Metabolism and Biological Potency of *retro*-Retinyl Acetate in the Rat

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1. *retro*-Retinyl acetate was shown to exert its biological activity by conversion into vitamin A. 2. When administered orally, *retro*-retinyl acetate was hydrolysed to *retro*-retinol in the intestine, isomerized to retinol and esterified before being transported to the liver for storage. 3. Administration of the compound at as high a dose as 4.0 mg./day for 4 days led to the accumulation of both vitamin A and *retro*-vitamin A in the liver. The amount of *retro*-vitamin A in liver gradually decreased until it was almost completely converted into vitamin A in 18 days. 4. Intraperitoneal administration of the compound led to the accumulation of both vitamin A and *retro*-vitamin A in liver and other tissues. No vitamin A was detected in any tissue of rats receiving *retro*-retinyl acetate intraperitoneally after enterectomy. 5. The small intestine is the major site of conversion of *retro*-vitamin A into vitamin A. The conversion could also be demonstrated by everted intestinal sacs. 6. The biological potency of *retro*-retinyl acetate determined by the rat-growth assay was 20.5% that of all-*trans*-retinyl acetate, when given orally.

A number of derivatives of vitamin A having the *retro* structure are present in fish-liver oils and pharmaceuticals (Balasundaram, Bamji, Cama, Sundaresan & Varma, 1958; Varma, Erdody & Murray, 1965a). Anhydrovitamin A ($\lambda_{\max}$ at 350, 370 and 390 m μ) was biologically 0.4% as active as vitamin A in the rat-growth assay (Shantz, Cawley & Embree, 1943) and was converted into rehydrovitamin A in rat and stored in the liver (Shantz, 1950). Similarly, anhydrovitamin A₂ when given to rats was deposited in the liver as rehydrovitamin A₂ (Bamji, Cama & Sundaresan, 1962). Rehydrovitamin A was recently shown to be a mixture of several compounds, some of which possessed one or two hydroxyl groups (Varma, Erdody & Murray, 1965b). Unlike rehydrovitamin A, the chemically synthesized *retro*-retinyl acetate (Beutel, Hinkley & Pollack, 1955), when given orally in high doses, gave rise to vitamin A and *retro*-vitamin A in the livers of vitamin A-deficient rats (Varma & Murray, 1963). Varma *et al.* (1965a) suggested that the major site of conversion of *retro*-vitamin A into vitamin A is the gastrointestinal tract, since there was little or no conversion of the derivative into vitamin A when administered subcutaneously.

The metabolism of *retro*-vitamin A both *in vivo* and *in vitro* and its biological assay, by the rat-growth assay method, are described in the present paper.

MATERIALS AND METHODS

retro-Retinyl acetate was prepared by treating synthetic all-*trans*-retinyl acetate with aqueous HBr as described by Beutel *et al.* (1955). *retro*-Retinol was prepared by alkaline hydrolysis of the acetate by using ethanolic 10% (w/v) KOH. It was chromatographed on 8% (v/w)-water-deactivated neutral alumina (Jungalwala & Cama, 1965); *retro*-retinol was eluted with 8% (v/v) diethyl ether in light petroleum (b.p. 40–60°). *retro*-Retinyl palmitate was prepared from synthetic retinyl palmitate according to the procedure of Beutel *et al.* (1955). The $E_{1\%}^{1\text{cm}}$ values of these *retro*-vitamin A compounds in the ultraviolet region as well as by the Carr–Price colour test agreed with those already reported (Beutel *et al.* 1955; Varma *et al.* 1965a). Though *retro*-vitamin A and rehydrovitamin A had similar ultraviolet spectroscopic properties, they have been shown to be distinctly different from one another by various criteria (Varma *et al.* 1965b).

Oral administration. For bioassay experiments, *retro*-retinyl acetate was freshly prepared every 3 days and administered daily in concentrations of 2.5 and 5.0 $\mu\text{g.}/0.05\text{ ml.}$ of refined deodorized groundnut oil containing 0.5% of α -tocopherol. All-*trans*-retinyl acetate was given in concentrations of 0.5 and 1.0 $\mu\text{g.}/0.05\text{ ml.}$ of diluent oil. For metabolic purposes, the compound was prepared just before use at a concentration of 2.0 mg./0.1 ml. of diluent oil containing 1.0 mg. of α -tocopherol.

