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Enzymic Oxidation of Vitamin A Aldehyde to Vitamin A Acid by Rat Liver

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Vitamin A acid, first synthesized by Van Dorp & Arens (1946*a*), was shown to have vitamin A activity in the rat (Van Dorp & Arens, 1946*b*); Sharman (1949) confirmed that the acid can support growth of rats. However, no vitamin A acid or alcohol could be detected in the liver of rats dosed with the acid (Van Dorp & Arens, 1946*b*; Sharman, 1949), which led Moore (1957) to suggest that the acid might be able to fulfil some of the functions of vitamin A, though not being able to replace it in the visual process. This suggestion was fully substantiated by Dowling & Wald (1960), who demonstrated conclusively that vitamin A acid can indeed replace the classical vitamin A in the general metabolism of the rat except perhaps in the visual cycle. More recent observations, however, indicate that vitamin A acid fails to maintain the reproductive performances of vitamin A-deficient male and female rats (Thompson, Howell & Pitt, 1961*a, b*).

It is being increasingly recognized that the classical vitamin A alcohol is not the actual active form of the vitamin for the general metabolic needs of the animals, and the consensus of opinion is that the active compound might be derived from vitamin A acid, if the acid by itself is not the active metabolite. Dmitrovsky (1961) has shown that the aldehyde oxidase prepared from pig liver can oxidize retinene to vitamin A acid, and a preliminary report by Elder & Topper (1961) claims that a NAD-linked aldehyde dehydrogenase in rabbit liver can catalyse the conversion of retinene into vitamin A acid. Further, several workers have shown that vitamin A aldehyde is distributed in the tissues of many species (Plack & Kon, 1961; Winterstein & Hegedus, 1960). It is now demonstrated that rat liver does contain retinene as well as two enzymes that independently catalyse the

reduction of retinene to vitamin A alcohol and the oxidation of retinene to vitamin A acid, and that the two enzymes can be separated by ammonium sulphate fractionation of rat-liver preparations. Since the properties of the alcohol dehydrogenase which catalyses the oxidation of vitamin A alcohol to retinene are well established (Bliss, 1951), in the present paper some of the properties of the retinene-oxidizing enzyme are described and the implications of these findings are discussed with respect to the possibility of the formation of vitamin A acid *in situ* for metabolic needs.

MATERIALS AND METHODS

Materials. Retinene was prepared from vitamin A alcohol and was purified by chromatography on 10% deactivated alumina according to Ball, Goodwin & Morton (1948). The purified product had an absorption maximum at 381 m μ in ethanol (Fig. 1*b*). GSH, FAD, *p*-chloro-mercuribenzoate, NAD, NADH₂ and NADP were from Sigma Chemical Co., St Louis, Mo., U.S.A., thiobarbituric acid from L. Light and Co. Ltd., Colnbrook, Bucks, and thiourea from Hopkin and Williams Ltd., London. Crystalline vitamin A alcohol and acid were from Hofmann-La Roche. The sources of other materials and reagents were as described by Mahadevan, Murthy, Krishnamurthy & Ganguly (1961).

Separation, identification and estimation of vitamin A alcohol, aldehyde and acid. Two methods, as described below, were used for the chromatographic separation of these components.

(*a*) Adsorption chromatography. When a mixture of these components (0.5 mg. each), dissolved in a small volume of light petroleum, was passed through a 10% deactivated alumina column (1 cm. $\times$ 5 cm.), the retinene could be first eluted with 20 ml. of 2% (v/v) acetone in light petroleum (b.p. 40–60°) and the alcohol with 20 ml. of 8% (v/v) ethanol in light petroleum (b.p. 40–60°). The column was

then extruded and the vitamin A acid was eluted from the adsorbent with aq. 30% (v/v) ammonia (sp.gr. 0.88) in methanol. The alkaline extract was acidified with acetic acid and re-extracted with light petroleum, when the vitamin A acid was quantitatively recovered in the light-petroleum layer.

