Oxidation of Vitamin A_1 Aldehyde and Vitamin A_2 Aldehyde to the Corresponding Acids by Aldehyde Oxidase from Different Species

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It is well known that vitamin A_1 predominates in mammals and birds (vitamin A_1 species), whereas vitamin A_2 is characteristic of freshwater fishes (vitamin A_2 species; Moore, 1957). So far, most of the bioassays of vitamin A compounds have been carried out on rats and the results have been assumed to apply to other species. However, Harris (1960) has pointed out that such approaches may prove erroneous. Thus Gillam, Heilbron, Jones & Lederer (1938) have shown that some of the freshwater-fish-liver oils may be toxic to rats, and Jensen, Shantz, Embree, Cawley & Harris (1943) have observed that, in massive doses, vitamin A_2 is not as well tolerated by rats as are equal doses of vitamin A_1. All this seems to imply that there are species differences in the metabolism of vitamin A_1 and vitamin A_2 and in their biological activities.

The two major pathways of metabolism of vitamin A_1 are (*a*) the conversion of vitamin A_1 alcohol into vitamin A_1 aldehyde, brought about by liver alcohol dehydrogenase, which has been studied by Bliss (1951) and by Zachman & Olson (1961), and (*b*) the oxidation of vitamin A_1 aldehyde to vitamin A_1 acid catalysed by aldehyde oxidase (Dmitrovsky, 1961; Futterman, 1962). Subsequent to our initial report (Bamji, Mahadevan, Lakshmanan & Murthy, 1962), Mahadevan, Murthy & Ganguly (1962) have also described the oxidation of vitamin A_1 aldehyde to vitamin A_1 acid by an enzyme from rat liver. Since the work of Dowling & Wald (1960), it is recognized that vitamin A_1 acid is nearer to 'active vitamin A', and several other workers (Van Dorp & Arens, 1946*a*; Redfearn, 1960; Thompson, Howell & Pitt, 1961*a*, *b*; Malathi, Subba Rao, Seshadri Sastry & Ganguly, 1963) have substantiated that vitamin A_1 acid has considerable biological activity in the rat. Aldehyde oxidase, which brings about the conversion of vitamin A_1 aldehyde into vitamin A_1 acid, may play a pivotal role in the metabolism of vitamin A. Thus a comparison of activities of the aldehyde oxidases from vitamin A_1 species and vitamin A_2 species might lead to a clearer understanding of the metabolism of these vitamins and of their biological activities. In the present paper are described the species specificities of hepatic aldehyde oxidases towards vitamin A_1 aldehyde and vitamin A_2 aldehyde, and some of the properties of the enzymes obtained from rat, pig, chicken, monkey and several freshwater fishes.

MATERIALS AND METHODS

Vitamin A_1 aldehyde was prepared by the oxidation of synthetic vitamin A_1 alcohol with manganese dioxide. It was purified by repeated chromatography on deactivated alumina columns according to the procedure of Ball, Goodwin & Morton (1948). The final product had an absorption maximum at 381 mμ in ethanol ($E^{1\%}_{1cm.}$ 1376). Vitamin A_2 was isolated from high-potency liver oil of the freshwater fish *Wallago attu* (Balasundaram, Cama, Sundaresan & Varma, 1956), and vitamin A_2 aldehyde was obtained in pure form by the oxidation of the alcohol (Cama *et al.* 1952). Its absorption maximum was at 397 mμ in ethanol ($E^{1\%}_{1cm.}$ 1410). Spectroscopically pure ethanol was prepared by refluxing rectified spirit with zinc dust and potassium hydroxide for 6 hr. and distilling it twice. Thiobarbituric acid and 2,3-dimercaptopropanol (BAL) were from L. Light and Co. Ltd., Colnbrook, Bucks.; thiourea was from Hopkin and Williams Ltd., London; FAD and *p*-chloromercuribenzoate were from Sigma Chemical Co., St Louis, Mo., U.S.A.; menadione, vitamin A_1 acid and vitamin A_2 acid were from Hoffmann–La Roche; GSH was from British Drug Houses Ltd., India, and was more than 95% pure as tested in our Laboratory. Other chemicals and reagents were from E. Merck Ltd.

