Studies on Metabolism of Vitamin A

THE EFFECT OF VITAMIN A STATUS ON THE SECRETION RATES OF SOME STEROIDS INTO THE OVARIAN VENOUS BLOOD OF PREGNANT RATS

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1. Rats raised on a vitamin A-deficient diet supplemented with either retinyl acetate or retinoic acid were mated and became pregnant. 2. The rates of secretion of progesterone, 20α -hydroxypregn-4-en-3-one, oestradiol- 17β and oestrone into the ovarian-venous blood of rats in these two groups were measured on days 9 and 15 of pregnancy. 3. Rates of secretion of progesterone and 20α -hydroxypregn-4-en-3-one, both on days 9 and 15, were lower for the rats given retinoic acid. No such differences were found in ovarian oestrogen secretion. 4. The implications of these results are discussed in the light of the previous demonstration that the activity of ovarian 3β -hydroxy- Δ^5 -steroid dehydrogenase was markedly less in pregnant rats given retinoic acid.

Juneja, Murthy & Ganguly (1964) and Thompson, Howell & Pitt (1964) showed that female rats given a vitamin A-free diet supplemented with retinoic acid grew normally and that ovulation, fertilization and blastocyst implantation were normal. Complete foetal resorption, however, invariably occurred, the first stage of which was necrosis of the periphery of the placenta at about day 16 (Thompson *et al.* 1964). Calastro & Lichton (1968) found that apparently normal placentae developed in such rats between days 11 and 13 of pregnancy (day of finding vaginal sperm counted as day 1), but placental degeneration then began in some rats and by day 18 was evident in all rats.

After some further time on the retinoic acid diet rats may develop the defect of vision typical of vitamin A deficiency but remain otherwise healthy. Rats given vitamin A-free diets supplemented with retinyl acetate are entirely normal. Thus it appears that vitamin A has a specific role not only in vision but also in foetal maintenance in rats.

Juneja, Moudgal & Ganguly (1969) found that foetal resorption in rats given retinoic acid is preventable by hormone therapy applied from day 8 of pregnancy. They also found that on day 13 in such rats, when luteal progesterone secretion in normal rats is approaching its maximum value

(three- to four-fold greater than the oestrous-cycle maximum according to Hashimoto, Henricks, Anderson & Melampy, 1968), the activity of the ovarian 3β -hydroxy- Δ^5 -steroid dehydrogenase is appreciably lower than normal. The activity of this enzyme was also similarly lowered during compensatory ovarian hypertrophy in non-pregnant rats given retinoic acid. The pituitary content of luteinizing hormone and follicle-stimulating hormone in such animals was normal.

Juneja *et al.* (1969) therefore concluded that the ovaries of rats given retinoic acid are unable to perform their steroid-secretory function fully at those times when this is normally at relatively high level.

To investigate further the nature of this apparent ovarian disorder the secretion rates of some ovarian steroids have been measured in two groups of pregnant rats, one given retinoic acid and the other retinyl acetate.

EXPERIMENTAL

Materials and apparatus. Tritium-labelled steroids, supplied by The Radiochemical Centre, Amersham, Bucks., U.K., were: [7α - ^3H]progesterone (batch 4, 0202385), 2.26 Ci/mmol; [$2,4,6,7$ - $^3\text{H}_4$]oestradiol- 17β (TRK 322, batch 1), 100 Ci/mmol; [$2,4,6,7$ - $^3\text{H}_4$]oestrone (TRK 321, batch 1), 100 Ci/mmol. 20α -Hydroxypregn-4-en-

3-one was supplied by CIBA Ltd., Basle, Switzerland. Beadlet preparations of retinyl acetate and retinoic acid encapsulated in gelatin were used (Hoffmann-La Roche and Co. Ltd., Basle, Switzerland). Silica gel for column chromatography (60–120 mesh) was supplied by BDH Chemicals Ltd., Poole, Dorset, U.K.; it was activated before use (105°C for 30 min). Silica gel for t.l.c. was Kieselgel GF₂₅₄ (E. Merck A.-G., Darmstadt, Germany). A Pye Unicam model 64 gas chromatograph with a heated flame ionization detector was used. It was necessary to use high-purity H₂ to achieve a stable base-line.

