The Effect of Vitamin A Deficiency on the Biosynthesis of Steroid Hormones in Rats

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1. The synthesis, both *in vivo* and *in vitro*, of various steroid hormones in the adrenals, testes and ovaries of rats was compared at various stages of vitamin A deficiency with the corresponding pair-fed controls. 2. The enzymic conversion of the Δ^5 - 3β -hydroxy steroids into the corresponding Δ^4 -3-oxo steroids was significantly decreased in these tissues even at the mild-deficiency stage, further loss taking place on prolonging the deficiency. 3. In all three tissues the loss in the production of androstenedione from dehydroepiandrosterone could be reactivated *in vitro* by retinol or retinoic acid, but only at the mild stage of the deficiency. 4. Of the various lipoidal materials tried, retinal, retinol, retinoic acid, α -tocopherol acetate, menadione, calciferol, lecithin, palmitic acid and cholesterol, only retinol and retinoic acid restored the lost activity. 5. Intraperitoneal injection of retinal, retinol or retinoic acid into the deficient rats, 24 hr. before they were killed, corrected the effect of the deficiency on this reaction. 6. Vitamin A deficiency markedly affected the synthesis of deoxycorticosterone and corticosterone from pregnenolone in the adrenals of rats, even at the mildly deficient stage, with further loss taking place at the acute stage; *in vitro*, retinol or retinoic acid could restore the loss at the mild-deficiency but not at the acute-deficiency stage. 7. The deficiency had no such effect on the synthesis of deoxycorticosterone from progesterone or of corticosterone from progesterone or deoxycorticosterone. 8. Compared with the pair-fed normals, the adrenals of deficient rats contained smaller amounts of deoxycorticosterone and corticosterone.

Observations from several Laboratories have repeatedly indicated that normal steroidogenesis may depend on the vitamin A-nutritional status of animals. Thus Ershoff (1952) suggested that vitamin A is probably required for the normal biosynthesis and secretion of glucocorticoids. Some support for this idea came from the work of Lowe, Morton & Harrison (1953), who demonstrated that in rat adrenals the zona fasciculata, the zone where glucocorticoid synthesis takes place, suffers histological damage during vitamin A deficiency. Johnson & Wolf (1960) confirmed these observations and claimed that some of the steps in the biosynthesis of glucocorticoids are depressed in vitamin A-deficient rats. In addition, the work of Grangaud and his colleagues (Grangaud & Conquy, 1958; Grangaud, Conquy & Nicol, 1959a,b,c; Grangaud, Nicol, Jouan & LeGall, 1963; Grangaud, Nicol, LeGall & Soussy, 1964) produced further evidence on the possible role of vitamin A in steroid biochemistry in rats. Juneja, Murthy & Ganguly

(1964a) demonstrated that during vitamin A deficiency the concentration of Na^+ in the blood plasma of rats increases and that of K^+ decreases, and suggested that the biosynthesis of the mineralocorticoids also may be affected by the deficiency.

Earlier classical nutrition work had led to the view that vitamin A is necessary for normal reproduction in both male and female rats. However, vitamin A deficiency usually leads to several pathological derangements of animal tissues (Moore, 1957), which obscure the true effects of the deficiency and the correct interpretation of the results. It has recently been shown that both male and female rats, when maintained on retinoic acid instead of retinol or its esters, fail to reproduce (Juneja, Murthy & Ganguly, 1964b; Thompson, Howell & Pitt, 1964). The retinoic acid-treated rats are apparently healthy and free of the pathological conditions that are usually encountered in vitamin A-deficient rats. These experiments have therefore shown that retinol or its esters are required for the normal reproduction of rats of both sexes. Several steroid hormones are necessary for normal repro-

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duction in both sexes, and it is therefore logical to assume that vitamin A deficiency may affect the biosynthesis of some of these steroids also.

Thus the literature reviewed above suggests that vitamin A deficiency may affect the biosynthesis of several steroid hormones. By using systems *in vitro* we show below that the activity of the apparently rate-limiting enzyme system, Δ^5 - 3β -hydroxy steroid dehydrogenase, which has been shown to be present in almost all the steroid-producing glands of many vertebrates (Samuels, 1960), is decreased in the tissues of vitamin A-deficient male and female rats, and that, at the earlier phases of the deficiency, the loss in its activity can be restored by the addition of either retinol or retinoic acid *in vitro*.

