Studies on Metabolism of Vitamin A

THE EFFECT OF HORMONES ON GESTATION IN RETINOATE-FED FEMALE RATS

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1. Female rats, raised to maturity on a vitamin A-deficient diet supplemented with retinoate as the sole source of vitamin A, conceived when allowed to mate with normal males, but the conception resulted in foetal resorption, beginning from day 14 or 15 of pregnancy. 2. Daily injections of pregnenolone or oestradiol-17 β , but not of progesterone, were fully effective, and transplantation of pituitary homografts under kidney capsules was 80% effective, in preventing resorption. 3. Unilateral ovariectomy, leading to compensatory hypertrophy of the remaining ovary, resulted in significantly fewer corpora lutea and in lower weight of the hypertrophied ovary of the retinoate-treated rats, as compared with the corresponding retinyl acetate-fed ones. 4. The activity of the enzyme system Δ^5 -3 β -hydroxy steroid dehydrogenase was significantly less in the ovaries of the retinoate-fed rats that were subjected to unilateral ovariectomy or were made pregnant, when compared with that of the corresponding controls. Also, the ovaries of the retinoate-treated rats were insensitive to both exogenous and endogenous gonadotrophin stimulus. 5. These results indicate that one of the reasons for foetal resorption in the retinoate-fed rats might be inadequate synthesis of steroid hormones such as pregnenolone and oestrogen.

Earlier nutritional work had led to the general conclusion that normal reproduction is dependent on the vitamin A nutritional status of both male and female rats. But the general pathological damages caused to many tissues by vitamin A deficiency had failed to pinpoint the true effects produced by the deficiency on reproduction (Moore, 1957). However, clearer evidence supporting absolute requirements of retinol in normal reproduction of both male and female rats has since become available. Thus in female rats reared on diets containing retinoate as the sole source of vitamin A, although they show normal oestrous cycle and conceive when mated with normal males, the conception is terminated by foetal resorption, beginning from day 14 or 15 of pregnancy; in the males retinoate supplementation results in an impairment of spermatogenesis and of the development of accessory glands (Juneja, Murthy & Ganguly, 1964; Thompson, Howell & Pitt, 1964; Coward, Howell, Pitt & Thompson, 1966).

However, little is known about the manner in which retinoic acid affects the reproductive processes, and an attempt was therefore made to study the mechanism by which retinoic acid interferes in the maintenance of full-term gestation in the pregnant rats. The observations now reported

suggest that one of the factors responsible for the resorption of foetuses in the retinoate-fed pregnant rats might be inadequate availability of steroid hormones such as pregnenolone and oestrogen. A preliminary report has already been published (Juneja, Ganguly & Moudgal, 1967).

MATERIALS AND METHODS

Materials. Pregnenolone (3 β -hydroxypregn-5-en-20-one), progesterone (pregn-4-ene-3,20-dione), oestrone [3-hydroxy-oestra-1,3,5(10)-trien-17-one], oestradiol-17 β [oestra-1,3,5(10)-triene-3,17 β -diol], dehydroepiandrosterone (3 β -hydroxyandrost-5-en-17-one), androstenedione (androst-4-ene-3,17-dione) and NAD⁺ were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.). Pregnant-mare's serum (Gestyl) was a product of Organon Laboratories (London, W.C. 2), ovine follicle-stimulating hormone, prolactin and luteinizing hormone were gifts from the National Institutes of Health (Bethesda, Md., U.S.A.) and human chorionic gonadotrophin was a gift from Leo Pharma (Hälsingborg, Sweden). Thyroxine was obtained from Mann Laboratories (New York, N.Y., U.S.A.). Crystalline retinyl acetate and retinoic acid were gifts from Hoffmann-La Roche (Basle, Switzerland).

Diets and animals. The vitamin A-free diet was composed of 20 parts of vitamin A-free casein, 60 parts of tapioca starch, 10 parts of cane sugar, 5 parts of groundnut oil and 5 parts of salt mixture (Steenbock & Nelson, 1923). The

vitamin A-free casein was obtained essentially as described by Malathi, Subba Rao, Seshadri Sastry & Ganguly (1963), except that the crude casein was refluxed twice, for 12 hr. each time, on a boiling-water bath with approx. 3 vol. of acidified ethanol (pH 4.5).

