	31.0	37.0
	Retinal	1193	188	48.0	12.0	15.0	2.2
12	Retinyl ester	—	2.0	5.7	55.0	31.0	893
	Retinol	—	2.5	2.2	2.5	33.0	15.0
	Retinal	17.0	8.0	3.5	0.75	16.0	4.6
24	Retinyl ester	—	1.5	2.5	50.0	31.0	950
	Retinol	—	1.3	2.0	2.5	42.0	25.0
	Retinal	3.0	2.1	2.6	0.3	9.0	5.0

Table 2. *Intracellular distribution of retinal, retinol and its esters in the intestinal mucosa of rats fed with retinal*

The mode of dosing, fractionation of the mucosa and estimation of the different forms of vitamin A are as described in the Materials and Methods section. All values are expressed as $\mu\text{g./total cell fraction}$. Values are averages of four.

Time-interval (hr.)		Fractions		
		Nuclear-mitochondrial	Microsomal	Supernatant
2	Retinyl ester	7.5	5.0	35.0
	Retinol	2.5	2.5	2.0
	Retinal	72.0	43.0	67.0
3	Retinyl ester	5.0	5.0	30.0
	Retinol	2.0	2.5	2.0
	Retinal	53.0	35.0	37.0
4	Retinyl ester	2.5	1.5	20.0
	Retinol	2.8	2.0	2.0
	Retinal	45.0	33.0	33.0

determine whether the aldehyde must also be reduced to the alcohol before it can enter the cell. For this purpose, the intracellular distribution of the alcohol, the esters and the aldehyde were studied from $\frac{1}{2}$ hr. to 24 hr. after a dose of the aldehyde. From the representative values given in Table 2 it will be seen that the aldehyde was diffusely distributed among the nuclear-mitochondrial, microsomal and supernatant fractions between the second and fourth hours. A similar trend was apparent at all other time-intervals. The supernatant fraction had relatively more of the ester, whereas the alcohol was more concentrated in the particulate materials.

Effect of protein depletion on the absorption of retinal

Rats were raised on diets containing 5, 10 and 20% of casein as described by Deshmukh *et al.* (1964) and were dosed with 5 mg. of retinal, after which the three forms of the vitamin were analysed in the various tissues. It is shown in Table 3 that in the rats belonging to all the three groups large amounts of the aldehyde appeared in the contents of the small intestine within 1 hr. of the dose and appreciable quantities of it persisted there till 6 hr. While there was no significant difference in the alcohol and the ester values of the intestinal contents of the rats receiving the three diets, at each time-interval the aldehyde concentration decreased with the increase in the protein content of the diet and this decrease was very apparent in the mucosa and muscles of the small intestine also. However, the concentrations of retinol and its esters showed a progressive increase. Increased protein intake also led to the appearance of more retinol (free and esterified) in the blood at all time-intervals. The aldehyde, however, did not display any uniform pattern. More significant was the picture in liver:

at all time-intervals not only were there increased amounts of the alcohol and its esters with increased protein intake, but at 24 hr. the livers showed marked differences in their content of the vitamin according to the diet. Here again, the aldehyde did not show any uniform pattern with respect to the time of administration or to the protein intake.

Identification of retinal in the tissues

With a spectrophotometric method Glover *et al.* (1948) could detect only the reduced form of retinal, and not the unchanged aldehyde, in the intestinal wall of rats 4–24 hr. after oral administration of this compound. But, as shown here, the concentration of the aldehyde was considerable in both mucosa and muscles of the small intestine up to 6 hr. after the dose, and it could also be detected in blood and liver. It therefore appeared essential to establish the identity of the aldehyde. Three methods were used for this purpose.

Reverse-phase chromatography on silicone-impregnated paper. The technique was essentially that of Mahadevan & Ganguly (1961). The samples were spotted on silicone-impregnated Whatman no. 3MM filter paper and the chromatogram was developed for 12–14 hr. in methanol-toluene-ammonia (sp.gr. 0.88) (8:1:1, by vol.), after which the retinol derivatives were identified as described by Mahadevan *et al.* (1962). In this system the R_f values for retinol and retinaldehyde are 0.6 and 0.4 respectively.

The retinal fractions of the tissues, as obtained after adsorption chromatography on alumina columns, were subjected to such chromatographic procedures, along with authentic samples of the aldehyde. The retinal of the tissue extracts moved at the same rate as the standard sample and on

Table 3. *Effect of dietary protein content on the tissue distribution of the three forms of vitamin A after a dose of retinal*

Experimental details are as described in the text. Values are expressed as in Table 1. Average liver weights were 3.4, 4.6 and 5.6g. in rats fed with 5, 10 and 20% of protein respectively.

