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Metabolism and Biological Potency of 5,6-Monoepoxyvitamin A Aldehyde in the Rat

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1. The metabolism of 5,6-monoepoxyvitamin A aldehyde in the rat was found to be identical with that of vitamin A aldehyde. It promptly alleviated all the symptoms of vitamin A deficiency and promoted the growth of the vitamin A-deficient rats. 2. When administered orally, 5,6-monoepoxyvitamin A aldehyde was reduced to the corresponding alcohol in the intestine and esterified before being transported to the liver for storage. 3. 5,6-Monoepoxyvitamin A aldehyde was not converted into the furanoid form, 5,8-monoepoxyvitamin A aldehyde, during passage through the stomach. 4. Intraperitoneal administration of 5,6-monoepoxyvitamin A aldehyde led to the accumulation of 5,6-monoepoxyvitamin A in the liver and other tissues. Subcutaneous administration of this compound alleviated all the symptoms of vitamin A deficiency. 5. The small intestine is the major, if not the only, site for the metabolic reduction of 5,6-monoepoxyvitamin A aldehyde and its subsequent esterification. 6. It was demonstrated that the rat possesses the necessary enzymes for the reduction and oxidation of 5,6-monoepoxyvitamin A aldehyde to the corresponding alcohol and acid as well as the esterification of 5,6-monoepoxyvitamin A alcohol to its palmitate. These metabolic conversions were shown to be as efficient as those of vitamin A aldehyde and alcohol. 7. 5,6-Monoepoxyvitamin A aldehyde possesses a biological potency 108% that of all-*trans* vitamin A acetate. 8. A new visual pigment with $\lambda_{max.}$ 480 mμ, along with natural rhodopsin, was isolated from the retinas of rats maintained on 5,6-monoepoxyvitamin A aldehyde. 9. Oral administration of 5,8-monoepoxyvitamin A aldehyde to vitamin A-deficient rats led to the accumulation of 5,8-monoepoxyvitamin A in the liver and other tissues. Enzymic reduction and oxidation of 5,8-monoepoxyvitamin A aldehyde to its alcohol and acid, as well as the esterification of the alcohol, were demonstrated.

Ever since vitamin A acid was shown to possess considerable biological activity (Van Dorp & Arens, 1946*b*; Redfearn, 1960; Thompson, Howell & Pitt, 1961*a*,*b*), in fact as high or somewhat higher than the alcohol or the aldehyde forms (Van Dorp & Arens, 1946*a*; Malathi, Subba Rao, Seshadri Sastry & Ganguly, 1963), the concept of an 'active form' of vitamin A has centred around the more-oxidized state of the vitamin. Epoxides of vitamin A with established structures have now been synthesized (Jungalwala & Cama, 1965), of which 5,6-monoepoxyvitamin A aldehyde (5,6-monoepoxyretinene) is the most stable. The present paper describes the metabolism of this compound, both in *in vivo* and *in vitro*, and the assay of its biological potency according to the more precise U.S.P. XIV on male albino rats, as compared with that of vitamin A acetate.

MATERIALS AND METHODS

5,6- and 5,8-Monoepoxyvitamin A aldehyde and alcohol were obtained as described by Jungalwala & Cama (1965). 5,6-Monoepoxyvitamin A palmitate was prepared by the same method starting from synthetic vitamin A palmitate and showed absorption maxima at 310 and 325 mμ ($E^{1\%}_{1cm.}$ 1767 and 1490 respectively) (in light petroleum). The $E_{325m\mu}/E_{310m\mu}$ ratio was 0·843, which is in good agreement with that observed for the corresponding acetate (Jungalwala & Cama, 1965). In the $SbCl_3$ colour test, 5,6-monoepoxyvitamin A palmitate also gave a pink colour, instantaneously changing to a stable yellow colour with $\lambda_{max.}$ 460 mμ ($E^{1\%}_{1cm.}$ 1437). Synthetic all-*trans* vitamin A acetate was obtained as a gift from Hofmann–La Roche, Basel, Switzerland, and $NADH_2$ was from Sigma Chemical Co., St Louis, Mo., U.S.A. For other reagents see Lakshmanan, Vaidyanathan & Cama (1964) and Jungalwala & Cama (1965).