MATERIALS AND METHODS

Materials. Pregnenolone,* 17α -hydroxypregnenolone, progesterone, 17α -hydroxyprogesterone, deoxycorticosterone, corticosterone, dehydroepiandrosterone, androstenedione, tetrazolium blue, glucose 6-phosphate, NAD and NADP were obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. Aldosterone was a gift sample from Ciba, Basle, Switzerland. Crystalline retinol, retinyl acetate and retinoic acid were gifts from Hoffmann-La Roche, Basle, Switzerland. Retinal was prepared and purified by the method of Ball, Goodwin & Morton (1948). The purified product had $\lambda_{\max}$ 381 m μ in ethanol. The dispersions of the vitamin A derivatives used for injection were prepared as described by Malathi, Subba Rao, Seshadri Sastry & Ganguly (1963).

Retinol is most unstable both in the crystalline state and in solution, even when antioxidants are present. Therefore extreme care had to be taken that the samples used for experiments *in vitro* were satisfactory, and retinol was therefore always freshly prepared from synthetic retinyl acetate enzymically (Subba Rao & Ganguly, 1966).

Malinckrodt silicic acid for chromatography (100 mesh) was ground in a ball mill for 8 hr. and passed through a 200-mesh sieve. The finer powder thus obtained was used for thin-layer chromatography. Alumina used for column chromatography was obtained from Sarabhai Merck Ltd., Bombay, India. The 4%-deactivated alumina, when used, was prepared by adding 4 ml. of water/100 g. of the alumina as supplied by the manufacturers.

Crude methanol and ethanol were kept over NaOH pellets at room temperature for 48 hr. They were then refluxed with zinc powder for 16 hr. and finally distilled at

60° and 78° respectively. Light petroleum (b.p. 40–60°) was purified as described by Ganguly, Krishnamurthy & Mahadevan (1959). Freshly distilled peroxide-free diethyl ether was used. *n*-Hexane, ethyl acetate, chloroform, acetone and benzene were from the British Drug Houses Ltd., Bombay, India, and methylene dichloride was from E. Merck A.-G., Darmstadt, Germany.

Animals. Rats of both sexes and of this Institute strain were made vitamin A-deficient according to the procedure of Malathi *et al.* (1963). The three stages of vitamin A deficiency were essentially similar to those of Subba Rao & Ganguly (1966), except that the mild-deficiency stage lasted for about 4–6 days, during which the rats lost about 8–10 g. in weight and the severely deficient rats lost about 15 g. At all the three stages of the deficiency, the activities of the enzyme systems of the deficient animals were compared with those of the corresponding pair-fed controls.

The adrenals and testes were obtained from the male rats, and ovaries from the female rats. The female rats were usually killed when they were in the oestrous phase of their oestrous cycle.

Preparation of enzymes and assay of their activities. In those experiments where only the step consisting in the conversion of the Δ^5 - 3β -hydroxyl group of the steroid into the Δ^4 -3-oxo group was studied, the enzymes from the adrenals and ovaries were prepared by the procedure of Rubin, Deane & Hamilton (1963), and those from the testes were obtained by homogenizing the decapsulated tissue in Krebs–Ringer phosphate buffer (pH 7.4, 0.2 M) in an all-glass Potter–Elvehjem homogenizer. The enzyme activities were assayed by the method of Rubin *et al.* (1963).

The activities of such enzyme systems have been assayed by Rubin *et al.* (1963) spectrophotometrically at 240 m μ . By using the solvent system chloroform–*n*-hexane (7:3, v/v), we could readily separate pregnenolone from progesterone by thin-layer chromatography (details given below), which therefore enabled us to estimate, again at 240 m μ , the actual amounts of progesterone formed in the reaction mixtures. The values obtained at 240 m μ before and after separation by thin-layer chromatography were comparable. Therefore the spectrophotometric procedure of Rubin *et al.* (1963) was used for routine work, and the thin-layer system was occasionally used to check these values.

In the experiments where the synthesis of deoxycorticosterone or corticosterone was also investigated, the endogenous steroids of the adrenal glands were first eliminated by preincubating the adrenal glands as described by Koritz & Peron (1959). The incubation medium was rejected. Further processing of the tissues, the incubation conditions and the subsequent extraction with methylene dichloride were as described by Peron (1964).

