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Studies in Vitamin A

6. CONVERSION IN VIVO OF VITAMIN A ALDEHYDE (RETINENE₁) TO VITAMIN A₁

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Retinene₁ is a fairly stable protein-free substance first obtained from rhodopsin (visual purple), the photolabile chromoprotein of the dark-adapted retina. It has recently been shown to be the aldehyde of vitamin A₁ (Morton & Goodwin, 1944; Ball, Goodwin & Morton, 1948). Chemical methods for its preparation have been described (Hunter & Hawkins, 1944; van Dorp & Arens, 1947), and a very simple method, in which a solution of vitamin A₁ alcohol in light petroleum is left to stand over powdered pyrolusite in the cold (Ball *et al.* 1948), has made it easily accessible.

The present paper is concerned with the fate of retinene₁ when presented to body tissues other than the retina. In particular, it was desired to find out whether it possessed *per se* vitamin A₁ activity. The substance, in a preparation containing no detectable vitamin A₁ but some 75% of retinene₁, has been administered orally and parenterally to vitamin A₁-depleted rats. It has been found to be easily convertible to vitamin A₁, particularly in the gut wall.

EXPERIMENTAL

Retinene₁

Preparation and storage. Vitamin A₁ aldehyde was prepared by the method of Ball *et al.* (1948). The material used had been purified by chromatography and contained about

75% of the pure substance, but unchanged vitamin A₁ could not be detected spectrophotometrically. It was stored as a solution in light petroleum at 0° in the dark, under which conditions it was quite stable.

Solution in oil. A known quantity of refined ground-nut oil was added to a measured volume of the stock retinene solution in light petroleum. The volatile solvent was removed by warming under reduced pressure. The resulting solution in oil was used for oral administration.

Solution in propylene glycol. A similarly prepared solution in propylene glycol was administered intraperitoneally.

Determination. The amounts of retinene₁ administered were determined by spectroscopic methods, using the absorption maxima at 368 mμ. (cyclohexane solution) and 664 mμ. (SbCl₃ colour test) following Ball *et al.* (1948). Similar methods using the ultraviolet maxima at 326 mμ. and the visible maximum at 617 mμ. (SbCl₃ colour test) were used for vitamin A₁. For the few extracts in which vitamin A₁ and retinene₁ occurred together, the correction procedure of Morton & Stubbs (1946) was applied to the ultraviolet absorption curves so as to determine both constituents.

Animals

Preparation for tests. Twenty-five newly weaned rats were depleted of vitamin A₁. Because of a shortage of rice starch, the diet low in vitamin A₁ which was previously used (Glover, Goodwin & Morton, 1947a) was modified by introducing boiled potatoes in place of rice starch. The cooked potatoes contained very small amounts of provitamin A (mainly as β-carotene). Careful analyses showed that each rat may have received the equivalent of 2-3 i.u./day of provitamin A. The

diet contained enough provitamin to ward off frank deficiency, but too little to delay depletion of the small liver reserves. After 21–28 days, the rats had become almost stationary in weight, and, when the two largest animals were killed, the lipids extracted from livers and intestines gave negative Carr-Price tests for vitamin A₁.

Administration of retinene₁. (a) *Oral.* Retinene₁ (1–8 mg.) in oil was given either by drops from a calibrated syringe into the back of the mouth, or through a fine rubber catheter, internal vol. 0.2 ml., direct into the stomach. For the more quantitative work exactly 1 ml. of oil solution was placed in a small flask, and c. 0.7 ml. was withdrawn into a 1 ml. syringe, which was then at once connected to the rubber catheter which had previously been inserted into the rat's stomach. As much as possible of the oil was expelled from the syringe, and the catheter was then carefully withdrawn while still connected to the syringe. The syringe and catheter were then washed with ether and the rinsings added to the 0.3 ml. in the flask. The retinene in the flask was then determined and the amount given to the animal calculated by difference. The method was convenient for administering c. 0.5 ml. of oily solution. The dose of retinene₁ was varied by adjusting the concentration of solute rather than the volume of solution. Groups of 3–5 animals were used at each dose level, and the individuals were killed at definite intervals of time after dosing.

(b) *Parenteral.* Each rat in two groups of three each was given 1–5 mg. retinene₁ in 0.5 ml. of oil by subcutaneous injection. Two rats were given 4.2 mg. retinene₁ in 0.5 ml. propylene glycol by intraperitoneal injection. All eight animals grew normally and showed no ill effects.