(b) Reverse-phase chromatography on silicone-impregnated filter paper. The technique is essentially that described by Mahadevan & Ganguly (1961) except that the solvent system was methanol-toluene-ammonia (8:1:1). Irrigation for 6 hr. gave complete separation of the three components. On inspection under ultraviolet light the vitamin A alcohol was identified by the characteristic fluorescence, whereas the other two adsorbed ultraviolet light. The retinene, however, could easily be differentiated on the paper by the intense pink colour developed when sprayed with the thiobarbituric acid reagent (Futtermann & Saslaw, 1961). The R_F values in this system for the vitamin A aldehyde, alcohol and acid were 0.4, 0.6 and 0.85 respectively.

For routine estimation column chromatography was more convenient, whereas paper chromatography was used for identifying these components as reaction products. After elution from the alumina column the vitamin A alcohol was estimated by the Carr-Price reaction and retinene by the thiobarbituric acid method (Futtermann & Saslaw, 1961). The acid was estimated in a Beckman model DU spectrophotometer at 350 m μ in ethanol by using the $E_{1\text{cm}}^{1\%}$ value of 1500 (Robeson *et al.* 1955).

Preparation of substrate. Murthy, Mahadevan, Seshadri Sastry & Ganguly (1961) successfully used ethanol as the dispersing agent in work on vitamin A esterases and cholesterol esterases, but it could not be used in the present system since ethanol itself would act as a substrate for the vitamin A alcohol dehydrogenase. Tween dispersions of retinene were, however, satisfactory. Aqueous dispersions of retinene with Tween 20 were prepared as described by Seshadri Sastry & Ganguly (1961); such dispersions usually contained 2 μ moles of retinene and 15 mg. of Tween 20/ml.

Animals. Male rats of this Institute strain, raised on normal diets with a restricted, but adequate, supply of vitamin A, were used when they weighed about 150 g. Their vitamin A reserves were then about 50 μ g./liver. They were killed under light ether anaesthesia by direct cardiac puncture. The livers were immediately removed and transferred into beakers, placed in crushed ice, for the preparation of the enzymes.

Enzyme assay. The reaction mixtures contained: 0.5 ml. of the enzyme, 0.5 ml. of substrate dispersion and 3 ml. of phosphate buffer ($\text{Na}_2\text{HPO}_4\text{--NaH}_2\text{PO}_4$; 0.1 M, pH 7.20). Water was added, to make the final volume 5 ml. Incubation was carried out for 1 hr. at 37°, when the reaction was stopped by the addition of 5 ml. of ethanol and the reaction mixture extracted twice with 20 ml. portions of light petroleum (b.p. 40–60°). The extracts were pooled and evaporated to dryness under reduced pressure and redissolved in a small amount of light petroleum. The final products were subsequently separated and estimated as described above.

RESULTS

Intracellular distribution. The cell fractions of rat-liver homogenates were isolated as described by Krishnamurthy, Seshadri Sastry & Ganguly (1958)

except that the microsomal fraction was sedimented by centrifuging at 85000g for 60 min. in a Spinco model L ultracentrifuge; fractions equivalent to 200 mg. of the original tissue were incubated with retinene. Table 1 shows that both the oxidizing and reducing enzymes are quantitatively localized in the soluble portion of the cytoplasm (see also Zachman & Olson, 1961).

Separation of the oxidizing and reducing enzymes. The supernatant fraction obtained from a 10% homogenate in water of rat liver by centrifuging it for 60 min. at 85000g in the Spinco ultracentrifuge was fractionated with solid ammonium sulphate, and the fractions that were precipitated in the ranges 0–45, 45–70 and 70–90% saturation were redissolved in water. A clear separation of the oxidizing and reducing enzymes of the supernatant was obtained (Table 2). The oxidizing enzyme was recovered in the 0–45% fraction, whereas the reducing enzyme was in the 45–70% fraction. The third fraction contained neither of these enzymes.