Extraction of incubation mixtures and whole adrenals. The incubation mixtures where only the activities of the enzyme system converting Δ^5 - 3β -hydroxy steroid into the Δ^4 -3-oxo steroid were assayed were extracted with ethyl acetate by the method of Rubin *et al.* (1963), whereas the others where the synthesis of deoxycorticosterone or corticosterone also was investigated were extracted with methylene dichloride.

With the whole adrenals, the glands were freed of the adhering fatty materials and then crushed in a mortar, after which 2–3 times their weight of water was added and the tissue was thoroughly ground. Methanol was now added in the proportion of twice the volume of the total

* Trivial names used: pregnenolone, 3β -hydroxypregn-5-en-20-one; 17α -hydroxypregnenolone, 3β , 17α -dihydroxypregn-5-en-20-one; progesterone, pregn-4-ene-3,20-dione; 17α -hydroxyprogesterone, 17α -hydroxypregn-4-ene-3,20-dione; deoxycorticosterone, 21-hydroxypregn-4-ene-3,20-dione; corticosterone, 11 β ,21-dihydroxypregn-4-ene-3,20-dione; dehydroepiandrosterone, 3β -hydroxyandrost-5-en-17-one; androstenedione, androst-4-ene-3,17-dione; aldosterone, 11 β ,21-dihydroxy-3,20-dioxopregn-4-en-18-al; tetrazolium blue, 3,3'-dianisolebis-4,4'-(3,4-diphenyl)-tetrazolium chloride.

homogenate. The mixture was thoroughly reground and then extracted with chloroform (2×4 vol.). The combined chloroform extracts were washed twice with water. The pooled aqueous washings were re-extracted with chloroform, after which the chloroform extract was added to the main extract. The combined extracts were dried over anhydrous Na_2SO_4 , evaporated to dryness under vacuum at $40-50^\circ$ and the residue was re-dissolved in diethyl ether for column chromatography on alumina as described below.

Purification and separation of the steroids. (a) Column chromatography. This step was not necessary for the extracts of the reaction mixtures, and was used only for the preliminary purification of the crude extracts obtained from the adrenals. The materials obtained from about 200mg. of adrenals were dissolved in 1.0ml. of diethyl ether and quantitatively transferred on to a column ($30\text{ cm.} \times 1\text{ cm.}$) of 10g. of 4%-deactivated alumina. After the adsorbed lipids had been washed out with 50ml. of 8% (v/v) diethyl ether in light petroleum, 70ml. of 20% (v/v) ethanol in light petroleum was added. The eluate was collected and evaporated to dryness, after which the residue was redissolved in 1.0ml. of chloroform for further purification by thin-layer chromatography.

(b) Thin-layer chromatography. A slurry made of 40g. of silicic acid- CaSO_4 (17:3, w/w) in 80ml. of water was spread over glass plates ($20\text{ cm.} \times 20\text{ cm.}$) to give a thickness of $250\text{ }\mu\text{m}$. The plates were dried in an oven at 100° for 1 hr., after which they were cooled and stored at room temperature. Just before use they were again heated in the oven at 100° for 1 hr.

Pregnenolone and progesterone from the incubation mixtures were readily separated by using the solvent system chloroform-*n*-hexane (7:3, v/v).

In the experiments where the synthesis of deoxycorticosterone or corticosterone was studied, the methylene dichloride extracts were directly subjected to thin-layer chromatography with ethyl acetate-*n*-hexane (1:1, v/v) as solvent. With this solvent system the steroids deoxycorticosterone, corticosterone, 18-hydroxydeoxycorticosterone and aldosterone were easily separated. For the adrenal extracts the materials obtained after the column chromatography were subjected to the same thin-layer system.

The resolved steroids were located under ultraviolet light ($253.7\text{ m}\mu$) before and after exposure to iodine vapour, and also, where desired, by spraying the plates with H_2SO_4 -ethanol (1:1, v/v) followed by heating at 100° for 15 min. or with 0.03% tetrazolium blue solution in 2*N*-NaOH. The steroids thus located were identified by comparing their R_F values with those of a set of similarly resolved standard steroids that were always used as reference on each plate.