Oral administration. The compounds under study were prepared in refined deodorized groundnut oil containing 0·5% of α-tocopherol (unless otherwise stated), and were stored at −20°. They were administered to the animals with an Agla micrometer syringe (Burroughs Wellcome). For bioassay experiments, 5,6-monoepoxyvitamin A aldehyde and vitamin A acetate were freshly prepared (concentrations 0·25, 0·5, 0·75 and 1·0 μg./0·05 ml. of diluent oil) every week. For metabolic purposes, the various compounds were prepared just before use (concentration 1·0 mg./0·1–0·2 ml. of diluent oil containing 0·5 mg. of α-tocopherol).

Preparation of tissue extracts. The procedure followed was essentially that described by Glover, Goodwin & Morton (1948).

Preparation of enzymes. Rat-liver alcohol dehydrogenase was prepared according to the modified method of Bonnichsen & Brink (1955), the enzyme obtained at the third step being used. An acetone-dried powder of rat pancreas, prepared as described by Murthy, Mahadevan, Seshadri Sastry & Ganguly (1961), served as a source of the enzyme for esterifying 5,6-monoepoxyvitamin A alcohol. Rat-liver aldehyde oxidase was prepared as described by Lakshmanan *et al.* (1964).

Enzyme assays. (*a*) Reductase reaction. The reaction mixture consisted of 250 mμmoles of 5,6- or 5,8-monoepoxyvitamin A aldehyde in 0·1 ml. of ethanol, 300 mμmoles of $NADH_2$, 1 μmole of GSH and 1–10 mg. of rat-liver alcohol dehydrogenase in a final volume of 2 ml. of 0·1 M-phosphate buffer, pH 6·0. After incubation for 1 hr. at 37° the reaction was stopped by adding 8 ml. of ethanol. The reaction mixture was extracted twice with diethyl ether, and the extracts were pooled, reduced in volume *in vacuo* and taken up in a small quantity of light petroleum.

(*b*) Esterase reaction. The reaction mixture was as described by Murthy *et al.* (1961), except that the substrate used was 5,6- or 5,8-monoepoxyvitamin A alcohol.

(*c*) Oxidase reaction. The reaction mixture was as described by Lakshmanan *et al.* (1964), except that the substrate used was either 5,6- or 5,8-monoepoxyvitamin A aldehyde.

The enzyme protein was determined as described by Lakshmanan *et al.* (1964), and the specific activities are defined as mμmoles of 5,6-monoepoxyvitamin A aldehyde utilized or 5,6-monoepoxyvitamin A alcohol esterified/mg. of protein after incubation for the time indicated.

Separation and identification of various epoxides of vitamin A. The following methods were used:

(*a*) Adsorption chromatography. The following mixtures (100 μg. of each component) were separated with quantitative recovery when chromatographed on 7%-(v/w)-water-deactivated alumina column as described by Jungalwala & Cama (1965): (i) 5,6-monoepoxyvitamin A palmitate, 5,6-monoepoxyvitamin A aldehyde and 5,6-monoepoxyvitamin A alcohol; (ii) 5,8-monoepoxyvitamin A palmitate, 5,8-monoepoxyvitamin A aldehyde and 5,8-monoepoxyvitamin A alcohol.

(*b*) Reverse-phase chromatography on paper impregnated with petroleum jelly (Jungalwala & Cama, 1962). The R_F values of the various compounds obtained by using this technique are as follows: (i) 5,6-monoepoxyvitamin A palmitate, 5,6-monoepoxyvitamin A aldehyde and 5,6-monoepoxyvitamin A alcohol, 0·13, 0·82 and 1·0 respectively; (ii) 5,6-monoepoxyvitamin A aldehyde and enzymically formed 5,6-monoepoxyvitamin A acid, 0·82 and 1·0 respectively; (iii) 5,8-monoepoxyvitamin A palmitate, 5,8-monoepoxyvitamin A aldehyde and 5,8-monoepoxyvitamin A alcohol, 0·19, 0·95 and 1·0 respectively; (iv) 5,8-monoepoxyvitamin A aldehyde and enzymically formed 5,8-monoepoxyvitamin A acid, 0·95 and 1·0 respectively.