Table 2 shows that efficient reduction of retinene can take place only when GSH and NADH_2 are added together; GSH, NAD or NADH_2 , or a combination of GSH and NAD, had no such effect on the same reaction. However, the unfractionated supernatant shows considerable activity without added NADH_2 . It is possible that some NADH_2 -generating system is present in this fraction to a limited extent, and this is probably removed during the ammonium sulphate fractionation.

The formation of the acid did not require GSH or NAD and thus the rat-liver enzyme would appear to be different from the rabbit-liver enzyme (Elder & Topper, 1961), which is NAD-dependent. However, NADH_2 was consistently found to activate this reaction, an observation difficult to explain.

Stoichiometry of the oxidation reaction and identification of the product. There was a strict stoichiometry between the appearance of the acid and the disappearance of the aldehyde (Table 3). Further,

Table 1. *Intracellular distribution of the oxidizing and reducing enzymes*

The homogenate and various cell fractions equivalent to 200 mg. of wet tissue were incubated with retinene under standard conditions for 60 min. at 37°. The vitamin A alcohol and the acid were estimated as described in the Materials and Methods section, and the results are given as μ m-moles formed/60 min./reaction mixture.

Fraction	Vitamin A acid	Vitamin A alcohol
Homogenate	162	86
Nuclear	14	8.2
Mitochondrial	3.4	2.6
Microsomal	7.0	4.0
Supernatant	134.6	58.4

Table 2. Separation of the oxidizing and reducing enzymes

The rat-liver supernatant was fractionated by ammonium sulphate and the enzyme activities were assayed as described in the text. NAD (1 μ mole), NADH₂ (1 μ mole) or GSH (2 μ moles) was added to the reaction mixture, individually or in combination, wherever indicated. The vitamin A alcohol and the acid were estimated as described in the Materials and Methods section, and the results are given as μ m-moles formed/60 min./reaction mixture.

Fraction	Protein/ reaction mixture (mg.)	None			GSH			NAD			NADH ₂			GSH + NAD			GSH + NADH ₂		
		Vitamin A		alcohol	Vitamin A		alcohol	Vitamin A		alcohol	Vitamin A		alcohol	Vitamin A		alcohol	Vitamin A		alcohol
		acid	acid		acid	acid		acid	acid		acid	acid		acid	acid		acid	acid	
Whole supernatant	13.2	63	127	0	64	126	0	66	125	0	113	175	0	65	132	0	115	182	0
0-45% (NH ₄) ₂ SO ₄ fraction	4.1	0	104	0	0	106	0	0	108	0	1	146	0	0	110	0	0	147	0
45-70% (NH ₄) ₂ SO ₄ fraction	4.6	11	2	0	13	2	0	13	4	0	19	7	0	15	3	0	110	5	0
70-90% (NH ₄) ₂ SO ₄ fraction	3.7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

paper chromatography failed to reveal any product other than vitamin A acid. The spectrum of the product in ethanol is given in Fig. 1(a). The λ_{max} . at 350 m μ agrees very closely with that reported in the literature for vitamin A acid and also with that of an authentic sample obtained from Hofmann-La Roche. Moreover, it had the same R_f as that of an authentic sample of vitamin A acid on the paper chromatogram. Because of the stoichiometry observed and the ease of the colorimetric estimation of retinene, it was more convenient to follow the progress of the reaction by measuring the amount of retinene that disappeared.

General properties of the oxidizing enzyme. The formation of the acid was proportional to the time of incubation up to 60 min. It increased with the amount of enzyme added. The activity increased with increasing pH up to 9.0 (0.1M-phosphate buffer), but there was no definite peak. In all the

Table 3. Stoichiometry of the oxidation reaction

Retinene was incubated under standard conditions with increasing amounts of the 0-45% saturated ammonium sulphate fraction. Retinene and vitamin A acid were estimated as described in the Materials and Methods section, and the results are expressed as μ m-moles present/reaction mixture after incubation for 60 min.