Radioactivity measurement. Radioactivity of tritium-labelled steroids was determined by liquid-scintillation counting.

Animals and diets. Hooded Norway rats of the Lister strain were used throughout.

The basal vitamin A-deficient diet (no. 1268) was diet no. 172a of Clarke & Todd (1957) except that maize starch replaced potato starch. This diet evidently contained little or no vitamin A, as female pups reared on it from 21 days of age ceased to grow after 3 weeks. Growth was promptly resumed when a supplement of retinoic acid was given. As noted by Malathi, Rao, Sastry & Ganguly (1963), this was associated with a marked increase in water intake.

Female pups were allocated at weaning (21 days) to the basal diet supplemented with either retinyl acetate or retinoic acid (2 µg/g of diet.). In addition they received once a week two drops of a solution of vitamin D and vitamin E in arachis oil (Malathi *et al.* 1963).

All rats were weighed weekly until mated, when they were weighed daily. They were approximately 130 days old and weighed about 220 g at mating. Three females were placed with one male, after which vaginal smears were examined every morning and the day when sperm were found was called day 1 of pregnancy. The mated females were kept three to a cage.

Collection of ovarian-vein blood sample. A hypodermic needle (0.80 mm external diam.) was specially prepared by cutting a slot (0.5 mm × 0.3 mm) in the wall of the needle about 1.5 mm back from the point on the side opposite the bevelled part. (This allowed blood to flow readily if the normal opening was occluded by the wall of the vein.) The shaft of the needle was then cut through transversely, 12 mm back from the point, and the cut end of the 12 mm portion was inserted into a polythene tube of internal diam. 0.75 mm.

Rats were anaesthetized with pentobarbitone sodium solution. (55 mg/kg body wt.); body temperature was maintained at 38°C. A triangular-shaped flap of skin, apex at the lower abdomen, was cut and folded back on its base near the diaphragm. Gauze, soaked in warm 0.9% NaCl, was placed on the exposed organs and as rapidly as possible all veins leading to the left renal vein were ligated except for the ovarian vein; a tie was also made round the branch of the ovarian vein collecting blood from the part of the uterus nearest the ovary. As the left lumbar vein frequently entered the renal vein, care was taken to tie this off. A loose ligature was placed round the renal vein, near the kidney and another, as near as possible to the vena cava. A dose of 1000 i.u. of heparin was given via the sublingual vein and as rapidly as possible the two renal ligatures were tightened, after which the hypodermic needle prepared as described above

was pushed into the renal vein. Blood was collected into a small tube standing in ice. The collection lasted 40 min and the mean volume of blood obtained was 2 ml. At the end of the collection the ovaries were removed and stored at –20°C. Of the 187 blood samples collected from pregnant rats, 104 were selected on the basis of near-average blood flow and closeness to the days of pregnancy chosen for study.

Extraction of steroids from blood and their separation. Each blood sample was added to acetone (10 vol.), left overnight at room temperature and the mixture filtered through sintered glass (porosity 1). The precipitate was then washed with acetone (5 × blood volume), and the combined filtrate stored at –20°C.

From the 104 filtrates, each from an individual blood sample, seven pairs of pooled filtrates (Table 1) were prepared. Corresponding to each pooled filtrate from samples from a group of retinoic acid-fed rats at a particular time in pregnancy, a second pool was prepared from samples from the same number of rats that had received the other diet but were in all other respects as similar as possible to the first group.

[³H]Oestradiol-17β, [³H]oestrone (each approx. 50 pg; 40 000 d.p.m.) and [³H]progesterone (approx. 2.2 ng; 40 000 d.p.m.) were added to each pooled filtrate, which was then evaporated to dryness on a rotary evaporator after the addition of three drops of octan-2-ol to prevent frothing. The residue was partitioned between diethyl ether (4 × 50 ml) and 1% (w/v) NaHCO₃ (30 ml). The ether solution (fraction A) then contained free steroids and the NaHCO₃ solution (fraction B) conjugated steroids.