The respective areas were marked with a pin and carefully scraped off with a fine scalpel. The collected powder was then transferred to a small chromatographic tube, at the bottom of which a thick cotton plug had previously been inserted. The adsorbed steroid was eluted with 20ml. of methanol. This procedure consistently gave a recovery of 80-90%. We have often found that the cotton plug gives very high blank values for the tetrazolium blue method of corticoid estimation. However, it was possible to remove the interfering materials by washing the column with 30ml. of 10% (v/v) acetone in light petroleum before the methanol was added.

(c) Paper chromatography. The spots resolved on the

thin-layer plates were, when desired, further identified by paper chromatography with reference standards. This procedure therefore gave additional evidence for the identification of the steroids.

Whatman no. 1 filter-paper strips were quickly passed through a solution of propylene glycol in methanol (1:1, v/v). The impregnating solution was then allowed to drip off for a few minutes, after which the paper strips were pressed between two sheets of filter paper. The papers were now ready for use. It has been our experience that, if the impregnated papers are used without blotting (Zaffaroni, Burton & Keutmann, 1950), spotting of the samples as well as the tracing of the solvent front becomes very difficult.

For separating pregnenolone, progesterone and cholesterol, *n*-hexane was used as the mobile phase; benzene was used for the separation of dehydroepiandrosterone and androstenedione, and toluene for aldosterone, corticosterone and deoxycorticosterone. In addition to these, the Bush (1952) systems A and B₁ were also occasionally used.

The spots were detected by inspection under ultraviolet light ($253.7\text{ m}\mu$), by passing the paper strips through a 10% (w/v) solution of phosphotungstic acid in ethanol followed by heating at 100° for 15 min., by passing the strips through 0.01% tetrazolium blue in 2*N*-NaOH, or by exposing them to iodine vapour.

Estimations. Progesterone and aldosterone were estimated spectrophotometrically at $240\text{ m}\mu$. Corticosterone and deoxycorticosterone were measured by the tetrazolium blue method of Elliott, Birmingham, Schally & Schonbaum (1954), as modified by Peron (1962). The method of Lewbart & Mattox (1961) was also used for measuring aldosterone. Proteins were determined by the biuret method of Gornall, Bardawill & David (1949).

RESULTS

Changes in the activities of the enzyme system converting the Δ^5 - β -hydroxy steroids into the corresponding Δ^4 -3-oxo steroids in the adrenals, testes and ovaries of vitamin A-deficient rats. This enzyme system acts on the steroids that contain the Δ^5 - β -hydroxyl group, giving rise to the Δ^4 -3-oxo steroids. The substrates used were 17α -hydroxypregnenolone, pregnenolone and dehydroepiandrosterone. The results in Table 1 demonstrate that the enzymes prepared from all three tissues were active against all three compounds, with dehydroepiandrosterone being the best substrate, pregnenolone coming next and 17α -hydroxypregnenolone being the least active. These results are in close agreement with the observations reported for the adrenals and ovaries of normal rats (Rubin *et al.* 1963), normal bovine adrenals and corpora lutea (Kowal, Forchielli & Dorfman, 1964) and normal bovine adrenals (Koritz, 1964).

Table 1 also shows that at the mildly deficient stage the activities of all the three tissues against all the substrates were decreased by nearly half. That no other compound was formed during the conversion of pregnenolone into progesterone was ascertained by occasional thin-layer chromato-

Table 1. *Effect of vitamin A deficiency on the conversion of the Δ^5 -3 β -hydroxy steroids into the Δ^4 -3-oxo steroids in the adrenals, testes and ovaries of rats*

Each reaction mixture contained 1–1.5 mg. of enzyme protein, 3 mg. of NAD, 0.2 ml. of propylene glycol and 500 μ g. of the steroid. The total volume was made up to 1.9 ml. with the respective buffer that was used for preparing the enzyme. The reaction mixtures for the adrenal enzymes were incubated at 37° for 30 min., whereas those for the ovarian and testicular enzymes were incubated at 37° for 20 min. The values are averages ($\pm$ s.e.m.) of six rats in each group.