Time-interval (hr.)		Intestine																		Blood						Liver		
		Contents						Mucosa						Muscles						5			10			20		
		Dietary protein (%).....	5	10	20	5	10	20	5	10	20	5	10	20	5	10	20	5	10	20	5	10	20	5	10	20		
0	Retinyl ester	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	8.5	6.0	3.5		
	Retinol	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	23.5*	30.5*	31.6*	—	—	—	1.5	1.5	1.5		
	Retinal	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—		
1	Retinyl ester	3.2	2.5	5.5	5.5	5.6	11.6	31.5	3.5	6.2	12.5	30.5	31.0	39.5	42.0	58.0	94.0	30.5	31.0	39.5	42.0	58.0	94.0	42.0	58.0	94.0		
	Retinol	2.5	3.2	4.2	4.2	7.5	8.5	9.2	2.5	3.4	5.3	16.0	16.9	21.0	3.5	4.5	7.5	16.0	16.9	21.0	3.5	4.5	7.5	3.5	4.5	7.5		
	Retinal	350	342	320	320	210	189	181	30.6	25.5	21.4	24.0	17.5	19.1	3.5	3.5	3.0	24.0	17.5	19.1	3.5	3.5	3.0	3.5	3.5	3.0		
2	Retinyl ester	2.5	2.9	3.1	3.1	11.5	21.5	35.0	5.3	11.0	16.0	34.5	39.5	45.6	62.5	85.0	161	34.5	39.5	45.6	62.5	85.0	161	62.5	85.0	161		
	Retinol	1.2	3.1	4.5	4.5	2.5	9.4	12.5	2.5	4.0	7.5	16.9	18.0	21.5	11.25	25.0	25.0	16.9	18.0	21.5	11.25	25.0	25.0	11.25	25.0	25.0		
	Retinal	293	281	279	279	211	128	116	20.0	18.0	14.0	25.0	21.5	21.9	4.0	3.0	2.5	25.0	21.5	21.9	4.0	3.0	2.5	4.0	3.0	2.5		
3	Retinyl ester	3.5	4.2	4.5	4.5	15.5	24.6	40.3	8.5	12.0	25.0	41.5	45.5	56.4	97.5	145	321	41.5	45.5	56.4	97.5	145	321	97.5	145	321		
	Retinol	1.0	1.5	2.2	2.2	3.4	6.5	8.5	2.5	3.8	8.8	17.3	18.8	21.4	27.5	25.0	29.0	17.3	18.8	21.4	27.5	25.0	29.0	27.5	25.0	29.0		
	Retinal	265	253	242	242	135	121	111	17.5	12.6	7.2	31.5	26.5	27.5	7.2	6.3	4.0	31.5	26.5	27.5	7.2	6.3	4.0	7.2	6.3	4.0		
4	Retinyl ester	1.2	1.5	1.8	1.8	10.25	18.0	30.0	11.3	12.5	31.3	21.3	34.3	51.3	154	290	515	21.3	34.3	51.3	154	290	515	154	290	515		
	Retinol	2.5	1.0	3.5	3.5	2.25	2.0	8.25	2.0	2.5	12.0	18.5	21.0	24.0	17.5	40.0	42.0	18.5	21.0	24.0	17.5	40.0	42.0	17.5	40.0	42.0		
	Retinal	288	245	224	224	151	123	103	21.0	16.4	12.0	34.0	25.3	17.3	5.2	4.5	3.5	34.0	25.3	17.3	5.2	4.5	3.5	5.2	4.5	3.5		
6	Retinyl ester	4.5	6.2	5.5	5.5	9.25	12.5	19.0	11.0	33.5	46.5	19.5	31.5	38.0	213	372	631	19.5	31.5	38.0	213	372	631	213	372	631		
	Retinol	2.1	2.2	3.2	3.2	5.2	7.2	9.5	2.0	2.5	2.5	21.2	26.4	29.5	16.2	17.8	28.2	21.2	26.4	29.5	16.2	17.8	28.2	16.2	17.8	28.2		
	Retinal	179	175	171	171	61.5	52.0	48.0	17.0	12.5	11.0	18.5	17.8	15.0	5.2	4.5	4.9	18.5	17.8	15.0	5.2	4.5	4.9	5.2	4.5	4.9		
24	Retinyl ester	1.5	1.5	2.0	2.0	1.0	2.5	4.0	20.0	37.5	44.0	17.5	21.5	31.5	252	411	851	17.5	21.5	31.5	252	411	851	17.5	21.5	31.5		
	Retinol	3.2	3.1	3.9	3.9	2.9	3.0	4.5	3.0	3.5	4.5	30.0	35.4	42.0	8.5	9.8	21.3	30.0	35.4	42.0	8.5	9.8	21.3	8.5	9.8	21.3		
	Retinal	2.1	2.3	0.8	0.8	2.8	2.5	2.0	0.5	0.2	0.2	12.5	10.5	8.0	6.5	2.5	5.5	12.5	10.5	8.0	6.5	2.5	5.5	6.5	2.5	5.5		

* Total vitamin A.