Animals. Normal albino rats of either sex, obtained from our Institute colony and maintained on a normal diet, were killed under light ether anaesthesia by direct cardiac puncture. Livers were perfused by passing ice-cold physiological saline (0·9% sodium chloride) through the inferior vena cava of the anterior side, the posterior side being cut open for outlet to the blood and injected saline. After perfusion, the organs were almost completely free of blood. They were immediately transferred to beakers kept in crushed ice for the preparation of the enzyme. Normal monkeys of the Institute colony were killed under light ether anaesthesia by puncture of the jugular vein and the livers were treated as for the rat livers. Normal chicken and pig livers were obtained fresh; freshwater-fish livers were procured fresh from the Fisheries Department, Hessaraghatta Lake, Bangalore.

Preparation of the enzyme. The method employed in the preparation of aldehyde oxidase was that of Mahler, Mackler & Green (1954). Enzyme prepared by the method of Rajagopalan, Fridovich & Handler (1962) gave concordant results.

Enzyme assay. The reaction mixture consisted of 0·1 ml. of enzyme (containing 0·1–1·0 mg. of protein) and 0·1 ml. of ethanolic vitamin A_1 aldehyde or vitamin A_2 aldehyde (0·1 μmole), the volume being made up to 2·0 ml. with 0·1 M-sodium phosphate buffer, pH 7·5, or with 0·1 M-sodium borate buffer for pH values above 8. After incubation for 30 min. at 37°, the reaction was stopped by adding 5·0 ml. of ethanol. The reaction mixture was extracted twice with light petroleum (b.p. 40–60°), and the pooled extracts were evaporated to dryness under reduced pressure and taken up in a small quantity of light petroleum. The unutilized aldehyde and the product were separated and estimated as described below. Protein was assayed either by the spectrophotometric method of Waddell (1956), as modified by Murphy & Kies (1960), or by the colorimetric procedure of Lowry, Rosebrough, Farr & Randall (1951). In all these experiments 0·1 M-sodium phosphate buffer, pH 7·5, was used unless otherwise stated. Specific activity is defined as μm-moles of vitamin A_1 acid or vitamin A_2 acid formed/mg. of enzyme protein during 30 min.

Separation, identification and estimation of vitamin A_1 aldehyde, vitamin A_1 acid, vitamin A_2 aldehyde and vitamin A_2 acid; (*a*) Adsorption chromatography. The mixture of either vitamin A_1 aldehyde and vitamin A_1 acid or vitamin A_2 aldehyde and vitamin A_2 acid (100 μg. of each) in light petroleum was separated by chromatography on a column (1 cm. × 10 cm.) of 10%-deactivated acid-washed alumina (Farrar, Hamlet, Henbest & Jones, 1952*b*). The aldehyde fraction was completely eluted with light petroleum, but the acid fraction was strongly adsorbed. The adsorbent was extruded from the column and the acid fraction eluted from it with aq. ammonia (sp.gr. 0·88)–ethanol (3:7, v/v). The alkaline extract was acidified with acetic acid and vitamin A acid quantitatively re-extracted with light petroleum.

(*b*) Reverse-phase chromatography on paper impregnated with petroleum jelly (obtained from Stanvac and Co., India). The technique was that described by Jungalwala & Cama (1962) which leads to the complete separation of either of the two mixtures mentioned above (R_F values: vitamin A_1 aldehyde, 0·55; vitamin A_1 acid, 0·65; vitamin A_2 aldehyde, 0·67; vitamin A_2 acid, 0·84). Both the acid and the aldehyde bands absorbed ultraviolet light. However, the aldehydes were identified by spraying with thiobarbituric acid (Futterman & Saslaw, 1961), when they gave an intense pink colour. Effective separation of these compounds could also be obtained with ethanol–acetic acid (3:1, v/v) as developing solvent.

Vitamin A_1 aldehyde and vitamin A_2 aldehyde were estimated in a Beckman model DU spectrophotometer by the method of Futterman & Saslaw (1961), by using 530 and 550 mμ respectively as their absorption maxima. Vitamin A_1 acid and vitamin A_2 acid were estimated from $E^{1\%}_{1cm.}$ values of 1500 at 350 mμ and 1395 at 370 mμ respectively (Kofler & Rubin, 1960).