Substrate	Activity (μ g. of oxo steroid formed/min./mg. of protein)					
	Pair-fed normals			Mildly deficient		
	Adrenals	Testes	Ovaries	Adrenals	Testes	Ovaries
17 α -Hydroxypregnenolone	0.50 $\pm$ 0.06	0.43 $\pm$ 0.05	0.38 $\pm$ 0.03	0.26 $\pm$ 0.02	0.18 $\pm$ 0.07	0.20 $\pm$ 0.02
Pregnenolone	0.72 $\pm$ 0.13	0.77 $\pm$ 0.08	0.66 $\pm$ 0.07	0.43 $\pm$ 0.05	0.40 $\pm$ 0.06	0.36 $\pm$ 0.04
Dehydroepiandrosterone	1.16 $\pm$ 0.10	1.38 $\pm$ 0.12	1.30 $\pm$ 0.21	0.76 $\pm$ 0.13	0.75 $\pm$ 0.22	0.83 $\pm$ 0.16

Table 2. *Effect of the addition of retinol or retinoic acid in vitro on the conversion of dehydroepiandrosterone into androstenedione at the various stages of vitamin A deficiency in the adrenals, testes and ovaries of rats*

The experimental conditions were the same as given for Table 1, except that retinol or retinoic acid, when added, was dispersed along with the steroid. Values are averages of six individual values from each group.

		Activity (μ g. of androstenedione formed/min./mg. of protein)						
		Normal (fed <i>ad lib.</i>)	Weight plateau		Mild deficiency		Acute deficiency	
			Pair-fed normal	Deficient	Pair-fed normal	Deficient	Pair-fed normal	Deficient
Tissues	Addition (5 μ g./ reaction mixture)							
	Adrenals							
	None	1.20	1.16	1.12	1.16	0.76	1.13	0.37
	Retinol	0.96	0.98	1.26	1.12	1.05	1.10	0.46
	Retinoic acid	1.06	1.03	1.14	1.09	1.23	1.10	0.51
Testes	None	1.42	1.40	1.06	1.36	0.75	1.36	0.28
	Retinol	1.26	1.30	1.32	1.30	1.20	1.28	0.26
	Retinoic acid	1.28	1.29	1.40	1.30	1.40	1.30	0.28
Ovaries	None	1.36	1.32	1.20	1.28	0.84	1.28	0.24
	Retinol	1.22	1.24	1.22	1.26	1.16	1.65	0.31
	Retinoic acid	1.30	1.27	1.42	1.23	1.32	1.26	0.28

graphy of the extracts of the reaction mixtures in various solvent systems. In no case could we detect any other compound except pregnenolone and progesterone. Moreover, the net amounts of pregnenolone plus progesterone recovered after the reactions always accounted for almost all of the pregnenolone added. Therefore the decreased synthesis of progesterone from pregnenolone shown here reflects the net effects of vitamin A deficiency on this particular reaction.

Since dehydroepiandrosterone was the best substrate and since the activity against it was almost proportionally affected in all the tissues, this compound was used as substrate in the subsequent experiments.

Effect of the three stages of vitamin A deficiency on the conversion of dehydroepiandrosterone into androstenedione by the adrenals, testes and ovaries of rats. It is shown in Table 2 that the stage of the deficiency

exerts a profound effect on the activity of the enzyme system, as well as on the ability of the added vitamin A derivatives to restore the lost activity. Thus, compared with the pair-fed normal rats, in all the three tissues tested there was slight loss of the activity at the weight-plateau stage, and at the mild-deficiency stage a further decrease in the activity took place, with the acute stage of the deficiency showing maximum effect. When added *in vitro*, both retinol and retinoic acid were effective in variable degrees in restoring the lost activity in all the three tissues at the mildly deficient stage, whereas in contrast no such restoration was possible at the acute stage of the deficiency.

The vitamin A derivatives were effective in the deficient tissues at a concentration of 5 μ g./reaction mixture, 10 μ g. causing considerable inhibition. The results in Table 2 show that the addition of even 5 μ g. of the vitamin A derivatives tended to

cause some inhibition of the enzyme systems of the normal tissues. Subba Rao & Ganguly (1966) made similar observations with respect to the loss of the ATP-sulphurylase activity of rat liver, as well as the ability of the vitamin A derivatives to restore the loss *in vitro*, at the three stages of vitamin A deficiency.