The ether solution was dried overnight (over Na₂SO₄) at 5°C, filtered and evaporated to dryness. The residue was taken up in 5 ml of toluene and placed on to a silica-gel column (1 cm diam.; 3 g of SiO₂) packed in light petroleum (b.p. 60–80°C). More toluene (5 ml) was added to the column. The resulting first eluate contained most of the triglycerides and cholesterol esters and was discarded. The hormonal steroids were eluted with acetone (30 ml), leaving phospholipids on the column. Evaporation of acetone gave a residue, which was taken up in 1 ml of ethanol, 25 ml of toluene and 25 ml of light petroleum (b.p. 40–60°C) and the solution washed with 0.4 M-NaOH (2 × 25 ml).

The solvent phase (fraction C) then contained neutral steroids and the NaOH phase (fraction D) any free oestrogen from the blood.

Assay of oestrogens. (a) The NaHCO₃ phase (fraction B, above). This solution, presumed to contain any conjugated oestrogens originally present, was brought to pH 7 (with conc. HCl); then 0.1 vol. of conc. HCl was added, followed by ether (20 ml) and [³H]oestradiol-17β (50 pg; 40 000 d.p.m.). The mixture was left for 22 h with occasional shaking. After final vigorous shaking, the phases were separated and the aqueous layer was again shaken with fresh ether (2 × 20 ml). The combined ether phase, after being washed with saturated NaHCO₃ solution (2 ml) and water (4 × 2 ml), was dried overnight (over Na₂SO₄), filtered and evaporated to dryness. The residue was dissolved in methanol (5 ml). Of this, 1 ml was taken for radioactivity determination and the remainder evaporated to dryness. The residue, in 200 µl of propane-1,2-diol–water (1:10, v/v), was assayed by the tetrazolium method as described below.

(b) The NaOH phase (fraction D, above). This was immediately acidified to pH1.0 and washed with ether (4×50 ml); the ether layer was washed with saturated aqueous NaHCO₃ (10 ml), dried (over Na₂SO₄), filtered and the ether evaporated. The residue was taken up in 100 µl of toluene and applied to a layer of silica gel (500 µm thick) (Storry & Tuckley, 1967). The central lane was used for markers, 10 µg each of megestrol acetate (17-acetoxy-6-methylpregna-4,6-diene-3,20-dione; BDH Research Ltd., Godalming, Surrey, U.K.) and norethisterone acetate (17-acetoxy-19-nor-17α-pregn-4-en-20-yn-3-one; Parke, Davis and Co. Ltd., Hounslow, Middx., U.K.), which absorb strongly at 254 nm and have *R_F* values near those of oestradiol-17β and oestrone. The solvent system used was modified by substituting toluene for benzene. The areas of silica gel estimated to contain oestradiol-17β and oestrone from the blood extracts were removed by suction on to a sinter (1 cm diam.) and the extracts eluted with acetone (3×5 ml). Each was then transferred to toluene solution, a portion taken for radioactivity counting and the remainder applied to Whatman no. 20 paper (3 cm-wide lane). The chromatogram was run for 5 h in Bush (1952) system B (1), with (as a marker) BDH universal indicator, which gives pink spots similar in *R_F* values to oestradiol-17β and oestrone. Each oestrogen area was cut out and the steroid eluted with acetone, which was then evaporated and the residue taken up in toluene. A portion was taken for radioactivity counting and the rest transferred to 0.5 ml of propane-1,2-diol-acetone (1:50, v/v). The acetone was evaporated and 100 µl of water added. After thorough mixing, 10 µl was removed for radioactivity counting and the remainder assayed biologically by the intravaginal tetrazolium-reduction assay of Martin (1960, 1964).

Assay of progesterone and 20α-hydroxypregn-4-en-3-one. The solvent phase (fraction C) was evaporated to dryness and the residue taken up in 50 ml of light petroleum (b.p.