Preparation of tissues for experiments in vitro. For experiments with the everted intestinal sacs, normal rats weighing 120–150 g. were starved for 24 hr. and killed by a blow on the head. The middle one-third portion of the

intestine was washed twice with ice-cold 0.9% NaCl, everted as described by Wilson & Wiseman (1954) and filled with 5.0 ml. of the Krebs-Ringer phosphate buffer, pH 7.4. The everted sac was put in 20 ml. of the same buffer containing 5.0 mg. of sodium taurocholate and 500 μ g. of GSH. The compound under study was dissolved in ethanol and dispersed in sodium phosphate buffer by using Tween 20.

For experiments with intestinal homogenates, the whole intestine was homogenized in ice-cold 0.1 M-sodium phosphate buffer, pH 7.2 (10 ml. of buffer being used/g. of tissue). Similarly, vitamin A-deficient rat liver, after thorough washing with ice-cold 0.9% NaCl, was homogenized in ice-cold 0.1 M-sodium phosphate buffer, pH 7.2 (10 ml. of buffer being used/g. of tissue).

Preparation of tissue extracts. The procedure followed for extraction of tissues was essentially the same as described by Glover, Goodwin & Morton (1948).

Separation of retro-retinol and retro-retinyl palmitate from the corresponding vitamin A compounds by thin-layer chromatography (John, Lakshmanan, Jungalwala & Cama, 1965). *retro*-Retinol and retinol were separated on 8% (v/v) acetone in light petroleum (b.p. 40–60°) solvent system in a 25 cm. $\times$ 12 cm. $\times$ 25 cm. chamber (R_F values: *retro*-retinol, 0.29; retinol, 0.24). *retro*-Retinyl palmitate, *retro*-retinyl acetate and retinyl palmitate were separated in the same way with 1% (v/v) acetone in cyclohexane as the solvent system (R_F values: *retro*-retinyl palmitate, 0.61; *retro*-retinyl acetate, 0.37; retinyl palmitate, 0.69). In the above systems, unlike retinol, which moves slower than *retro*-retinol, retinyl palmitate moves faster than *retro*-retinyl palmitate.

Estimations. Vitamin A or *retro*-vitamin A, when present in tissue extracts as a single component, was estimated by the SbCl₃ colour test method by using the $E_{1\text{cm}}^{1\%}$ values 4420 at 620 m μ for vitamin A and 3910 at 575 m μ for *retro*-vitamin A. However, when these two compounds were present together, the extinction of the mixture at 325 m μ was measured and the relative amounts of the two were calculated from the standard graph similar to the one employed by Varma & Murray (1963) showing the composition of vitamin A against E_{325}/E_{347} for mixtures of vitamin A and *retro*-vitamin A of known compositions.

For bioassay and metabolic studies, male albino rats of this Institute strain, made deficient in vitamin A, were used (Lakshmanan, Jungalwala & Cama, 1965).

RESULTS

Oral administration

Four vitamin A-deficient rats were given *retro*-retinyl acetate orally for 7 days (1.0 mg./rat/day). The average weight gain of the rats during this period was 12 g. They were killed 24 hr. after the last dose and the various tissues pooled and analysed. All the tissues, except stomach, showed the presence of vitamin A (Table 1).

The lipid extract from liver showed the characteristic properties of vitamin A. This was further confirmed by saponification of the lipid extract and conversion of the alcohol into anhydro-vitamin A by ethanolic hydrochloric acid as well

Table 1. *Recovery of vitamin A and retro-vitamin A from various tissues of rats given retro-retinyl acetate (1.0 mg./rat/day) for 7 days orally and intraperitoneally*

Tissue	Recovery (% of orally administered dose)		Recovery (% of intra- peritoneally administered dose)	
	<i>retro</i> - Vitamin A	Vitamin A	<i>retro</i> - Vitamin A	Vitamin A
Stomach	0.634	—	—	—
Intestine	—	0.143	0.803	—
Liver	—	2.380	1.050	0.280
Blood	—	0.006	—	0.010
Kidneys	—	0.044	0.260	0.140
Spleen	—	0.120	0.220	0.150
Lungs	—	0.038	0.151	0.140

as by the oxidation of the alcohol to retinal by manganese dioxide.