(*c*) Thin-layer chromatography on kieselgel (E. Merck, Darmstadt, Germany) (Stahl, 1958, 1959). Chromatograms were developed with 6% (v/v) acetone in light petroleum (200 ml.) for about 2 hr. The R_F values were: 5,6-monoepoxyvitamin A acid, 5,6-monoepoxyvitamin A alcohol, 5,6-monoepoxyvitamin A aldehyde and 5,6-monoepoxyvitamin A palmitate, 0, 0·072, 0·44 and 0·84 respectively; 5,8-monoepoxyvitamin A acid, 5,8-monoepoxyvitamin A alcohol, 5,8-monoepoxyvitamin A aldehyde and 5,8-monoepoxyvitamin A palmitate, 0, 0·07, 0·42 and 0·81 respectively (John, Lakshmanan, Jungalwala & Cama, 1965). Compounds were identified by comparing their R_F values with those of authentic samples, as well as by co-chromatography with the authentic samples, in the above systems. They also gave their characteristic absorption spectra after their elution from the chromatograms, and the specific $SbCl_3$ colour test (Jungalwala & Cama, 1965) on spraying with $SbCl_3$.

Estimations. 5,6-Monoepoxyvitamin A aldehyde was estimated by the thiobarbituric acid procedure (Futterman & Saslaw, 1961) in a Beckman model DU spectrophotometer, by using 480 mμ as the absorption maximum. 5,6-Monoepoxyvitamin A palmitate and the alcohol were estimated by the $SbCl_3$ colour test method, by using $E^{1\%}_{1\,cm.}$ values of 1437 and 1270 respectively at 460 mμ. In some extracts where 5,6-monoepoxyvitamin A aldehyde and alcohol could not be separated by chromatography, the former was estimated by the thiobarbituric acid procedure (Futterman & Saslaw, 1961) and the latter by the $SbCl_3$ colour test by applying the correction formula of Cama, Collins & Morton (1951). Vitamin A, if any, was also estimated by the $SbCl_3$ colour test with the correction procedure of Cama *et al.* (1951).

For bioassay and metabolic studies, male albino rats of this Institute strain, when 15 days old, were placed on a diet free from milk, carrots and shark-liver oil. When 4 weeks old and weighing approx. 30–40 g., they were placed on a vitamin A-free diet and water *ad lib.*: casein (refluxed with ethanol and extracted with ether), 18·0%; starch, 63·0%; sucrose, 10·0%; refined groundnut oil, 5·0%; salt mixture (Hawk & Oser, 1931), 4·0%. The following vitamins were also added per kg. of diet: α-tocopherol, 100 mg.; 2 methyl-1,4-naphthaquinone, 5 mg.; calciferol, 5 mg.; thiamine, 5 mg.; riboflavine, 5 mg.; nicotinic acid, 50 mg.; pyridoxine, 5 mg.; pantothenic acid, 50 mg.; biotin, 0·5 mg.; folic acid, 0·5 mg.; inositol, 100 mg.; choline chloride, 1 g.; ascorbic acid, 1 g. Aqueous solutions of water-soluble vitamins were prepared and mixed with the diet every day. Generally the rats maintained on this diet were depleted of vitamin A reserves within about 28 days (Bliss & György, 1951). At the depletion period, the rats reached an average constant weight of 80–90 g. The depleted animals were assigned to various groups according to a 4 × 4 latin-square design, and four such latin squares were designed for the complete bioassay experiment.

For the visual-pigment experiments, eight of the bioassay rats maintained on 5,6-monoepoxyvitamin A aldehyde

were given the same compound for a further 10 days at 1 mg./rat/day before they were killed for the extraction and isolation of visual pigments (Bamji, Cama & Sundaresan, 1961).

RESULTS

Oral administration. Four vitamin A-deficient rats were given 5,6-monoepoxyvitamin A aldehyde by mouth (1 mg./rat/day) for 10 days. The average weight gain of all the rats during this period was 28 g. They were killed 9 hr. after the last dose, and 5,6-monoepoxyvitamin A recovered from the various tissues was determined (Table 1). The extracts from the contents of the stomach and small intestine gave the characteristic absorption spectra of unabsorbed 5,6-monoepoxyvitamin A aldehyde, and no trace of 5,6-monoepoxyvitamin A alcohol or ester could be detected in either tissue. The extract from the intestinal wall showed a typical spectrum of 5,6-monoepoxyvitamin A alcohol or ester (Jungalwala & Cama, 1965). Traces of 5,6-monoepoxyvitamin A aldehyde could also be detected. About 75% of the vitamin was in the ester form, probably the palmitate.