RESULTS

The results obtained for monkey-liver aldehyde oxidase are presented in Tables 1 and 3–8.

Stoicheiometry of the oxidase reaction and identification of the product. There was strict relationship between the utilization of aldehyde and formation of acid (Table 1). Moreover, depending on the substrate used, the formation of any product other than vitamin A_1 acid or vitamin A_2 acid could not be detected by paper or column chromatography. The products had absorption maxima at 350 and 370 mμ respectively, which agree closely with those of authentic samples of vitamin A_1 acid and vitamin A_2 acid (Kofler & Rubin, 1960). Since the enzyme reaction was strictly stoicheiometric, the progress of the reaction was always followed by measuring the amount of the aldehyde that was utilized, which was more accurate and easier than the estimation of vitamin A acid.

Activities of aldehyde oxidases from different species. The specific activities of liver aldehyde oxidases from rat, pig, chicken, monkey and several freshwater fishes are presented in Table 2. The rate of acid formation was linear during the incubation period of 30 min. The pH optimum for the enzyme varied according to species from 7·5 to 10·0, although in most of the cases there was no definite peak (Table 2.)

Effect of inhibitors, metal ions and activators on the oxidase reaction. Inhibitions by *p*-chloromercuribenzoate and arsenite (Table 3) indicate

Table 1. *Stoicheiometry of the oxidation of vitamin A aldehydes to the appropriate acids by the aldehyde oxidase of monkey liver*

Vitamin A_1 aldehyde and vitamin A_2 aldehyde (1 μmole of each) were each incubated with increasing concentrations of the enzyme under standard conditions. The aldehydes and the corresponding acids were estimated as described in the Materials and Methods section and the results are expressed as μm-moles/reaction mixture after incubation for 30 min.

Enzyme protein added (mg.)	Vitamin A_1 aldehyde utilized	Vitamin A_1 acid formed	Vitamin A_2 aldehyde utilized	Vitamin A_2 acid formed
0	0	0	0	0
0·5	95	95	106	104
1·0	196	192	209	208
1·5	288	285	315	312
2·0	382	378	422	420
2·5	479	480	528	525

Table 2. *Specific activities of the aldehyde oxidase from livers of rat, pig, chicken, monkey and freshwater fishes*

Vitamin A_1 aldehyde or vitamin A_2 aldehyde was incubated with each enzyme preparation in duplicate at its optimum pH under standard conditions. The results are expressed as μm-moles of acid formed/mg. of protein after incubation for 30 min. Each value represents the mean ± S.E. for six animals, except for freshwater fishes where single values are given.

Species	Activity with vitamin A_1 aldehyde	Optimum pH	Activity with vitamin A_2 aldehyde	Optimum pH
Rat	273·2±1·56	8·1	133·4±1·4	8·3–8·6
Pig	154·5±1·16	7·9–10·0	151·6±0·73	7·9–10·0
Chicken	22·8±0·57	7·5–9·0	23·7±0·45	7·5–9·0
Monkey	193·6±0·98	7·5–8·8	220·3±1·53	7·5–8·8
Freshwater fishes				
Ophiocephalus muralius (i)	46·6	9·1–9·5	109·3	9·1–10·0
O. muralius (ii)	53·5	9·1–9·5	115·3	9·1–10·0
Labeo calbasu (i)	30·6	9·1–9·5	63·8	9·1–10·0
L. calbasu (ii)	36·4	9·1–9·5	76·6	9·1–10·0
Cyprinus carpio communis (i)	20·1	9·1–9·5	38·3	9·1–10·0
C. carpio communis (ii)	21·8	9·1–9·5	46·8	9·1–10·0

Table 3. *Effect of inhibitors, heavy-metal salts and activators on the aldehyde oxidase of monkey liver*

Enzyme containing 0·2 mg. of protein was preincubated with the indicated final concentration of the compounds for 10 min. at 37° before the substrate was added. The rest of the procedure was as described in the Materials and Methods section.