Male and female rats of this Institute strain were put on the respective diet when they were 18–20 days old; at this age they usually weighed about 20–25 g. The rats were given a dough obtained by thoroughly kneading a mixture of the dry vitamin A-free diet, the desired supplements of the B-group vitamins (Malathi *et al.* 1963) dissolved in water and an oily solution of retinyl acetate or a buffered dispersion of retinoic acid as its sodium salt, and water. The vitamin A supplements were mixed in such a proportion that each rat received daily 15–20 µg. of the vitamin. Supplementation with the other fat-soluble vitamins was as described by Malathi *et al.* (1963), except that they were given twice a week. The diet was given to the rats until they were 4–5 months old, at which age they were used.

The vitamin A-deficient rats were obtained as described by Juneja, Murthy & Ganguly (1966).

Female mice of this Institute strain, 21–25 days old, were used for the assay of total gonadotrophic potency of pituitary and plasma of rats.

Mating designs. The rats were of comparable body weight and age. One male was placed with three females irrespective of the phase of the oestrous cycle. The vaginal smears were examined every morning thereafter and the presence of sperms in the smear on any one morning was taken as day 1 of pregnancy. The mated females were then caged individually and their body weights were recorded daily. Where necessary, the rats were laparotomized on days 8 and 19 of pregnancy for noting the number of implantation sites and live pups respectively.

Administration of hormones. The hormones were injected subcutaneously twice a day, the steroids being injected in 0.1 ml. of groundnut oil, other hormones being given in 0.1 ml. of 0.9% NaCl.

Assay of the total gonadotrophin in the pituitary and blood plasma of rats. The total gonadotrophin contents of the pituitary and plasma of rats were determined by the mouse uterine assay method of Rosemberg & Engel (1961).

Plasma and pituitary obtained from individual rats were

pooled within groups and stored at -20° until further use. At the time of assay 1.0 ml. of plasma or a saline homogenate of pituitary equivalent to 10 mg. of the tissue was injected/mouse twice a day over the 3-day period of assay. The rest of the procedure was as described by Rosemberg & Engel (1961), and the results are expressed as gain in uterine weight/100 g. body wt. of the mouse.

In the present work comparative values of the gonadotrophin in the pituitary and plasma of different groups of similarly treated rats were obtained, and therefore there was no necessity for using a standard preparation of the total gonadotrophin.

RESULTS

Effect of injections of different hormones on the maintenance of pregnancy in the retinoate-supplemented rats. The earlier observations (Juneja *et al.* 1964; Thompson *et al.* 1964), that female rats raised on a vitamin A-deficient diet and supplemented with retinoate instead of retinyl acetate conceive when mated with normal males, and that the conception results in foetal resorption beginning from day 14 or 15 of pregnancy, were fully confirmed during the present investigations.

An intricate interplay of various hormones is essential for successful gestation in animals, and attempts were therefore made to investigate whether the foetal resorption in the retinoate-treated rats is due to inadequate availability of hormones. In some experiments the female rats were brought to the weight-plateau stage of vitamin A deficiency, after which they were simultaneously given retinoate and the steroid hormones. The results of one set of such experiments (Table 1) show that, of the hormones tested, pregnenolone not only prevented the resorption, but also promoted development of the fetuses to full term. All of the 12 rats receiving such treatment littered, the average litter size and litter weights being 8.0

Table 1. *Effect of prolonged treatment with steroid hormones on pregnancy in vitamin A-deficient rats given retinoate*

Female rats (18–20 days old) were placed on the vitamin A-deficient diet until they started losing weight, showed irregular oestrous cycle, xerophthalmia etc. They were then changed over to the retinoate-supplemented diet and were given on every fourth day a subcutaneous injection of oestradiol benzoate, progesterone or pregnenolone (100 µg./rat) for a period of 40 days. The control group received groundnut oil only. The rats were then allowed to mate with normal bucks and the steroid supplementation was continued during the pregnancy. Gains in body weight are given as means $\pm$ S.D.

Treatment	No. of rats	No. of rats conceived	No. of rats resorbing fetuses	Mean gain in body wt. during pregnancy (g.)	Average litter size	Average litter wt. (g.)
None	6	6	6	35* (± 4.0)	—	—
Oestradiol benzoate	12	0	—	—	—	—
Progesterone	12	10	10	38* (± 4.6)	—	—
Pregnenolone	12	12	0	70 (± 8.2)	8.0	42

* Gain until the beginning of resorption.

Fig. 1. Growth curves for individual rats receiving the following treatments: ●, retinyl acetate alone; △, retinoate alone; ▲, retinoate and progesterone; ○, retinoate and pregnenolone; ○, retinoate and oestradiol benzoate. The rats were from the experiments described in Table 1.

and 42g. respectively. However, the pups died soon after birth. In contrast, although progesterone did not prevent the resorption, oestradiol benzoate at the dose tried prevented conception. Representative growth curves of one rat from each group receiving these treatments during pregnancy are given in Fig. 1.