The extract from the livers gave the characteristic spectrum of 5,6-monoepoxyvitamin A alcohol or ester. On very careful examination, a small fraction could be eluted from the alumina column just before the major 5,6-monoepoxyvitamin A palmitate was eluted; this fraction gave a faint blue colour with the antimony trichloride reagent. However, it failed to give any characteristic spectrum of vitamin A alcohol or ester. There was no trace of vitamin A alcohol. If this fraction is vitamin A, its quantitative estimation gave a value of 0·18 μg./g. fresh wt. of tissue. To confirm whether vitamin A-deficient rats themselves contain such minute quantities of vitamin A, four vitamin A-deficient rats at the plateau stage were killed and the lipid extract of the pooled livers was analysed as before. A fraction corresponding to vitamin A palmitate was eluted from the alumina column, and this gave distinctly a faint blue colour with the antimony trichloride reagent and absolutely no spectrum of vitamin A. The quantitative estimation of this fraction gave a value of 0·2 μg./g. fresh wt. of tissue.

The extract from the kidneys gave the typical spectrum of 5,6-monoepoxyvitamin A, whereas those from the blood sera and spleen failed to give any spectra of 5,6-monoepoxyvitamin A or vitamin A. However, with the antimony trichloride colour test, all the tissue extracts gave a yellow colour and they moved just like 5,6-monoepoxyvitamin A alcohol on thin-layer and reverse-phase chromatography systems.

To investigate the active absorption of 5,6-monoepoxyvitamin A aldehyde and its probable site of conversion into 5,6-monoepoxyvitamin A, time-distribution studies were carried out with vitamin A-deficient rats. The results of these experiments showed a rapid increase of 5,6-monoepoxyvitamin A during the first 3 hr. of absorption, followed by a progressive increase in the livers during the next 9 hr. (Table 2).

Intraintestinal administration. Rats were anaesthetized and, after an incision had been made along the abdomen, the middle one-third of the small intestine was ligated at both the ends. 5,6-Monoepoxyvitamin A aldehyde (1 mg.) was injected directly into the intestine just below the upper ligature, the abdominal incision was sutured and

Table 1. *Recovery of 5,6-monoepoxyvitamin A from various tissues of four vitamin A-deficient rats after oral or intraperitoneal administration of 5,6-monoepoxyvitamin A aldehyde for 10 days at a dose rate of 1·0 mg./rat/day*

Tissue	Recovery of 5,6-monoepoxyvitamin A (% of administered dose)	
	After oral administration	After intraperitoneal administration
Control	—	—
Experimental		
Liver		
Total	25·6	22·9
5,6-Monoepoxyvitamin A palmitate	23·3	20·7
5,6-Monoepoxyvitamin A alcohol	2·3	2·2
Intestinal wall		
Total	0·38	0·2
5,6-Monoepoxyvitamin A palmitate	0·28	—
5,6-Monoepoxyvitamin A alcohol	0·09	—
Kidneys	0·18	0·08
Spleen	—	—
Lungs	—	—
Blood (serum)	—	—

Table 2. *Conversion of 5,6-monoepoxyvitamin A aldehyde into 5,6-monoepoxyvitamin A alcohol in the intestine of the rat after the oral administration of 1·0 mg. of 5,6-monoepoxyvitamin A aldehyde*

Rat no.	Time between administration and death (hr.)	5,6-Monoepoxyvitamin A aldehyde recovered (μg.)			5,6-Monoepoxyvitamin A alcohol and ester recovered		Total recovery of 5,6-monoepoxyvitamin A aldehyde, alcohol and ester (% of administered dose
		Stomach	Intestinal washings	Intestine	Intestine	Liver	
1 (Control)	—	0	0	0	0	0	0
2 (Control)	—	0	0	0	0	0	0
3	½	395·4	1·5	5·1	0	0	40·2
4	½	439·0	3·0	4·3	0	0	44·6
5	1	221·3	4·5	5·8	0	0	23·2
6	1	243·9	13·0	5·6	0	0	26·3
7	3	115·3	8·0	9·0	38·2	140·3	31·1
8	3	387·6	8·2	19·0	46·6	170·4	63·1
9	6	181·1	4·3	9·0	12·5	196·1	40·3
10	6	41·0	2·9	7·7	17·2	194·2	26·3
11	12	15·2	3·0	6·3	19·3	290·7	32·5
12	12	31·9	2·2	6·7	15·9	294·2	35·1
13	24	7·5	11·7	0	0	222·4	24·2
14	24	21·5	10·2	0	0	246·3	27·8