To investigate the active absorption of *retro*-retinyl acetate and its probable site of conversion into vitamin A, time-distribution studies were carried out with vitamin A-deficient rats. Vitamin A was detected in the liver as early as 1 hr. after administration and was also present in trace amounts in the intestine. A considerable amount of vitamin A was found in the liver up to 24 hr. No *retro*-vitamin A was detected in liver at any time after the administration of the compound (Table 2).

However, Varma *et al.* (1965a) detected small amounts of *retro*-vitamin A in liver of rats that were given 4.0 mg. of *retro*-retinyl acetate/rat/day for 3 days. Hence, to study the metabolic fate of *retro*-vitamin A after reaching the liver, high doses of *retro*-retinyl acetate (4.0 mg./rat/day for 4 days) were administered to vitamin A-deficient rats. The animals were killed at various time-intervals after being starved for the previous 24 hr. The blood and livers were analysed for vitamin A and *retro*-vitamin A. No *retro*-vitamin A was detected in the blood. The lipid extract from each liver was chromatographed on a column (2 cm. $\times$ 25 cm.) of 25 g. of 7%-(v/w)-water-deactivated alumina. The mixture of esters of vitamin A and *retro*-vitamin A was eluted with light petroleum (b.p. 40–60°), whereas that of the alcohols was eluted with 10–15% (v/v) diethyl ether in light petroleum and estimated as described in the Materials and Methods section. The amount of *retro*-vitamin A in liver continuously decreased until it reached a negligible concentration after 18 days. Meanwhile, the amount of vitamin A in the liver was maintained at a fairly constant level throughout this period (Table 3).

The ratio of *retro*-retinol to *retro*-retinyl ester found in liver was comparatively higher than that of retinol to retinyl ester. This may well be because of

Table 2. Conversion of retro-retinyl acetate into vitamin A in the intestine of the rat after oral feeding of 2.0mg. of retro-retinyl acetate

Rat no.	Time between administration and death (hr.)	retro-Vitamin A recovered (μg.)					Vitamin A recovered (μg.)					Total recovery of vitamin A + retro-vitamin A (% of dose)
		From stomach	From intestinal washings	From intestine			Ester	From intestine		From liver		
				Ester	Alcohol	Total		Alcohol	Total			
1	(Control)	—	—	—	—	—	—	—	—	—	—	
2	1	1000.0	43.7	—	—	—	291.4	Trace	—	13.6	67.4	
3	3	304.2	21.1	—	1.3	49.4	50.7	22.7	Trace	13.2	20.6	
4	5	201.2	26.5	—	41.1	79.8	120.9	36.5	Trace	111.9	24.9	
5	12	154.3	93.0	—	—	—	77.7	—	—	107.3	25.6	
6	24	—	—	—	—	—	—	—	—	5.7	13.4	
										262.6		

Table 3. Recovery of retro-vitamin A and vitamin A from liver and blood of rats given oral doses of retro-retinyl acetate (4.0mg./rat/day) for 4 days