Preparation of tissue extracts

The liver, stomach and small intestine were removed from the anaesthetized animal. The stomach and the small intestine were separately flushed with saline, and the contents with the washings were retained for examination. The tissues (e.g. liver) were ground in a mortar with c. 5 times their weight of anhydrous Na₂SO₄ and a little acid-washed silver sand, and thoroughly extracted with redistilled ether (Glover *et al.* 1947*a*). Retinene₁ is unstable in the presence of concentrated alkali, and saponification has therefore to be avoided. In the extraction of macerated and dried intestinal tissue, addition of a little ethanol promotes filtration through the sintered glass funnel (G4), which is otherwise liable to become clogged.

The contents of the stomach and intestines were transferred to a mortar and triturated with ethanol (10–20 ml.). The mixture was then extracted with ether in a separatory funnel.

The lipid extracts were always freed from ether and taken up in a known volume of chloroform or cyclohexane.

RESULTS

Oral administration

In the animals which received the retinene₁ by mouth, the contents of the stomach and the small intestine often contained unabsorbed and unchanged vitamin A₁ aldehyde but no trace of vitamin A₁. On the other hand, the intestinal wall and the liver con-

tained vitamin A₁ with no trace of retinene₁. These results indicate that the vitamin A₁ aldehyde was reduced to vitamin A₁ alcohol immediately on absorption into the intestinal mucosa. The reduction must have taken place extremely rapidly, as no trace of the retinene was detected in the extract from the intestinal tissue.

Fig. 1. Absorption spectra of lipid extracts (in cyclohexane) of tissues of a rat killed 24 hr. after dosing with 8.0 mg. retinene₁ in 1.0 ml. oil. — Retinene₁ (max. 368 mμ): (1) stomach contents; (2) contents of small intestine. --- Vitamin A₁ (max. 328 mμ): (3) gut wall; (4) liver.

Fig. 1, which records the absorption spectra of the various extracts in a typical experiment, shows the clear-cut nature of the results. The absorption curves for the liver and small intestine show maxima at 328 mμ., characteristic of vitamin A₁, whilst those for the contents of the stomach and intestine have maxima at 368 mμ., characteristic of vitamin A₁ aldehyde. Table 1 summarizes the results obtained in this series of experiments. The excellent recoveries of vitamin A₁ in the animal as shown by the quantitative experiments indicate that retinene₁ is stable in the lumen of the gut and its reduction to vitamin A₁ alcohol *in vivo* must be a very efficient process.

The site of absorption of the retinene₁ into the intestine was briefly investigated. A rat was given a dose of retinene₁ by stomach tube, and was killed after an interval of 15–17 hr. The small intestine was ligatured at the pyloric sphincter and caecum, and was then cut into four parts corresponding as near as could be judged with the duodenum, upper half of jejunum, lower half of jejunum and ileum. Each portion of tissue was examined for vitamin A₁ and the lumen contents checked for the presence of retinene₁. The results are presented in Table 2.

Table 1. *Conversion of retinene₁ into vitamin A₁ in the intestine of the rat*

Experiment	Rat no.	Dose of retinene ₁ (μg.)	Time between dosing and test (hr.)	Retinene ₁ recovered from		Vitamin A ₁ (μg.) recovered from		Total recovery of retinene ₁ + vitamin A ₁	
				Stomach (μg.)	Contents of small intestine (μg.)	Wall of small intestine	Liver	(μg.)	Percentage of dose
1*	1)	Nil	Controls	—	—	Nil	Nil	Nil	—
	2)			—	—	—	—	—	—
	3			Nil	Nil	551	1072	1623	41
2*	4	8000	4	4610	91	18	98	4817	60
	5	8000	7.5	4183	4	80	438	4705	59
	6	8000	16	2063	240	141	1619	4063	51
	7	8000	24	799	141	724	2405	4069	51
3*	8	2000	5	152	69	103	518	842	42
	9	2000	10	4	2	60	557	623	31
	10	2000	15	21	0	93	689	803	40
	11	2000	20	1	0	47	725	773	39
	12	2000	25	1	0	43	539	533	29
4†	13	1054	5	821	0	10	64	895	85
	14	1887	8	1080	0	126	545	1751	93
	15	1732	15	0	4	175	1070	1249	72

* Retinene administered orally as drops of oil solution.

† Retinene introduced by stomach tube.