Table 3. *Recovery of 5,6-monoepoxyvitamin A aldehyde and 5,6-monoepoxyvitamin A from various tissues of rats given 5,6-monoepoxyvitamin A aldehyde (1·0 mg./rat) by various routes*

Rat no.	Route	Time between administration and death (hr.)	Tissue	Recovery (% of administered dose) 5,6-Monoepoxyvitamin A aldehyde	5,6-Monoepoxyvitamin A alcohol (and ester)	Total of 5,6-monoepoxyvitamin A aldehyde and alcohol (and ester)	
1 (Control)	—	—	—	0	0	0	
2 (Control)	—	—	—	0	0	0	
3	Stomach	½	Stomach	78·6	0	78·6	
4	Stomach	½	Stomach	72·1	0	72·1	
5	Intraintestinal	2	Middle third of small intestine	2·2	5·64	7·84	25
		2	Liver	0	17·2	17·2	
6	Intraintestinal	2	Middle third of small intestine	3·1	8·8	11·9	31·4
		2	Liver	0	19·5	19·5	

the animals were returned to their cages. They were killed after 2 hr. and the intestines between the two ligatures and livers were analysed. Both the intestines and the livers contained 5,6-monoepoxyvitamin A alcohol and ester (Table 3).

Stomach experiments. Two vitamin A-deficient rats were anaesthetized and, after an incision had been made in the abdomen, the stomach was ligated at the duodenal and oesophageal ends. 5,6-Monoepoxyvitamin A aldehyde (1 mg.) was injected directly into the stomach, the incision was sutured and the rats were returned to their cages. After 30 min. both rats were killed and the stomach contents analysed. Although 5,6-monoepoxyvitamin A aldehyde is readily converted into its furanoid form, 5,8-monoepoxyvitamin A aldehyde, in the presence of traces of mineral acids (Jungalwala & Cama, 1965), the extracts failed to show any spectrum characteristic of 5,8-monoepoxyvitamin A aldehyde; only 5,6-monoepoxyvitamin A alde-

hyde was recovered (Table 3). Further, chromatography of the extracts either on a water-deactivated alumina column or on the reverse-phase system did not reveal the presence of 5,8-monoepoxyvitamin A aldehyde.

Parenteral administration. Four vitamin A-deficient rats were given 5,6-monoepoxyvitamin A aldehyde (1mg./rat/day) intraperitoneally for 10 days. All the rats on an average gained about 16g. in weight during this period. The animals were killed 9hr. after the last dose. The results showed that the metabolism of 5,6-monoepoxyvitamin A aldehyde when administered intraperitoneally is similar to that when given orally (Table 1).

Enterectomy. Two vitamin A-deficient rats were anaesthetized with urethane and, after the abdomen had been opened up, the small intestine was ligated at the duodenal and caecal ends. The blood vessels with the mesentery leading to the intestines were tied together to prevent bleeding and then the intestine in between the two ligatures was cautiously removed. The abdomen was then sutured and 5,6-monoepoxyvitamin A aldehyde (1mg.) was injected intraperitoneally, and the rats were returned to their cages. After 3hr. they were killed and the various tissues were analysed. None of the tissues showed the presence of 5,6-monoepoxyvitamin A or the injected compound, as tested by their absorption spectra, antimony trichloride colour test and chromatography on thin-layer and reverse-phase systems. However, the extract from the peritoneal cavity distinctly gave the spectrum of 5,6-monoepoxyvitamin A aldehyde.

Subcutaneous administration. In a single experiment, a vitamin A-deficient rat was given 1mg. of 5,6-monoepoxyvitamin A aldehyde subcutaneously. The rat was relieved of all the symptoms of vitamin A deficiency within a day or two and gained about 70g. in weight in 3 weeks, after which it was killed. Analysis of the livers and the subcutaneous tissue at the site of injection showed the presence of neither 5,6-monoepoxyvitamin A alcohol nor the injected compound.

Enzymic reduction of 5,6-monoepoxyvitamin A aldehyde to the corresponding alcohol by rat-liver alcohol dehydrogenase. The formation of 5,6-monoepoxyvitamin A alcohol was stoicheiometric with the disappearance of 5,6-monoepoxyvitamin A aldehyde. The reduced product was characterized by its absorption spectrum, antimony trichloride colour test and chromatographic behaviour. The activity of the enzyme towards 5,6-monoepoxyvitamin A aldehyde was 14·06mμmoles/mg. of enzyme protein/hr., compared with 12·86mμmoles/mg./hr. towards vitamin A aldehyde.