Enzyme protein added (mg.)	Retinene	Vitamin A acid
0	1062	0
1	1012	49
2	955	108
3	912	150
4	874	189
5	846	214

Fig. 1. (a) Absorption spectrum of vitamin A acid in ethanol. —, Authentic sample; ---, isolated from the enzyme reaction. (b) Absorption spectrum of vitamin A aldehyde in ethanol. —, Prepared by oxidizing vitamin A alcohol; ---, isolated from rat liver.

experiments with this enzyme, 0.1M-phosphate buffer, pH 7.2, was used.

Effect of dialysis. Dialysis against water or 0.01N-ammonia for 18 hr. in the cold resulted in a loss of about 22 % of the activity. The cofactors, as indicated in Table 4, had a more pronounced effect on the enzyme dialysed against aqueous ammonia. Thus the addition of FAD or sodium molybdate or both restored the entire activity. There was a slight activation by Fe^{2+} ions. However, Fe^{3+} ions inhibited the undialysed as well as the dialysed enzymes and this inhibition was enhanced by the addition of FAD. Neither NAD nor NADP could restore the lost activity.

Effect of metal chelators, metal ions and sulphhydryl reagents on the oxidation of retinene. At a final concentration of 1 mM most of the metal chelators inhibited the activity considerably (Table 5). However, it was consistently observed that azide has no such effect. The inhibition by arsenite and by *p*-chloromercuribenzoate indicated the sulphhydryl nature of the enzyme, whereas the inhibition by Atabrine should indicate the involvement of FAD (Hass, 1944; Mahler, Mackler & Green, 1954). This is further supported by the fact that added FAD can reverse the inhibition caused by Atabrine (Table 6). However, FMN and riboflavin were ineffective. These observations would therefore indicate the possibility of the enzyme being a metalloflavoprotein.

The above experiments show that metals are associated with this enzyme and the effects of various metal ions were therefore tested (Table 7). The action of these ions on the enzyme previously incubated with diethyldithiocarbamate is also included in Table 7. The enzyme was activated considerably by Fe^{2+} ion, at the lower concentration used, whereas the activation by molybdate and Mg^{2+} ions was not so pronounced. In contrast, Cu^{2+} ion was a powerful inhibitor. Whereas the inhibition by diethyldithiocarbamate was not reversed by any of the other metal ions tested, Co^{2+} ion fully restored the activity.

Other sources of the oxidizing enzyme. Besides the liver, small intestine and kidneys of the rat had considerable activity. Pigeon-liver homogenates oxidized retinene to the acid and, as with rat liver, the activity was localized in the supernatant fraction and could be precipitated by a similar ammonium sulphate (0.45 % saturation) concentration. The specific activities of the purified enzymes were 80 and 77 μm -moles of acid formed/mg. of protein/hr. for rat and pigeon liver respectively. Since pigeon liver does not contain xanthine oxidase (Morgan, 1926; Richert & Westerfeld, 1951), this enzyme is not responsible for the oxidation of retinene.

Presence of retinene in normal rat liver. The occurrence of retinene in the retina and its role in

the visual process is well known. However, the possibility of its occurrence in other tissues of different animals has received attention only recently. Thus Plack & Kon (1961) screened various tissues of different species of animals and presented data on their retinene contents. However, the presence of retinene in rat liver does not seem to have been conclusively established because, though Winterstein & Hegedus (1960) claimed that rat liver contains 2 μg . of retinene/g., Futterman &

Table 4. *Effect of dialysis and cofactors on the oxidation of retinene*

Samples of the 0.45 % saturated ammonium sulphate fraction were dialysed against aq. 0.01N-ammonia or water for 18 hr. at 2–5°, then against 0.1M-tris buffer, pH 8.1, for 3 hr. Suitable portions of the undialysed and dialysed enzyme solutions were assayed for the oxidation of retinene in the presence or absence of 0.2 μm ole of each of the compounds/digest. Results are expressed as μm -moles of vitamin A acid formed/60 min./reaction mixture.