Rat no.	Time between administration and death (days)	Blood vitamin A (μg./100ml.)	Liver						Vitamin A in liver as % of total liver storage of vitamin A + <i>retro</i> -vitamin A	Vitamin A + <i>retro</i> -vitamin A % of administered dose
			<i>retro</i> -Vitamin A (μg.)			Vitamin A (μg.)				
			Total	Ester	Alcohol	Total	Ester	Alcohol		
1 (control)	—	0	0	0	0	0	0	0	0	
2 (control)	—	0	0	0	0	0	0	0	0	
3	1	5.4	688.0	569.7	118.3	317.1	287.0	30.1	31.6	6.3
4	1		386.3	323.4	62.9	208.2	174.3	33.9	34.8	3.7
5	3	7.9	641.7	574.2	67.5	334.5	309.2	25.3	34.3	6.1
6	3		326.2	298.5	27.7	284.9	270.0	14.9	46.6	3.8
7	6	5.2	188.8	157.3	31.5	311.5	286.3	25.2	62.3	3.1
8	6		342.4	303.9	38.5	395.3	370.7	24.6	53.6	4.6
9	9	6.3	156.3	145.3	11.0	335.0	315.9	19.1	68.2	3.1
10	9		117.2	98.3	18.9	311.2	287.2	24.0	72.7	2.7
11	12	5.0	81.7	54.4	27.3	390.6	363.9	26.7	82.7	2.9
12	12		72.8	35.2	37.6	331.1	316.5	14.6	81.9	2.5
13	18	5.1	0	0	0	386.0	332.2	53.8	100.0	2.4
14	18		4.0	0	4.0	308.1	258.9	49.2	98.7	1.9

the need of preferential utilization of retinol from liver for metabolic needs of the animal. The *retro*-retinyl ester found in liver seems to be mainly the palmitate, as shown by its identical behaviour with synthetic *retro*-retinyl palmitate on thin-layer and reverse-phase chromatography systems (John *et al.* 1965; Jungalwala & Cama, 1962).

Intraintestinal administration

The experiment was performed as described by Lakshmanan *et al.* (1965). A 2.0mg. sample of *retro*-retinyl acetate was administered to a vitamin A-deficient rat and the animal was killed 3½ hr. later. The lipid extracts from both liver and intestine showed the presence of vitamin A.

Parenteral administration

Four vitamin A-deficient rats were given *retro*-retinyl acetate intraperitoneally (1.0mg./rat/day) for 7 days; the average weight gain of these rats during the period was 12g. They were killed 24hr. after the last dose and both vitamin A and *retro*-vitamin A were detected in most of the tissues (Table 1).

Enterectomy

A vitamin A-deficient rat was kept under light ether anaesthesia and the intestine was removed as described by Lakshmanan *et al.* (1965). Then 2.0mg. of *retro*-retinyl acetate was injected intraperitoneally, and the rat was returned to the cage and killed 8hr. later. The lipid extract from liver contained *retro*-retinyl acetate and *retro*-retinol; however, vitamin A was not detectable. About 4.0% of the administered dose was present in liver. Lungs, spleen and kidneys showed the presence of traces of *retro*-vitamin A. However, no vitamin A was detectable anywhere in the body.

Subcutaneous administration

Two vitamin A-deficient rats were each given 2.0mg. of *retro*-retinyl acetate subcutaneously. The rats were relieved of all the symptoms of vitamin A deficiency within a day or two and gained on an average about 20g. in weight in 3 weeks, after which they were killed. Analyses of the livers and the subcutaneous tissue at the site of injection failed to show the presence of vitamin A or *retro*-vitamin A, which confirms the observations of Varma *et al.* (1965a).

Conversion of retro-vitamin A into vitamin A in vitro

Experiments with everted intestinal sac. The experiment was conducted as described in the

Materials and Methods section. Either 100µg. of *retro*-retinol or *retro*-retinyl acetate was added to the buffer medium containing the everted intestinal sac and kept in a metabolic shaker at 37° for 2hr. The sacs from four reaction flasks were washed with ethanol and the serosal fluid and intestinal wall analysed. The lipid extract from serosal fluid showed the typical spectrum of vitamin A and exhibited the characteristic fluorescence of vitamin A. It gave two spots on thin-layer and reverse-phase chromatography corresponding to retinol and retinyl higher fatty acid ester. Another spot corresponding to unchanged *retro*-retinyl acetate was also observed when *retro*-retinyl acetate was used as the substrate. Both vitamin A and *retro*-vitamin A were present in the lipid extract from the intestinal wall. In general, the extent of conversion of *retro*-vitamin A into vitamin A was very low in all these experiments, namely 4–6% when *retro*-retinol was the substrate and 0.5–1.0% when *retro*-retinyl acetate was the substrate.