Final concn. of compound	Activity (%) 1 mM	0·1 mM	0·01 mM
p-Chloromercuri-benzoate*	20	20	65
Sodium arsenite	30	30	68
Sodium azide	90	100	100
Sodium cyanide*	30	30	60
Atabrine	0	55	90
Menadione	15	28	60
p-Benzoquinone	20	20	58
Copper sulphate	10	60	92
Cadmium chloride	0	58	85
Sodium molybdate	110	100	100
Sodium arsenate	105	100	100
GSH	105	100	100
2,3-Dimercaptopropanol	103	100	100

* In these experiments, the concentrations of the inhibitor were 2 mM, 0·2 mM and 0·02 mM respectively.

Table 4. *Protection by reduced glutathione against the inhibition by* p-*chloromercuribenzoate of the aldehyde oxidase of monkey liver*

The conditions were as described in Table 3 except that the enzyme was preincubated with the indicated concentration of the protecting agent before the inhibitor was added.

Concn. of *p*-chloromercuri-benzoate (mM)	Concn. of GSH (mM)	Vitamin A_2 acid formed (μm-moles/reaction mixture/30 min.)
0	0	42
0·2	0	8·5
0·2	0·5	20·0
0·2	1·0	39·0

the thiol nature of the enzyme. The inhibition by *p*-chloromercuribenzoate could be completely prevented by GSH (Table 4). Since the inhibition by arsenite could be prevented only by very high concentrations of 2,3-dimercaptopropanol (Table 5) and as the complete inhibition by Cd^{2+} ions could not be prevented by 2,3-dimercaptopropanol (Table 6), the involvement of dithiol groups in the enzyme is uncertain. Inhibition by Atabrine (Table 3) indicates the involvement of FAD (Haas, 1944; Mahler *et al.* 1954), and this is further supported by the fact that FAD could protect the enzyme against the inhibitory action of Atabrine (Table 7). However, FMN and riboflavin were ineffective. Inhibition by cyanide could be completely prevented by sodium molybdate, but other metal salts were ineffective, except tungstate which had a slight protecting effect (Table 8). These observations imply the involvement of a heavy metal in the activity of the enzyme. The inhibition by menadione (Table 3) is very striking in that it differentiates this enzyme from partially purified milk xanthine oxidase which can also catalyse the conversion of vitamin A_1 aldehyde into vitamin A_1 acid (Futterman, 1962) but which is unaffected by menadione (Rajagopalan *et al.* 1962).

DISCUSSION

Vitamin A_1 acid possesses as high or somewhat higher biological activity than either the alcohol or aldehyde forms (Van Dorp & Arens, 1946*b*; Malathi *et al.* 1963). Hence most of the functions of vitamin A can be completely fulfilled by vitamin A acid or by some of its derivatives except probably its functions in vision and reproduction (Thompson *et al.* 1961*a*, *b*). Thus the aldehyde oxidase which is responsible for the metabolic oxidation of vitamin A aldehyde to vitamin A acid may be of importance

Table 5. *Protection by 2,3-dimercaptopropanol against the inhibition by arsenite of the aldehyde oxidase of monkey liver*

The conditions were as described in Table 4.

Concn. of sodium arsenite (mM)	Concn. of 2,3-dimercapto-propanol (mM)	Vitamin A_2 acid formed (μm-moles/reaction mixture/30 min.)
0	0	42·5
0·1	0	12·6
0·1	1·0	19·0
0·1	10·0	41·8

Table 6. *Protection by 2,3-dimercaptopropanol against the inhibition by cadmium ions of the aldehyde oxidase of monkey liver*

The conditions were as described in Table 4 except that 0·1 M-tris buffer, pH 8·0, was used.

Concn. of cadmium chloride (mM)	Concn. of 2,3-dimercapto-propanol (mM)	Vitamin A_2 acid formed (μm-moles/reaction mixture/30 min.)
0	0	41·4
0·1	0	24·0
1·0	0	0
0·1	1·0	33·0
0·1	10·0	40·0
1·0	10·0	6·0

Table 7. *Protection by flavin–adenine dinucleotide against the inhibition by Atabrine of the aldehyde oxidase of monkey liver*

The conditions were as described in Table 4.

Concn. of Atabrine (mM)	Concn. of FAD (mM)	Vitamin A_2 acid formed (μm-moles/reaction mixture/30 min.)
0	0	209
0·1	0	95
0·1	0·05	136
0·1	0·10	172
0·1	0·20	178
1·0	0·20	100

Table 8. *Protection by molybdate against and the effect of other metal salts on the inhibition by cyanide of the aldehyde oxidase of monkey liver*

The conditions were as described in Table 4.