Of all the lipid-soluble compounds tested, retinol and retinoic acid were the only substances capable of restoring the activity lost as a result of the deficiency (Table 3). Here also, somewhat similar results were reported by Subba Rao & Ganguly (1964) with regard to the reactivation of the ATP-sulphurylase activity in the liver of deficient rats, except that in their hands retinal was slightly effective.

The results of Table 4 show that an injection of the vitamin A derivatives into the rats about 24 hr. before they were killed corrected the effect of the deficiency. Here, all the three vitamin A derivatives

were about equally effective, with retinal showing a little less effect.

Effect of the deficiency on the biosynthesis of corticosterone or deoxycorticosterone from pregnenolone, progesterone and deoxycorticosterone in rat adrenals. The work of Vandyke, Wolf & Johnson (1960) and of Wu-Chang & Willis (1963) had shown that the overall synthesis of corticosterone from cholesterol is decreased in the adrenals of vitamin A-deficient rats and pigs. One of the steps in the synthesis of corticosterone from cholesterol is the conversion of pregnenolone into progesterone, and the experiments described above demonstrated that this step is affected in the three tissues of vitamin A-deficient rats. The results in Table 5 show that when pregnenolone is used as the starting material a significant loss in the synthesis of corticosterone occurs in rat adrenals even at the mild stage of the deficiency, and that the loss becomes more pronounced on prolonging the deficiency to the acute stage. Only

Table 3. *Effect of the addition of various lipid-soluble compounds in vitro on the conversion of dehydroepiandrosterone into androstenedione in the adrenals, testes and ovaries of the mildly vitamin A-deficient rats*

Experimental conditions were the same as described for Table 2. The method of adding the lipid-soluble materials was similar to that for retinol or retinoic acid described in Table 2. Values are averages of four separate trials in each group.

Status of rats	Addition (5 µg.)	Activity (µg. of androstenedione formed/min./mg. of protein)		
		Adrenals	Testes	Ovaries
Normal	None	1.16	1.42	1.30
Vitamin A-deficient	None	0.77	0.75	0.83
	Retinol	1.06	1.22	1.12
	Retinoic acid	1.18	1.38	1.26
	Retinal	0.83	0.78	0.86
	α-Tocopherol acetate	0.85	0.80	0.90
	Menadione	0.79	0.73	0.84
	Calciferol	0.86	0.83	0.80
	Cholesterol	0.78	0.83	0.82
	Palmitic acid	0.86	0.79	0.87
	Lecithin	0.88	0.76	0.85

Table 4. *Effect of a single intraperitoneal injection of retinal, retinyl acetate or retinoic acid on the conversion of dehydroepiandrosterone into androstenedione by the adrenals, testes and ovaries of vitamin A-deficient rats*

The procedure for the injections was that of Malathi *et al.* (1963), except that each animal received 100 µg. of the vitamin A derivative 24 hr. before being killed. The enzymes were assayed as described in Table 1. Values are averages of four individual trials in each group.

Status of rats	Substance injected	Activity (µg. of androstenedione formed/min./mg. of protein)		
		Adrenals	Testes	Ovaries
Normal	None	1.26	1.47	1.40
Vitamin A-deficient	None	0.85	0.90	0.94
	Retinal	1.06	1.20	1.14
	Retinyl acetate	1.16	1.42	1.26
	Retinoic acid	1.24	1.37	1.30

Table 5. *Effect of vitamin A deficiency on the synthesis of corticosterone from pregnenolone, progesterone and deoxycorticosterone in rat adrenals*

Each reaction flask contained 3.36 mg. of NaHCO_3 , 3.32 mg. of glucose, 800 μg . of NADP, 2.10 mg. of glucose 6-phosphate, 40 μg . of CaCl_2 , 60 μg . of the respective steroid dissolved in 0.02 ml. of ethanol and the enzyme preparation in 0.3 ml. of 0.154 M-KCl. The final volume was made up to 2 ml. with 0.154 M-KCl. Where indicated, retinol or retinoic acid was added in 0.02 ml. of ethanol along with the steroid. The incubations were for 1 hr. at 37°. Values are expressed as means of six different experiments.