Fig. 1. Absorption curves in ethanol solution of the retinal samples obtained from tissues of rats 4 hr. after the administration of retinal. The purification of the tissue retinal samples is described in the text. $\circ$, Blood; $\square$, liver; $\bullet$, intestinal muscles; $\blacktriangle$, intestinal mucosa; $\triangle$, intestinal contents; $\odot$, authentic sample of retinal.

chromatography as a mixture they could not be resolved.

Thin-layer chromatography. John, Lakshmanan, Jungalwala & Cama (1965) have described a thin-layer chromatographic procedure for the separation of retinaldehyde from other retinol derivatives. In this method glass plates are coated with silicic acid-calcium sulphate ($\frac{1}{2}\text{H}_2\text{O}$) (85:15, w/w), and the chromatograms are developed in a solvent system consisting of 6% (v/v) acetone in light petroleum. In such a system the tissue retinal and the authentic samples moved together.

Spectral analysis. After purification by adsorption chromatography the tissue retinal samples were examined in the Beckman model DU spectrophotometer. The intestinal samples gave the typical spectrum of retinal, but those obtained from blood and liver did not show the characteristic peak at $380\text{m}\mu$ in ethanol, which was probably due to the presence of large amounts of impurities. Samples from blood and liver were rechromatographed on alumina columns, after which they were subjected to the thin-layer chromatography. The retinal spots were eluted and again read in the spectrophotometer, when spectra characteristic of the aldehyde were obtained (Fig. 1).

DISCUSSION

Our results have demonstrated that unchanged retinaldehyde is absorbed by rats. Thus between $\frac{1}{2}$ hr. and 6 hr. after oral administration of the aldehyde large amounts of it were found in the small intestine. Six hours after the dose the aldehyde was present in appreciable amounts in the intestinal contents, the mucosa and the muscles. Its presence in considerable quantities in the intestinal muscles establishes that some of it escapes the mucosal cells unchanged, and therefore its reduction in the intestine, during absorption, is not too rapid a process.

The aldehyde was rather evenly distributed among the nuclear-mitochondrial, microsomal and supernatant fractions of mucosal cell homogenates. Since the microsomal fractions probably contain the cell membrane together with the membranes of the endoplasmic reticulum (Palade & Siekewitz, 1956), it seems that some proportion of the aldehyde associated with this fraction would be attached to the endoplasmic reticulum. That the bulk of the retinal does enter the cell unchanged is proved by the presence of large amounts in the nuclear-mitochondrial and supernatant fractions. Our earlier work on the absorption of retinol and its esters (Mahadevan *et al.* 1963*a,b*) showed that only retinol, and not its esters, can enter the mucosal cells and that the ester requires to be hydrolysed before it can be absorbed. However, the present work shows that retinal also can enter the mucosal cell of rat intestine.

We have provided strong evidence that the compound isolated from the tissues, after the oral administration of retinal, was indeed the aldehyde. It is difficult to explain how Glover *et al.* (1948) failed to notice the presence of large amounts of retinal in the intestinal wall of rats after massive doses of the aldehyde.

The reduction of retinal is catalysed by liver alcohol dehydrogenase (Bliss, 1951; Zachman & Olson, 1961; Mahadevan *et al.* 1962); until now the presence of this enzyme has not been unequivocally demonstrated *in vitro* in the mucosal cell of rats (Lutwak-Mann, 1938; Leloir & Munoz, 1938; Lubin & Westerfeld, 1945; Jacobsen, 1952). We failed to obtain any appreciable reduction of retinal *in vitro* with whole homogenates of the mucosal cell or its subcellular fractions. Furthermore, we have been unable to obtain reduction of retinal when tissue preparations were supplemented with either NAD or NADP together with isocitrate, lactate, malate, fumarate or pyruvate (S. K. Murthy, S. Mahadevan, D. S. Deshmukh & J. Ganguly, unpublished work).

In rats the intestinal conversion of β -carotene into retinol is drastically affected by reduced protein intake (Deshmukh & Ganguly, 1964). It is now clear that one of the steps in the conversion of

carotene into retinol, the reduction of retinal to the alcohol, is considerably depressed in protein insufficiency. It is possible that the enzyme responsible for the reduction of the aldehyde suffers a loss in activity during protein depletion; thus similar losses in protein-deficient animals have been observed with xanthine oxidase and aldehyde oxidase of rat liver (Richert & Westerfeld, 1957), cytochrome *c* reductase, choline esterase and D-amino acid oxidase of human liver (Scrimshaw & Behar, 1961), succinate dehydrogenase, glutamate dehydrogenase, arginase and uricase of rat liver (Muramatsu & Ashida, 1962) and retinene oxidase of rat liver and vitamin A esterases of rat intestinal mucosa (Deshmukh *et al.* 1964).

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