Concn. of sodium cyanide (mM)	Metal salt (2 mM) added	Vitamin A_2 acid formed (μm-moles/reaction mixture/30 min.)
0	None	42·2
0·2	None	12·5
0·2	Sodium molybdate	39·4
0·2	Sodium tungstate	17·2
0·2	Ferrous sulphate	12·6
0·2	Copper sulphate	4·0
0·2	Manganous sulphate	12·8
0·2	Magnesium chloride	12·5
0·2	Cobalt nitrate	12·9

in vitamin A metabolism. Our results show that, whereas the enzyme from rat liver is twice as active with vitamin A_1 aldehyde as with vitamin A_2 aldehyde, that from freshwater fishes is more active with vitamin A_2 aldehyde. This implies that the rat is unable to metabolize vitamin A_2 aldehyde and related compounds as efficiently as vitamin A_1 aldehyde and other compounds. It may also explain why the biological activities of vitamin A_2 compounds are considerably less than those of vitamin A_1 compounds when rats are the test animals (Shantz, 1948; Farrar, Hamlet, Henbest & Jones, 1952*a*; Sundaresan & Cama, 1961). These conclusions are in agreement with those of Gillam *et al.* (1938), Jensen *et al.* (1943) and Harris (1960). It may well be that vitamin A_2 compounds would be as biologically potent as vitamin A_1 compounds if bioassayed on freshwater fishes, monkeys or pigs. Quite strikingly, the enzymes from the livers of pig and monkey are as active with vitamin A_2 aldehyde as with vitamin A_1 aldehyde. The enzyme from chicken liver is also equally active with vitamin A_1 aldehyde and vitamin A_2 aldehyde but its specific activities are very low with both.

SUMMARY

1. Aldehyde oxidase was isolated from rat, pig, chicken, monkey and several freshwater-fish livers and shown to catalyse the oxidation of the aldehydes of both vitamin A_1 and vitamin A_2 to the corresponding acids.

2. Whereas the enzyme from rat liver was more active with vitamin A_1 aldehyde than with vitamin A_2 aldehyde, that from the freshwater-fish livers was more active with vitamin A_2 aldehyde. The enzymes from pig, chicken and monkey livers were equally active with both.

3. Unlike xanthine oxidase, which can also catalyse the same oxidation, the enzyme is inhibited by menadione.

4. The role of aldehyde oxidase in the metabolism of vitamin A_1 and vitamin A_2 in different species is discussed.

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A Comparison of the Electrophoretic Characteristics of the Human Normal and Sickle Erythrocyte

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The process of sickling in which red cells from sickle-cell-anaemia or sickle-cell-trait subjects lose their biconcave discoid form and become crescent-shaped is now thought to be primarily due to the properties of an abnormal haemoglobin (haemoglobin S) within the cell. Haemoglobin S synthesis *in vivo* is probably under the control of a single gene (the sickle-cell gene) which has undergone mutation (Neel, 1949). Ingram (1957) has shown that the genetically conferred difference between normal and sickle-cell haemoglobin resides in the replacement of glutamic acid by valine in a peptide moiety of the sickle-cell globin, which also accounts for the difference in mobilities of the haemoglobins by paper electrophoresis. Secondary physicochemical differences between normal and sickle cells have been reported in the literature. The net exchange of Cs^+, K^+ and Na^+ ions increases in the order: normal cell, promeniscocyte and sickle cell (Tosteson, Carlsen & Dunham, 1955; Tosteson, 1955). Prankerd (1955) has reported a decrease in [^{32}P]orthophosphate uptake by sickle cells and a diminution in the concentration of adenosine triphosphate. The reduced glutathione (GSH) content of sickle cells is also significantly greater than that in normal cells. Prolonged dialysis against physiological saline will remove the GSH from the red cells and render them incapable of sickling (Schneider, Levin & Haggard, 1949; Kass, Ingbar, Harris & Ley, 1951). Electrophoretic studies on the plasma and serum proteins in sickle-cell anaemia have

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