40–60°C); this was washed with 80% (v/v) methanol (4×50 ml), leaving the cholesterol in the light-petroleum layer. From the 80%-methanol phase a portion was taken for radioactivity counting and the rest applied to a layer of silica gel (400 µm thick) and run in the same solvent system as for the oestrogens. The areas containing progesterone and 20α-hydroxypregn-4-en-3-one, visible in u.v. light (254 nm) as dark spots, were sucked off; the steroids were eluted with acetone and transferred to toluene solution, portions of which were taken for radioactivity counting. The steroids were then determined by g.l.c., with solid injection (Menini & Norymberski, 1965) on a column (180 cm×0.4 cm) of 3% (w/v) FSI (fluoroalkylsiloxane polymer) at 230°C. Individual observation differed on average from means of duplicates by 2% for progesterone and by 8% for 20α-hydroxypregn-4-en-3-one.

The mean recoveries of [³H]progesterone, [³H]oestradiol-17β and [³H]oestrone were 64.3, 53.7 and 47.7% respectively. No radioactive 20α-hydroxypregn-4-en-3-one was used as tracer and recovery of this steroid was assumed to be the same as that of progesterone.

RESULTS

For both progesterone and 20α-hydroxypregn-4-en-3-one differences in estimates (Table 1) are certainly greater than the experimental error of the analytical method.

For retinyl acetate groups the secretion rates of both steroids are higher at day 15 than at day 9. This is in agreement with studies with normal pregnant rats by Eto, Masuda, Suzuki & Hosi (1962), Fajer & Barraclough (1967) and Hashimoto *et al.* (1968).

Table 1. *Secretion rates of progesterone and 20α-hydroxypregn-4-en-3-one in ovarian-venous blood near days 9 and 15 of pregnancy of rats given a vitamin A-deficient diet supplemented with retinoic acid or retinyl acetate*

Experimental details are given in the text.

Pairs of pooled extracts	Supplement	No. of rats	Day of pregnancy		Progesterone		20α-Hydroxypregn-4-en-3-one	
			Mean	Range	(ng/h per ovary)	'(Ratio retinoic acid'/retinyl acetate)'	(ng/h per ovary)	'(Ratio retinoic acid'/retinyl acetate)'
1	Retinoic acid	4	8	0	350	0.61	230	0.36
	Retinyl acetate	4	8	0	570		640	
2	Retinoic acid	10	8.2	8–9	1890	0.56	790	0.66
	Retinyl acetate	10	8.2	8–9	3390		1200	
3	Retinoic acid	4	9	0	500	0.45	200	0.29
	Retinyl acetate	4	9	0	1100		700	
4	Retinoic acid	10	10.3	9–11	2450	1.11	720	0.85
	Retinyl acetate	10	9.8	9–11	2200		850	
5	Retinoic acid	10	15.3	15–17	1680	0.46	2630	0.88
	Retinyl acetate	10	13.8	13–15	3660		2990	
6	Retinoic acid	4	15	0	1900	0.38	1140	0.31
	Retinyl acetate	4	15	0	4970		3740	
7	Retinoic acid	10	15.4	15–16	4330	0.83	4140	1.23
	Retinyl acetate	10	15.3	15–16	5200		3360	

Table 2. *Secretion rates of oestrogens in ovarian-venous blood near days 9 and 15 of pregnancy of rats given a vitamin A-deficient diet supplemented with retinoic acid or retinyl acetate*

Experimental details are given in the text. The 95% confidence limits for the secretion rates are given in parentheses.