Table 2. *Distribution of the absorption of retinene₁ along the small intestine of a rat killed 17 hr. after receiving 1 mg. in oil by stomach tube*

	Retinene ₁ in intestinal contents	Vitamin A ₁ in intestinal wall (μg.)
Duodenum	0	6
Jejunum: Upper half	0	15
Lower half	0	38
Ileum	Trace	129

It was further of interest to determine if the vitamin A₁ formed in the gut wall became esterified on its way into the blood stream. Light petroleum solutions of the lipid extracts from the intestinal tissue of rats 14 and 15 were examined chromatographically using a bone-meal column. In the extract from rat 14 there were 6 parts of esterified vitamin A₁ to 1 part of free vitamin A₁; and in rat 15, 3 parts of ester to 1 part of alcohol.

Parenteral administration

The three vitamin A₁-depleted rats in the first group injected with 4.2 mg. of retinene concentrate dissolved in 0.5 ml. of refined ground-nut oil were killed after intervals of 1 day, 5 days and 5 weeks respectively after dosing. In each case *only* vitamin A₁ was found in the liver. This suggested, on first thought, that the liver is the locus of conversion of injected retinene₁, but an examination of the site of injection proved otherwise. The surrounding subcutaneous tissues were not examined in detail for

rats 17 and 18, but from their bright yellow colour obviously contained large amounts of unabsorbed retinene₁. The tissue near the point of injection in rat 19 had in 5 weeks become pale yellow. The pigmented portion of tissue was therefore dissected out and extracted. Analysis of the extract by the Carr-Price reaction showed that *only* vitamin A₁ was present. There was no trace of a reaction product with an absorption band at 664 mμ., i.e. retinene₁ was absent. This observation clearly indicates the presence in the subcutaneous tissue of an enzyme capable of converting vitamin A₁ aldehyde into vitamin A₁ alcohol.

The rats in the second group of three were each given 3.5 mg. of retinene₁ concentrate. Two of the animals were killed 3 weeks after dosing and the third 4 weeks later. The subcutaneous tissue and liver were in each case examined as before for residual retinene₁ and for vitamin A₁. The results are summarized in Table 3 and confirm the conclusion already reached. In the case of rat 21, both retinene₁ and vitamin A₁ were found in the subcutaneous tissue. The ultraviolet absorption spectrum of the extract in cyclohexane is recorded in Fig. 2 (curve 1). Using the vitamin A-correction procedure (Morton & Stubbs, 1946), the true contribution of vitamin A₁ in the extract was calculated (curve 2). Subtracted from the whole (curve 1) this gave the absorption due to retinene₁ plus some irrelevant absorption (curve 3). The co-existence of retinene₁ and vitamin A₁ in the subcutaneous tissue is clear proof that the reduction of the aldehyde to the alcohol can take place there.

Table 3. Reduction of retinene₁ (vitamin A₁ aldehyde) to vitamin A₁ alcohol in subcutaneous tissue of vitamin A₁-depleted rats, esterification in situ and liver storage

Experi- ment	Rat no.	Dose of retinene, injected in oil (μg.)	Time between injection and test (days)	Recovery from subcutaneous tissue				Vitamin A ₁ in liver (μg.)
				Retinene ₁ (μg.)	Vitamin A ₁ (μg.)			
					Total	Ester	Free	
6	17	4200	1	Observed as deeply stained tissue 0	—	—	—	54
	18	4200	5		—	—	—	600
	19	4200	35		446	—	—	1518
7	20	3500	21	0	311	214	71	739
	21	3500	21	291	714	631	71	1264
	22	3500	49	0	200	190	200	1175

Portions of the subcutaneous tissue extracts from this series of rats were dissolved in light petroleum, and examined chromatographically using a bone-meal column. A considerable portion of the vitamin A₁ was found in the esterified form. Thus, the subcutaneous tissue fluid contains, in addition to a reductase, an esterase or lipase capable of esterifying

DISCUSSION

In Exp. 3, Table 1, where smaller doses of retinene₁ were administered (rats 8–12), the amount of vitamin A₁ in the gut wall diminished rapidly after absorption from the lumen was complete. This fact, together with the failure to detect retinene₁ in either the gut wall or the liver, shows that the retinene₁ is immediately converted to vitamin A₁ on absorption into the gut.

The accumulation of vitamin A₁ in the gut wall during active absorption (rats 4–7, Table 1) shows that entry of retinene₁ into the mucosa, and its conversion to vitamin A₁, are rapid processes compared with the transport of the vitamin away from the gut wall.