Substrate used	Activity (μg . of corticosterone formed/hr./mg. of protein)			
	Mild deficiency		Acute deficiency	
	Pair-fed normal	Deficient	Pair-fed normal	Deficient
Pregnenolone	10.8	5.6	9.6	3.1
Pregnenolone + retinol (5 μg .)	10.5	8.7	9.7	3.6
Pregnenolone + retinoic acid (5 μg .)	10.6	9.1	9.7	3.6
Progesterone	14.2	13.3	12.3	9.6
Deoxycorticosterone	16.7	15.9	14.8	11.5

Table 6. *Effect of vitamin A deficiency on the biosynthesis of deoxycorticosterone by rat adrenals in the absence of Ca^{2+}*

The experimental conditions were the same as described in Table 5, except that Ca^{2+} was not added. The values are means of six different experiments.

Substrate	Activity (μg . of deoxycorticosterone formed/hr./mg. of protein)			
	Mild deficiency		Acute deficiency	
	Pair-fed normal	Deficient	Pair-fed normal	Deficient
Pregnenolone	9.2	4.1	8.7	2.8
Pregnenolone + retinol (5 μg .)	8.8	7.5	8.4	3.1
Pregnenolone + retinoic acid (5 μg .)	9.1	7.7	8.4	3.1
Progesterone	15.9	15.3	14.6	11.3

at the mild stage of the deficiency could both retinol and retinoic acid restore *in vitro* the loss to an appreciable extent. In contrast, when progesterone or deoxycorticosterone was used no such decrease in the synthesis was noticed at the mildly deficient stage, whereas at the acute stage appreciable loss took place, which, however, could not be restored by retinol or retinoic acid *in vitro*.

The step from deoxycorticosterone to corticosterone involves hydroxylation at C-11, and Peron (1964) has shown that Ca^{2+} stimulates this reaction. When Ca^{2+} was included in the reaction flasks (Table 5) we found little or no deoxycorticosterone, but only corticosterone, in all the samples. However, when Ca^{2+} was withheld (Table 6) deoxycorticosterone accumulated. These observations of ours therefore agree with the findings of Peron (1964). Table 6 further shows that here also, at the mild stage of the deficiency, a marked loss in the production of deoxycorticosterone from pregnenolone occurs which became more pronounced on prolonging the deficiency. Here again both retinol and retinoic acid were active in restoring the loss, but again only at the mild stage of the deficiency.

The results in Table 6 also show that when pregnenolone was replaced by progesterone the deficiency exerted no such effect on the synthesis of deoxycorticosterone at the mildly deficient stage, some loss taking place at the acute stage, however.

Deoxycorticosterone, corticosterone and aldosterone in the adrenals of weanling rats raised on the vitamin A-free diet and supplemented with retinyl acetate, retinoic acid or none. Weanling male rats were raised on the vitamin A-free diet of Malathi *et al.* (1963) till they reached the weight-plateau stage, after which each rat was daily supplemented with 5 μg . of retinyl acetate or retinoic acid, while a corresponding deficient group remained unsupplemented. This dietary regimen was continued till the unsupplemented rats lost about 15 g. in

Table 7. *Total endogenous tetrazolium blue-positive materials, corticosterone, deoxycorticosterone and aldosterone in the adrenals of normal, retinoic acid-fed and vitamin A-deficient rats*

The tissues were extracted and chromatographed on alumina columns, followed by thin-layer chromatography in the solvent system chloroform-*n*-hexane (7:3, v/v) as described in the Materials and Methods section. In this thin-layer chromatography system, the tetrazolium blue-positive substances stayed at the origin. Portions of these tetrazolium blue-positive materials were estimated, and other portions were subjected to thin-layer chromatography in the solvent system ethyl acetate-*n*-hexane (1:1, v/v). The values are means of two separate experiments, where the adrenals from 12 rats were pooled in each group.

Corticoid	Corticoid content ($\mu\text{g./100 mg. wet wt. of adrenals}$)		
	Normal	Retinoic acid-fed	Acutely deficient
Total tetrazolium blue-positive corticoids*	33	35	26
Corticosterone	7	8	4
Deoxycorticosterone	1.2	1.6	0.9
Aldosterone	1.9	2.1	3.2
18-Hydroxydeoxycorticosterone	Not detectable	Not detectable	Detectable

* These values are expressed in terms of corticosterone.

weight (in about 10–12 days). The animals were then killed and the adrenals removed and pooled within groups so that each group contained the adrenals from a total of 12 rats. The results in Table 7 show that the total tetrazolium blue-positive corticoids, as well as the concentrations of corticosterone and deoxycorticosterone, showed a net decrease in the deficient animals. The decrease in the concentrations of corticosterone and deoxycorticosterone is to be expected from the results of the experiments *in vitro* described above. The increase in the concentrations of aldosterone and 18-hydroxydeoxycorticosterone cannot, however, be explained on the basis of the same experiments.