Enzymic esterification of 5,6-monoepoxyvitamin A alcohol to the corresponding palmitate by rat-pancreas esterase. When incubated with esterase, 902mμmoles of 5,6-monoepoxyvitamin A alcohol were esterified/mg. of enzyme protein/½hr.

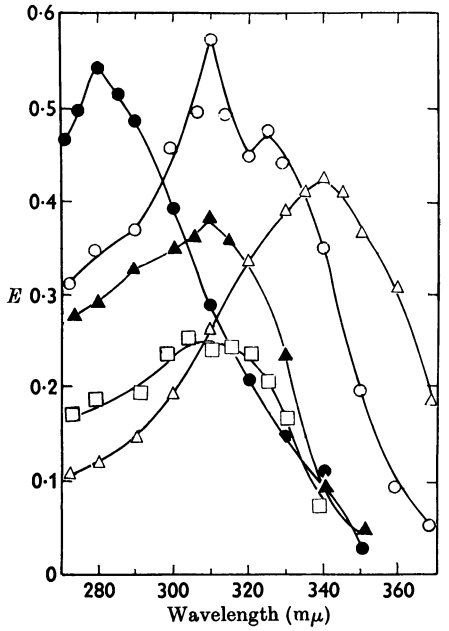

Fig. 1. Ultraviolet-absorption spectra, in light petroleum, of various products formed enzymically from 5,6- and 5,8-monoepoxyvitamin A aldehyde: ○, 5,6-monoepoxyvitamin A alcohol; ●, 5,8-monoepoxyvitamin A alcohol; △, 5,6-monoepoxyvitamin A acid; ▲, 5,8-monoepoxyvitamin A acid; □, 5,8-monoepoxyvitamin A acid formed from 5,6-monoepoxyvitamin A acid after treatment with ethanolic HCl.

Enzymic oxidation of 5,6-monoepoxyvitamin A aldehyde to the corresponding acid by rat-liver aldehyde oxidase. The amount of 5,6-monoepoxyvitamin A aldehyde that disappeared was proportional to the concentration of the enzyme protein, the specific activity being 283·3mμmoles/mg. of enzyme protein/hr. No product other than 5,6-monoepoxyvitamin A acid was revealed either by thin-layer or reverse-phase chromatography. The product of the enzymic reaction was characterized by its absorption spectrum (Fig. 1), with $\lambda_{max.}$ 337mμ, which agreed very well with the value deduced theoretically by analogy with those of vitamin A_1 acid and vitamin A_2 acid (Kofler & Rubin, 1960).

Thus the rat was shown to possess the necessary enzymes for the metabolic reduction and oxidation of 5,6-monoepoxyvitamin A aldehyde as well as for the esterification of 5,6-monoepoxyvitamin A alcohol.

Biological potency of crystalline 5,6-monoepoxyvitamin A aldehyde. The growth rate (g./week) over

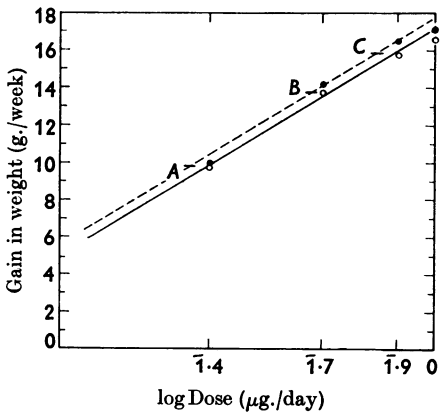

Fig. 2. Response of male albino rats to all-*trans* vitamin A acetate (○) and 5,6-monoepoxyvitamin A aldehyde (●) given orally, plotted in terms of gain in weight (g./week) against the logarithm of the daily dose. *A*, *B* and *C* represent the projections of the growth of the rats receiving 0·25, 0·5 and 0·75 μg. of all-*trans* vitamin A acetate on to the growth curve for 5,6-monoepoxyvitamin A aldehyde.