Experiments with intestinal homogenate. A sample (100µg.) of *retro*-retinyl acetate was incubated with the rat-intestinal homogenate for 1hr. at 37°. The lipid extract from the reaction mixture showed spots corresponding to both retinol and retinyl higher fatty acid ester in addition to *retro*-retinol and unchanged *retro*-retinyl acetate on thin-layer chromatography; however, they could not be characterized conclusively.

Hydrolysis of retro-retinyl acetate by rat-liver homogenate. A sample (100µg.) of *retro*-retinyl acetate was incubated with rat-liver homogenate for 1hr. at 37°. Analysis of the lipid extract from the reaction flask by thin-layer chromatography showed the presence of only *retro*-retinol.

Biological potency of retro-retinyl acetate. The bioassay was carried out essentially as described by Bliss & György (1951). The growth rates (g./week) over 4 weeks of individual litter mates receiving daily oral doses of 2.5 and 5.0µg. of *retro*-retinyl acetate and 0.5 and 1.0µg. doses of all-*trans*-retinyl acetate were measured. All the negative controls lost considerable weight and most of them died before the end of the assay. The mean weekly gain in weight plotted against the logarithm of dose gave lines for the standard and test doses that were parallel to each other. The biopotency of *retro*-retinyl acetate as compared with that of all-*trans*-retinyl acetate was 20.5%.

DISCUSSION

retro-Vitamin A has been shown to be isomerized to vitamin A in the rat. When administered orally to vitamin A-deficient rats at 1.0mg. dosage (Table 1), it was stored in the liver only in the form of vitamin A and no trace of *retro*-vitamin A was

detectable. At the same time, all the rats were alleviated of their vitamin A-deficiency symptoms and grew well. This implies that unlike retinoic acid or the epoxides of vitamin A, which exert their biological activities as such and not through vitamin A (Van Dorp & Arens, 1946; Lakshmanan *et al.* 1965), *retro*-vitamin A exerts its biological activity after its conversion into vitamin A.

The presence of large amounts of *retro*-vitamin A and vitamin A in the intestine even 12 hr. after administration (Table 2) shows that the absorption of *retro*-vitamin A is a comparatively slower process than those for retinal, 5,6-monoepoxyretinal and 3-dehydroretinal (Glover *et al.* 1948; Lakshmanan *et al.* 1965; John, Lakshmanan & Cama, 1966), and the subsequent isomerization of *retro*-retinol to retinol may also be a slow process.

When *retro*-retinyl acetate is administered intraperitoneally, it is taken up directly by various tissues and then probably undergoes isomerization in the intestine (Table 1). Thus it is easy to explain the presence of both *retro*-vitamin A and vitamin A in liver and other tissues. However, when the compound is administered intraperitoneally after enterectomy, no vitamin A is detected anywhere in the body in spite of the fact that *retro*-vitamin A is taken up by all these tissues; thus the intestine is the main site for the conversion of *retro*-vitamin A into vitamin A.

When *retro*-retinyl acetate is administered by the oral route at a concentration of 4.0 mg./rat/day, it escapes isomerization and can be detected in the liver as the palmitate or similar higher fatty acid ester (Table 3). Consequently, there seems to be a requirement of optimum concentration of *retro*-vitamin A beyond which the intestine loses its efficiency of isomerizing *retro*-retinol to retinol. At the same time, the fact that not even a trace of *retro*-retinyl acetate could be detected in the liver even at such a high dosage of oral administration (Table 3), as well as the presence of high amounts of *retro*-retinol in the intestinal wall (Table 2), indicates that *retro*-retinyl acetate is probably hydrolysed first to its alcohol form before being isomerized to retinol (Table 3). This is further supported by our observation in the everted-sac experiments that *retro*-retinol serves as a better precursor than *retro*-retinyl acetate for the formation of vitamin A. The retinol and the unisomerized *retro*-retinol may then be esterified, transported and stored in the liver, thus accounting for the presence of both retinyl palmitate and *retro*-retinyl palmitate in the liver.