The mode of absorption of retinene₁ appears to be very similar to that of vitamin A₁ (Popper & Volk, 1944). Absorption can occur readily along the whole length of the small intestine, but normally does so in the duodenum. In the experiment referred to in Table 2 the retinene₁ in 0.5 ml. oil was fed to the fasting animal by stomach tube, and 17 hr. after dosing a small amount of unabsorbed fat containing a little retinene₁ had passed well down into the ileum. Under these conditions retinene₁ is apparently very stable in the digestive fluids. This was confirmed by the excellent recovery of vitamin A₁ in rats 13 and 14, Table 1, which received accurately measured doses of retinene₁.

In these quantitative experiments, where retinene₁ was fed by stomach tube, the recovery as vitamin A₁ was exceedingly high (70–90 %). This is very much better than the recovery expected after dosing with vitamin A₁ itself. According to Reifman, Hallman & Deuel (1943) the recovery of vitamin A₁ at low dosage levels is only c. 30 %. Gray & Cawley (1942) recovered from 40 to 70 % of different preparations of vitamin A₁ administered to rats in moderate daily doses (c. 100–500 i.u.). A control experiment was carried out to estimate the recovery of single doses of vitamin A₁ ester given by stomach tube at doses matching those in Exp. 4, Table 1. Each of four young rats (not vitamin A₁-depleted but with low

Fig. 2. Absorption spectrum (1) of the lipid extract (in cyclohexane) from the subcutaneous tissue of a rat, 21 days after injection with 3.5 mg. retinene₁ in 0.5 ml. ground-nut oil, together with the corrected vitamin A₁ curve (2) and difference curve (3) with maximum at 368 mμ. (retinene₁).

free vitamin A₁. This is comparable to the findings of Schönheimer & Yuasa (1929), who had previously noted that free cholesterol injected subcutaneously becomes esterified on absorption. As with the administration of retinene₁ by mouth there is no indication that the liver has any opportunity to effect the reduction.

With intraperitoneal administration the result was much the same. The livers of the two rats, killed 24 and 36 hr. after injection, were found to contain 491 and 635 μg. of vitamin A₁ respectively. Unabsorbed retinene₁ was observed in the peritoneum.

Table 4. *Recovery of vitamin A₁ ester administered in single doses by stomach tube*

Rat no.	Dose of vitamin A ₁ in 0.5 ml. oil (μg.)	Vitamin A ₁ recovered from		Percentage of dose recovered	
		Intestine (μg.)	Liver (μg.)	Total	Liver only
23	1513	22	519	35.7	34.3
24	1699	14	267	16.5	15.7
25	1451	20	482	34.6	33.2
26	1686	38	579	36.6	34.3

liver stores, 30 μg./rat) was dosed with c. 1.5 mg. of vitamin A₁ ester concentrate in 0.5 ml. oil. The recovery of the vitamin from the animals killed 25 hr. after dosing ranged from 17 to 37 % (Table 4). It is worth noting, therefore, that if large liver stores are a therapeutic objective, retinene₁ is better than vitamin A₁ for attaining that end.

The absorption of retinene₁ from the subcutaneous tissue resembles that from the gut wall, except that it is very much slower. Retinene₁ has been detected 21 days after subcutaneous injection and vitamin A₁ after as long as 49 days. This is not unexpected, as the turn-over of tissue fluid (lymph) in a small piece of skin is much slower than in the intestinal wall, which is specially adapted for absorption. The fact that the vitamin A₁ alcohol, formed from the retinene₁ in the skin, underwent esterification before absorption is suggestive that the latter took place mainly via the lymphatic system as from the gut wall. The similarity in the modes of absorption from the skin and the intestine suggest that the enzymes, responsible for the reduction and esterification processes, are widespread in the body tissues.

Bliss (1947) has reported the isolation of a soluble protein from frogs' retinas capable of reducing retinene to vitamin A. Now that tissues other than the retina have been shown to contain the same or a similar enzyme, it may be possible to learn more regarding the nature of the enzyme in the eye. It is probably a reductase, because (a) the excellent recovery of vitamin A₁ from retinene₁ suggests that one molecule of the aldehyde gives rise to one molecule of the alcohol, and (b) it is not likely to be an aldehyde mutase which catalyzes reactions of the Cannizzaro type, producing the alcohol and the acid, since vitamin A₁ aldehyde contains an activated H atom in the α-position to the carbonyl group. The fact that the aldehyde is reduced easily to the alcohol, instead of being oxidized to the acid, favours the view that the alcohol grouping is necessary for vitamin A₁ activity.