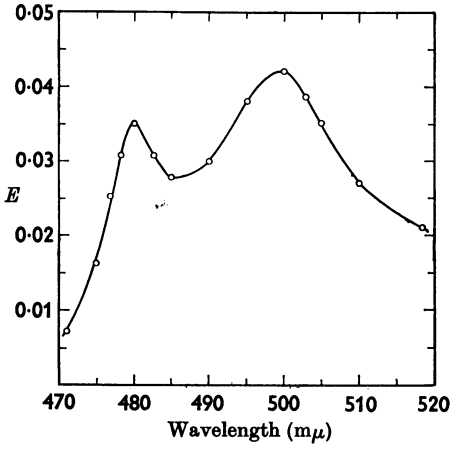

Fig. 3. Difference spectrum, in 1% digitonin, of the visual pigment isolated from the retinas of eight rats subsisting on 5,6-monoepoxyvitamin A aldehyde as sole source of vitamin A-like substances.

4 weeks of individual litter-mate rats receiving crystalline 5,6-monoepoxyvitamin A aldehyde or vitamin A acetate was measured, and closely similar responses were obtained. Out of the eight negative controls, only one survived till the end of the bioassay period, and even this rat lost 5g. in weight/week on an average. The mean weekly gain in weight plotted against the logarithm of the dose gave lines for the standard and the test doses that were parallel and close to each other (Fig. 2).

The results for rats receiving 1 μg. of either 5,6-monoepoxyvitamin A aldehyde or vitamin A acetate/day seem to be well outside the critical range of bioassay. The biopotency of 5,6-monoepoxyvitamin A aldehyde as compared with that of vitamin A acetate, calculated from these results, was 108%.

Visual pigments from the retinas of rats given 5,6-monoepoxyvitamin A aldehyde. The difference spectrum of the visual pigment isolated from eight vitamin A-deficient rats maintained on 5,6-monoepoxyvitamin A aldehyde for about 5 weeks seems to indicate a new absorption maximum at 480 mμ, along with that at 500 mμ due to rhodopsin (Fig. 3).

Metabolic studies on 5,8-monoepoxyvitamin A aldehyde. 5,8-Monoepoxyvitamin A aldehyde, when fed to vitamin A-deficient rats, was converted into 5,8-monoepoxyvitamin A alcohol and ester and stored in the livers. Enzymic reduction of 5,8-monoepoxyvitamin A aldehyde to the corresponding alcohol by rat-liver alcohol dehydrogenase and the esterification of the latter to its palmitate by rat-pancreas esterase were demonstrated. Rat-liver aldehyde oxidase brought about the conversion of 5,8-monoepoxyvitamin A aldehyde into its corresponding acid with a spectrum (Fig. 1) identical with that obtained when enzymically formed 5,6-monoepoxyvitamin A acid was treated with ethanolic hydrochloric acid (Fig. 1).

DISCUSSION

5,6-Monoepoxyvitamin A aldehyde was shown to alleviate all the symptoms of vitamin A deficiency in the rat, promoting the normal growth of the animal and fulfilling all the functions of vitamin A. It was metabolized in the same manner as vitamin A aldehyde (Glover *et al.* 1948). Since there was a rapid increase of 5,6-monoepoxyvitamin A in the intestine during the first 3hr. of absorption, followed by a progressive increase in the 5,6-monoepoxyvitamin A content of the liver in the next 9hr. (Table 2), it appears that 5,6-monoepoxyvitamin A aldehyde was rapidly reduced to 5,6-monoepoxyvitamin A and that the transport of the latter (as ester) to the liver was a comparatively slow process.

Intraperitoneal administration of 5,6-monoepoxyvitamin A aldehyde also led to the accumulation of 5,6-monoepoxyvitamin A ester in the liver and other tissues, and it could also be detected even in the intestine (Table 2). In contrast, no 5,6-monoepoxyvitamin A alcohol or ester could be detected in the liver or other tissues of rats that received 5,6-monoepoxyvitamin A aldehyde intraperitoneally after enterectomy. Thus the small intestine is the most active, if not the only, site

for the metabolic conversion of 5,6-monoepoxyvitamin A aldehyde into 5,6-epoxyvitamin A. By parenteral route, it is very likely that 5,6-monoepoxyvitamin A aldehyde is absorbed *in situ* and reaches the intestine through the blood stream before it is absorbed through the intestine. The administration of 5,6-monoepoxyvitamin A aldehyde subcutaneously was effective in the rat, since it alleviated all the symptoms of vitamin A deficiency and restored growth. The fate of the compound by this route is still obscure; it may well be that it is utilized so rapidly for the metabolic needs of the animal that it escapes detection in various tissues. In general, it may be concluded that irrespective of the route of administration, 5,6-monoepoxyvitamin A aldehyde is first reduced to 5,6-monoepoxyvitamin A alcohol and then subsequently esterified in the intestine of the rat before being transported to the liver for storage, only to be utilized as and when the metabolic needs of the animal demand.