Treatment	Undialysed	Dialysed against water	Dialysed against ammonia
Enzyme alone	345	270	265
Enzyme + FAD	335	240	330
Enzyme + Fe^{2+}	390	270	285
Enzyme + sodium molybdate	330	255	330
Enzyme + Fe^{3+}	210	180	165
Enzyme + NAD	330	270	265
Enzyme + NADP	360	270	250
Enzyme + FAD + Fe^{2+}	390	240	345
Enzyme + FAD + Fe^{3+}	120	105	90
Enzyme + FAD + sodium molybdate	330	240	330

Table 5. *Effect of metal chelators and sulphhydryl reagents*

The enzyme (0.5 ml. of 0.45 % saturated ammonium sulphate fraction containing 4.1 mg. of protein), phosphate buffer (3 ml.) and the solution of the inhibitor (0.5 ml.) were preincubated at 37° for 15 min., after which the reaction mixture was completed. The rest of the procedure was as described in the Materials and Methods section.

Final concn. of inhibitor ...	Inhibition (%)		
	1 mM	0.1 mM	0.01 mM
Inhibitor			
Sodium arsenite	70	30	10
Sodium cyanide	67	60	60
Sodium azide	10	0	0
Atabrine	90	50	10
<i>o</i> -Phenanthroline	50	30	20
8-Hydroxyquinoline	50	10	0
α -Bipyridyl	60	10	0
Sodium diethyldithiocarbamate	70	50	50
<i>p</i> -Chloromercuribenzoate	80	20	0

Table 6. *Reversal of Atabrine inhibition by flavin-adenine dinucleotide*

The conditions were as described in Table 5 except that the indicated amounts of FAD were added after preincubation of the enzyme (0-45% saturated ammonium sulphate fraction) with Atabrine for 15 min.

Atabrine (0.1 mM)	FAD (μ mole)	Vitamin A acid formed (μ m-moles/ 60 min./reaction mixture)
-	0	225
+	0	115
+	0.1	150
+	0.2	170
+	0.4	175
+	0.5	175

Table 7. *Effect of metal ions*

The conditions were as described in Table 5. In the experiments where the reversal of the diethyldithiocarbamate inhibition was tested (column 4), the enzyme (0-45% saturated ammonium sulphate fraction) and buffer were preincubated with the chelator (final concn. 0.1 mM) for 10 min. and then the given metal salts were added so as to give a final concentration of 1 mM.

Final concn. of compounds	Activity retained (%)		Chelator, 0.1 mM, + metal, 1 mM
	1 mM	0.1 mM	
Treatment			
None	100	100	
Sodium diethyl- dithiocarbamate	30	43	
Ferrous sulphate	108	143	40
Sodium molyb- date	100	114	44
Copper sulphate	21	21	0
Manganous sul- phate	100	104	40
Magnesium chloride	100	118	50
Cobalt nitrate	107	107	100

Saslaw (1961) failed to find any, and Wolf, Kahn & Johnson (1957) could not find retinene in rat liver after the injection of radioactive vitamin A into the rat.

When rat liver (35 g. of wet tissue) was extracted with light petroleum and ethanol in a Waring Blendor (Ganguly, Krishnamurthy & Mahadevan, 1959) and chromatographed on weakened alumina, the characteristic retinene band was clearly visible in the column, between the vitamin A alcohol and the ester bands, on inspection under ultraviolet light. The ester was separately eluted with light petroleum, after which the retinene could be eluted with 2% (v/v) acetone in light petroleum. The latter fraction gave the characteristic spectrum of retinene (Fig. 1b) and also the total amount was found to be 40 μ g./35 g. On further chromatography on

silicone-impregnated filter paper, it moved as a single band with the same R_F as that of a known retinene sample and gave a pink band when sprayed with the thiobarbituric acid reagent. It thus seems that rat liver contains definite, though small, amounts of retinene. It is possible that Futterman & Saslaw (1961) used too small a quantity of rat-liver tissue (1 g.) to obtain enough of the compound for identification. However, our attempts at the identification of vitamin A acid in rat liver have not been successful.