The oxidation of β-carotene *in vitro* yields a small amount of retinene₁ (Hunter & Hawkins, 1944), which can be reduced to vitamin A₁ in a subsequent stage.

The recent work of Sexton, Mehl & Deuel (1946),

Glover *et al.* (1947b), Thompson, Ganguly & Kon (1947), Mattson, Mehl & Deuel (1947), and Wiese, Mehl & Deuel (1947) has shown that the small intestine is the main site of the conversion of β-carotene into vitamin A₁. The earlier work of Goodwin, Dewar & Gregory (1946) also suggests that in the goat the intestine is capable of carrying out the conversion. In the light of all this evidence, together with the present observations, there is no longer any difficulty in accepting the suggestion that the transformation of β-carotene to vitamin A *in vivo* can take place by oxidation of the former to vitamin A₁ aldehyde, which is subsequently reduced to the vitamin A₁ alcohol. Such a process of oxidation and reduction is more probable than a hydrolytic fission.

SUMMARY

1. Retinene₁ (vitamin A₁ aldehyde) administered to rats orally, subcutaneously, or intraperitoneally is converted into vitamin A₁ during absorption. This suggests that the alcohol group (either free or esterified) of vitamin A₁ is essential for its transport and storage.
2. After oral administration the conversion takes place in the gut, and after subcutaneous injection in the subcutaneous tissues. The site of conversion after intraperitoneal injection is uncertain.
3. There is no direct evidence that the liver can convert retinene₁ to vitamin A₁.
4. The conversion represents a simple reduction of the aldehyde.
5. The extracellular spaces of subcutaneous tissue contain an enzyme capable of esterifying vitamin A₁ alcohol.
6. It is suggested that the transformation of β-carotene into vitamin A₁ *in vivo* is more likely achieved by oxidation of the former to retinene₁, which is then rapidly reduced to vitamin A₁, rather than by hydrolytic fission.

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The Composition and Reactivity of Medullated Keratins

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One of the features which distinguishes the coarser wools from the finer merino types is the presence of a medulla. Whereas the finer wools consist only of a cortex and outer scaly structure, the coarser wools contain also a medulla. The chemistry of wool and other keratins has been fairly intensively studied, but, apart from the considerable evidence which suggests a low sulphur content, relatively little is known about the composition of the medulla. Barritt & King (1931), from a comparison of the sulphur contents of medullated and non-medullated wools, calculated that the medulla substance contained little, if any, sulphur. Jordan Lloyd & Marriott (1933) found that the medullary cells of goat hair, after separation by treatment with 4*N*-sodium hydroxide at room temperature, contained only 0.23 % sulphur. Bekker & King (1931) showed that the medulla dissected from porcupine quill contained less sulphur than the cortex. The absence of cystine from the medulla was inferred from the appearance of cross-sections of the quill stained with sodium plumbite.

Bailey (1937) observed that scoured samples of kemp, which is a heavily medullated fibre, and to a much lesser extent wool, had a low cystine content. One such sample of kemp contained less than 2 % of cystine. He concluded that the cystine originally present had been destroyed, probably during the

scouring process. The cystine of kemp would therefore appear to be much more readily attacked by mild alkalis, i.e. the scouring liquor, than that of other keratins.

Stoves (1946*a, b*) has recently studied the medulla cells of a number of animal fibres, isolating them from bleached horse hair and other keratin fibres by chemical treatment. Observation of the change in appearance of the cells when treated with a 1 % tannic acid solution, 0.1 % sodium oleate or 1 % saponin suggested that the surface of the medulla cells contained a lipoprotein-cholesterol combination. Stoves further showed that when the medulla cells were refluxed with ethanolic sodium hydroxide the solution gave a positive reaction for phosphate, whilst a chloroform extract of the solution gave a positive reaction for sterols.

In the present paper the sulphur analyses of wool and kemp which had been scoured in the laboratory, and wool which had been scoured on a commercial scale, are compared with those of the same wool which had been degreased with solvents. The results of phosphorus determinations on a number of medullated and non-medullated keratins, and the examination of hydrolysates of medulla by two-dimensional partition chromatography are also given.