Table 3 shows that *retro*-vitamin A which has reached the liver is slowly and irreversibly converted into vitamin A. Such a conversion is probably brought about by enterohepatic circulation of the compound to the intestine, where it undergoes isomerization to vitamin A. It may well be that,

as the metabolic needs of the animal are drained away from the liver vitamin A, more of the *retro*-vitamin A is converted into vitamin A in the body, thus maintaining the liver retinyl palmitate at a somewhat constant concentration until all the liver *retro*-vitamin A is converted into vitamin A. However, the utilization of vitamin A from the liver is much more than could be accounted for by the metabolic needs of the animal (Table 3).

The biological potency of *retro*-retinyl acetate measured by the vaginal-smear assay method was 12% that of retinol when given orally and 11% by the liver-storage method, whereas when injected subcutaneously the activity was 4% that of retinol (Varma *et al.* 1965a). However, the biological potency of *retro*-retinyl acetate, determined by the rat-growth assay in the present investigations, was comparatively higher than the values obtained by the other methods. In addition, the biological potency of *retro*-retinyl acetate was greater than that observed for rehydrovitamin A by the rat-growth assay (Shantz, 1950).

The mechanism of conversion of *retro*-vitamin A into vitamin A is not known. Varma *et al.* (1965b) have shown that hydroxylation mechanisms are involved in the utilization of anhydrovitamin A by vitamin A-deficient rats. On the basis of our observation that both in absorption as well as in everted-intestinal-sac studies the rate of conversion of *retro*-vitamin A into vitamin A is extremely slow, it is likely that the reaction may involve a series of hydroxylation and dehydration steps, resulting in the ultimate formation of the vitamin A molecule.

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REFERENCES

- Balasundaram, S., Bamji, M. S., Cama, H. R., Sundaresan, P. R. & Varma, T. N. R. (1958). *J. biol. Chem.* **223**, 827.
- Bamji, M. S., Cama, H. R. & Sundaresan, P. R. (1962). *J. biol. Chem.* **237**, 2747.
- Beutel, R. H., Hinkley, D. F. & Pollack, P. I. (1955). *J. Amer. chem. Soc.* **77**, 5166.
- Bliss, C. I. & György, P. (1951). In *Vitamin Methods*, 1st ed., p. 41. Ed. by György, P. New York: Academic Press Inc.
- Glover, J., Goodwin, T. W. & Morton, R. A. (1948). *Biochem. J.* **43**, 109.
- John, K. V., Lakshmanan, M. R. & Cama, H. R. (1966). *Biochem. J.* **99**, 312.
- John, K. V., Lakshmanan, M. R., Jungalwala, F. B. & Cama, H. R. (1965). *J. Chromat.* **18**, 53.
- Jungalwala, F. B. & Cama, H. R. (1962). *J. Chromat.* **8**, 535.
- Jungalwala, F. B. & Cama, H. R. (1965). *Biochem. J.* **95**, 17.

- Lakshmanan, M. R., Jungalwala, F. B. & Cama, H. R. (1965). *Biochem. J.* **95**, 27.
- Shantz, E. M. (1950). *J. biol. Chem.* **182**, 515.
- Shantz, E. M., Cawley, J. D. & Embree, N. D. (1943). *J. Amer. chem. Soc.* **65**, 901.
- Van Dorp, D. A. & Arens, J. F. (1946). *Nature, Lond.*, **158**, 60.
- Varma, T. N. R., Erdody, P. & Murray, T. K. (1965a). *J. Pharm., Lond.*, **17**, 474.
- Varma, T. N. R., Erdody, P. & Murray, T. K. (1965b). *Biochim. biophys. Acta*, **104**, 71.
- Varma, T. N. R. & Murray, T. K. (1963). *Biochim. biophys. Acta*, **78**, 556.
- Wilson, T. H. & Wiseman, G. (1954). *J. Physiol.* **123**, 116.