DISCUSSION

Bliss (1951) concluded that the liver alcohol dehydrogenase is responsible for the NAD-dependent oxidation of vitamin A alcohol to retinene and that it is a reversible reaction; Zachman & Olson (1961) have confirmed this.

We have now demonstrated that the alcohol dehydrogenase of rat liver can be separated from another enzyme that catalyses the oxidation of retinene to vitamin A acid. The pig-liver aldehyde oxidase, prepared according to Mahler *et al.* (1954), can carry out the oxidation of retinene to the acid (Dmitrovsky, 1961). Aldehyde oxidase is a molybdo-flavoprotein (Mahler, 1956); our results show that retinene oxidase also is probably a molybdo-flavoprotein, because the Atabrine inhibition could be reversed by FAD, sodium molybdate reactivated the dialysed enzyme and ferrous sulphate stimulated the undialysed enzyme. These properties seem to be common to the aldehyde oxidase and retinene oxidase. None the less, there is a slight difference between their properties, since in aerobic reactions metal chelators inhibit the oxidation of retinene (Table 5), though the aldehyde oxidase is not inhibited under similar conditions (Mahler *et al.* 1954). These slight differences do not appear to be enough to draw any conclusions about the specificity of retinene oxidase; however, xanthine oxidase (yet another molybdo-flavoprotein) is not the enzyme responsible for this reaction, since we have demonstrated that pigeon liver, which is free of xanthine oxidase (Richert & Westerfeld, 1951), rapidly oxidizes retinene. Irrespective of whether retinene oxidase is specific or not, this enzyme assumes an important physiological role in the oxidation of retinene to the acid, not only because the acid supports growth of rats, but also because it is even more potent than the alcohol in this respect (P. Malathi, K. Subba Rao, P. Seshadri Sastry & J. Ganguly, unpublished work). We have also now demonstrated the presence of retinene in rat liver, which could readily provide the substrate for such an enzyme.

The only known major function of retinene in animal physiology is in the visual system, but our

results assign yet another equally important role to this compound in that it can act *in situ* as the precursor for the acid. If it were assumed that vitamin A acid or some other compound derived from it is the hidden 'active' form of vitamin A, it would appear that retinene occupies a pivotal position in the metabolic pathway of vitamin A, because it can be directly utilized in the visual system, and at the same time it can be converted into the 'active' vitamin A. It therefore follows that the vitamin A alcohol dehydrogenase and retinene oxidase are of vital importance for controlling the metabolism of vitamin A.

However, we were unable to detect vitamin A acid in normal rat liver, possibly owing to the inadequacy of the techniques in the detection of trace amounts of the acid. Since the equilibrium of the vitamin A alcohol dehydrogenase reaction is far towards the alcohol (Bliss, 1951), resulting in the formation of very low amounts of retinene, it does appear reasonable to expect low concentrations of the acid in tissues. In addition to the slow rate of formation of the acid, it is also possible that once it is formed it is rapidly metabolized or degraded by a mechanism involving decarboxylation. It is also possible that it might be esterified with some endogenous alcohol. These processes singly or together should be able to remove any excess of vitamin A acid, if formed, and thus prevent the development of toxicity, because vitamin A acid is very toxic in higher doses (Thompson & Pitt, 1960).

SUMMARY

1. Enzymes catalysing the oxidation and reduction of retinene to vitamin A acid and alcohol respectively were present in rat-liver supernatant and were separated by ammonium sulphate fractionation.

2. The oxidase was inhibited by *p*-chloro-mercuribenzoate, arsenite, Atabrine and by a number of metal-chelating agents. Whereas Fe^{2+} ion activated the undialysed enzyme, FAD and sodium molybdate reactivated the enzyme dialysed against aqueous ammonia.

3. The oxidase activity was also present in the kidneys and small intestine of rats as well as in pigeon liver.

4. It was conclusively proved by isolation, absorption spectrum, colour reaction and paper chromatography that retinene occurs in normal rat liver. The role of retinene, of alcohol dehydrogenase and of retinene oxidase in the metabolism of vitamin A in the rat is discussed.

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