DISCUSSION

As indicated above, considerable direct and indirect evidence has appeared in the literature suggesting some possible role of vitamin A in steroidogenesis. Thus Vandyke *et al.* (1960) demonstrated, *in vitro*, that the net synthesis of corticosterone from cholesterol in the adrenal homogenates of rats and pigs is decreased during vitamin A deficiency, and that the addition of retinol or retinoic acid is effective in correcting the loss. Later Wu-Chang & Willis (1963) confirmed these findings.

However, it was not clear which of the several steps involved in the synthesis of corticosterone is affected during the deficiency. The conversion of pregnenolone into progesterone is one of the steps in the biosynthesis of corticosterone, and, as stated above, the same enzyme system converts pregnenolone, 17α -hydroxypregnenolone and dehydroepiandrosterone into the corresponding oxo compounds, namely progesterone, 17α -hydroxy-

progesterone and androstenedione respectively. We have shown here that this reaction is markedly depressed even at the mild stage of vitamin A deficiency in the adrenals, testes and ovaries of rats. On prolonging the deficiency the conversion of dehydroepiandrosterone into androstenedione was further diminished in all the three tissues, and the addition of retinol or retinoic acid *in vitro* was effective in restoring the lost activity only at the mild stage of the deficiency.

In addition to pregnenolone, progesterone and deoxycorticosterone are further intermediates in the biosynthesis of corticosterone in rat adrenals. The conversion of deoxycorticosterone into corticosterone *in vitro* is stimulated by Ca^{2+} , in the absence of which deoxycorticosterone accumulates (Peron, 1964). By running such reactions in the presence or absence of Ca^{2+} it has been shown here that the synthesis of corticosterone or deoxycorticosterone from pregnenolone is markedly depressed in vitamin A deficiency and that at the mild stage of the deficiency this loss can be almost fully restored by the addition of retinol or retinoic acid *in vitro*. In contrast, when progesterone or deoxycorticosterone was used as the precursor substrate, at the mild stage of the deficiency no such effect on the synthesis of deoxycorticosterone or corticosterone could be demonstrated. This difference in the behaviour between pregnenolone on the one hand and progesterone and deoxycorticosterone on the other in serving as the precursors for corticosterone can readily be explained by our present observation that the enzymic activity involving the conversion of pregnenolone into progesterone suffers a major loss during vitamin A deficiency. Such a conclusion is further supported by our observation that the deoxycorticosterone and corticosterone contents of

the adrenals of deficient rats are lower. However, the aldosterone content was higher, and it is difficult to explain this at present.

These results of ours are in close agreement with the findings of Grangaud and his associates, who have shown from their experiments with whole animals that the conversion of pregnenolone into progesterone and of dehydroepiandrosterone into androstenedione is affected during vitamin A deficiency in rats (Grangaud & Conquy, 1958; Grangaud, Conquy & Nicol, 1959a,b,c, 1960a,b,c,d). Also, Grangaud, Nicol & Delaunay (1965) showed a 28% decrease *in vitro* in the conversion of pregnenolone into progesterone in the adrenals of vitamin A-deficient rats.

It thus appears that most of the existing information on the possible relationship between vitamin A metabolism and steroid biosynthesis can be fitted into a coherent pattern. As against such a general agreement, Boguth, Soliman & Weiser (1964) and Balke, Boguth & Staudinger (1965) seem to have found no changes in the production of corticosterone in vitamin A-deficient rats. However, the plan of their experiments is quite different; for example, they used hypophysectomized female rats. These rats were hypophysectomized when they weighed 120–140 g., and 5–6 weeks after the hypophysectomy they were changed over to the vitamin A-deficient diet. These experimental conditions were therefore so different that it is not possible to compare their results with ours as well as those of others.

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