Supplement	No. of rats	Day of pregnancy		Oestradiol-17 β * (ng/h per ovary)	Oestrone† (ng/h per ovary)
		Mean	Range		
Retinoic acid	10	8.2	8-9	0.390 (0.150-0.830)	0.079 (0.014-0.179)
Retinyl acetate	10	8.2	8-9	0.500 (0.214-1.081)	0.172 (0.054-0.363)
Retinoic acid	10	10.3	9-11	0.360 (0.206-0.582)	0.102 (0.024-0.219)
Retinyl acetate	10	9.8	9-11	0.330 (0.188-0.534)	0.072 (0.013-0.162)
Retinoic acid	10	15.3	15-17	0.305 (0.163-0.470)	0.070 (0.0035-0.194)
Retinyl acetate	10	13.8	13-15	0.350 (0.198-0.567)	0.033 (0.00054-0.109)
Retinoic acid	10	15.4	15-16	0.640 (0.359-1.103)	0.123 (0.036-0.260)
Retinyl acetate	10	15.3	15-16	0.160 (0.076-0.288)	0.090 (0.019-0.198)

* (2, 2) assays.

† (2, 1) assays.

If comparisons are made within pairs of pooled extracts (Table 1) it is seen that in 12 cases out of 14 the steroid-secretion rate (progesterone or 20 α -hydroxypregn-4-en-3-one) is lower for the rats given retinoic acid. Differences exist between pairs (for both steroids) that cannot be accounted for.

Pooled extracts of samples from ten rats were needed to provide sufficient material for oestrogen assay (Table 2). No significant differences were observed in the secretion rates of either oestradiol-17 β or oestrone in relation to diet or time in pregnancy; the secretion rate of oestradiol-17 β was always considerably greater than that of oestrone. Secretion rates of both oestrogens were very similar to those found by Waynforth, Pope & Hosking (1971) for normal pregnant Wistar rats. The mild acid hydrolysis of the conjugated steroid fraction yielded no further oestrogenic activity.

The adrenal glands and ovaries from 16 rats at day 21 of pregnancy, eight given retinyl acetate and eight given retinoic acid, contained very similar amounts of ascorbic acid per animal (181 and 176 μ g/adrenal glands and 40 and 35 μ g/ovaries respectively), suggesting that there is no profound disorganization of adrenal and ovarian function at this time in rats fed retinoic acid.

DISCUSSION

The present study has shown that from day 9 of pregnancy the ovarian secretion rates of progesterone and 20 α -hydroxypregn-4-en-3-one are usually lower in rats given retinoic acid. This finding is in general agreement with the results of studies *in vitro* by Juneja *et al.* (1969), with rats given retinoic acid, and of other studies, with vitamin A-deficient rats,

by Juneja, Murthy & Ganguly (1966) and Grangaud, Nicol & Desplanques (1969).

What is the biochemical mechanism whereby the vitamin A deficiency might lead to the lowering of the enzymically controlled production of progesterone and other 3-oxo- Δ^4 -steroids and, for the rats given retinoic acid, does the lowered progesterone secretion actually cause the subsequent foetal resorption? Evidence provided by Juneja *et al.* (1966) for ovarian tissue and by Grangaud *et al.* (1969) for the adrenal tissue of rats deficient in all forms of vitamin A is that the capacity of tissue homogenates to synthesize the 3-oxo- Δ^4 -steroids may be restored to normal by the addition of vitamin A in one or other of its chemical forms. The lowered ovarian secretion of progesterone and 20 α -hydroxypregn-4-en-3-one by rats given retinoic acid at day 9 of pregnancy and later may therefore be due to a lowered availability of vitamin A in the necessary chemical form at the site of synthesis in the corpora lutea.

The lowered ovarian secretion must be considered as a possible reason for the eventual foetal resorption in these animals. Support for this comes from those hormone-replacement experiments of Juneja *et al.* (1969), in which prolactin was either injected directly, or (it may be presumed) was secreted from transplanted pituitaries or was secreted in enhanced quantity after oestradiol-17 β administration (see Alloiteau & Mayer, 1967; Chen & Meites, 1970). In these experiments enhanced secretion of progesterone from the corpora lutea would be expected and foetal resorption was in fact prevented (the limited efficacy of the injected prolactin may have been due to its not being rat prolactin). The implications of the results of the other steroid-replacement experiments of Juneja *et al.* (1969) require further studies.

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