Only traces of vitamin A were detected in the livers of rats maintained on 5,6-monoepoxyvitamin A aldehyde or on a vitamin A-deficient diet. Shantz, Embree, Hodge & Wills (1946) in their studies on the replacement of vitamin A_1 by vitamin A_2 in the rat, as well as Dowling & Wald (1960) on the biological function of vitamin A acid, also report the presence of micro quantities of vitamin A in the livers of vitamin A-deficient rats, which may well be the 'immobilized' form of vitamin A; the trace amounts of vitamin A found were not likely to have arisen from 5,6-monoepoxyvitamin A aldehyde. It is therefore likely that these epoxides of vitamin A exert their activity themselves and not through an intermediate formation of vitamin A.

Further, the rat seems to metabolize 5,6-monoepoxyvitamin A aldehyde preferentially for all its metabolic needs, since it is very efficiently metabolized and transported to various tissues. Hence 5,6-monoepoxyvitamin A aldehyde exerts a 'sparing effect' on the 'immobilized' liver vitamin A, unlike vitamin A acid, which has no such effect (Dowling & Wald, 1960).

The rat has been demonstrated to possess very efficient mechanisms for the conversion of 5,6-monoepoxyvitamin A aldehyde into its corresponding acid and the alcohol *in vitro* as well as the esterification of the alcohol to its palmitate. The biological potency of 5,6-monoepoxyvitamin A aldehyde has been shown to be 108% compared with that of all-*trans* vitamin A acetate. By analogy with the biological activities of vitamin A_1 aldehyde and vitamin A_2 aldehyde, which are more or less equally as potent as vitamin A_1 alcohol and A_2 alcohol respectively (Wendler, Rosenblum & Tishler, 1950; Ames, Swanson & Harris, 1955; Farrar, Hamlet, Henbest & Jones, 1952; Sundaresan & Cama, 1961), 5,6-monoepoxyvitamin A may also be expected to possess as much biological potency as 5,6-monoepoxyvitamin A aldehyde.

Our preliminary studies show the formation of a new visual pigment, with $\lambda_{max.}$ 480mμ, in the retinas of rats subsisting on 5,6-monoepoxyvitamin A aldehyde, along with the natural rhodopsin, with $\lambda_{max.}$ 500mμ (Fig. 3), which is probably tenaciously held (Shantz *et al.* 1946). 5,6-Monoepoxyvitamin A aldehyde has one conjugated double bond less than vitamin A_1 aldehyde and two double bonds less than vitamin A_2 aldehyde. Hence, on comparison of the absorption maxima of rhodopsin and porphyropsin (500 and 520mμ respectively), the $\lambda_{max.}$ 480mμ for this new pigment is in good agreement with the expected value for a visual pigment having 5,6-monoepoxyvitamin A aldehyde as its chromophore. All the rats showed normal visual behaviour, unlike rats subsisting on vitamin A acid.

We have demonstrated that the rat possesses the necessary enzymes for the metabolic formation of 5,6-monoepoxyvitamin A acid, and the spectroscopic and acidic properties of enzymically formed 5,6-monoepoxyvitamin A acid (Fig. 1) seem to agree well with those of an 'active vitamin A compound' isolated by Wolf, Bergan & Sundaresan (1963) after feeding vitamin A acid to vitamin A-deficient rats. These observations imply that 5,6-monoepoxyvitamin A acid is far ahead of vitamin A acid itself in the proposed scheme of the general metabolism of vitamin A (Dowling & Wald, 1960).

The present investigations, as well as previous work from our Laboratory on the biopotency of anhydrovitamin A_2 (Balasundaram, Bamji, Cama, Sundaresan & Varma, 1958; Bamji, Cama & Sundaresan, 1962), agree with the suggestion by Morton (1960) and Wolf & Johnson (1960) that it is not the β-ionone ring but the conjugated system of four double bonds that is essential for any vitamin A-like compound to fulfil all the functions of vitamin A.

Similar studies on the less stable 5,8-monoepoxyvitamin A aldehyde show that it promotes the growth of vitamin A-deficient rats to a smaller extent than 5,6-monoepoxyvitamin A aldehyde, although it is metabolized in a similar manner.

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