Whereas in the above trials the animals were given the hormones before and during pregnancy, in another set of experiments the retinoate-fed female rats were treated with the hormones only during pregnancy. The results (Table 2) show that, not only pregnenolone, but oestradiol-17 β also was effective in promoting full-term gestation. Laparotomy on day 19 of pregnancy showed that almost all the foetuses were alive; these groups of rats had pregnancy indices of 0.86 and 0.94 respectively. However, although the rats receiving pregnenolone showed normal gestation, they bled profusely during littering and their mammary glands were not prominent in appearance. Their litter sizes and litter weights were comparable with those of the corresponding group receiving retinyl acetate, but the pups, although normal in appearance, died soon after birth. In contrast, in the oestradiol-17 β -treated group, the gestation continued to term, but the pups had to be delivered by caesarean operation, and the development of the mammary glands in this group was comparable with

that of the normal rats. Laparotomy of the corresponding progesterone-treated rats on day 19 of pregnancy showed the presence of live foetuses in only two out of six rats, and both littered. The mammary glands of these two rats were not well developed and the pups either were still-born or died soon after birth. The combined hormonal therapy with progesterone and oestradiol benzoate supported normal gestation and littering in only one out of six rats.

These results therefore indicated that the availability of pregnenolone or oestradiol-17 β or both might be decreased in the retinoate-fed rats. However, it is known that the pituitary is affected during deficiencies of protein (Leathem, 1958; Srebnik & Nelson, 1957), vitamin B₆ (Wooten, Nelson, Simpson & Evans, 1955), thiamine (Evans & Simpson, 1930; Srebnik & Nelson, 1963), riboflavin (Forker & Morgan, 1955), folic acid (Salhanick, Hisaw & Zarrow, 1952), vitamin A (Mason & Wolfe, 1930; Sutton & Brief, 1939; Hodgson, Hall, Sweetman, Wiseman & Converse, 1946; Erb, Andrews, Hauge & King, 1947; Serfaty & Oliverau, 1955) and vitamin E (Rowlands & Singer, 1936; P'an, Van dyke, Kaunitz & Slanetz, 1949; Griesbach, Bell & Livingston, 1957). Therefore the possibility that retinoic acid might affect the proper functioning of the pituitary glands, which in turn might influence the availability of pregnenolone or oestradiol-17 β or both, could not be ruled out. Further investigations were therefore undertaken.

The results (Table 2) show that, whereas administration of 20 i.u. of pregnant-mare's serum/rat resulted in successful gestation in 16% of the rats, injection of 20 μ g. of prolactin/day prevented foetal resorption in 40% of the rats; even the small number of rats that went to full term did not litter. Treatments with follicle-stimulating hormone, human chorionic gonadotrophin and luteinizing hormone, on the other hand, failed to prevent resorption. Administration of either a saline homogenate of two fresh pituitaries of adult female rats or a saline suspension of 2mg. of an acetone-dried powder of sheep pituitaries had no effect on the maintenance of pregnancy. However, when endogenous prolactin secretion was increased by transplanting pituitary homografts under the kidney capsule by the procedure of Everett (1956) 80% of the rats showed successful gestation, but none of them could litter and live pups could be obtained only by caesarean operation on day 21 of pregnancy. These rats had a mean pregnancy index of 0.87 and had an average of 6.4 live foetuses/rat on day 19 of pregnancy; their mammary development appeared as prominent as in the normal rats.

Compensatory hypertrophy of the ovaries of the retinoate- or retinyl acetate-fed rats. It is well known

Table 2. *Effect of hormone supplementation on the maintenance of pregnancy in retinoate-treated rats*

Weanling rats were raised to maturity on the vitamin A-deficient diet supplemented with 15–20 μg . of retinyl acetate or retinoic acid/rat/day. Further details of the experiments are described in the text. Pregnancy index is given by:

$$\frac{\text{Live foetuses/rat on day 19 of pregnancy}}{\text{Implantation sites/rat on day 8 of pregnancy}}$$

Vitamin A supplement	No. of rats	Treatment from day 8 to day 19 of pregnancy (dose/rat/day)	Implanta- tion sites/ rat on day 8 of pregnancy	Live foetuses/ rat on day 19 of pregnancy	Pregnancy index	Rats resorbing foetuses (%)	Successful littering (%)	Litter size	Litter wt. (g.)	Remarks
Retinyl acetate	6	None	7.2	6.8	0.94	0.0	100.0	6.8	40	
Retinoate	6	None	7.6	0.0	0.0	100.0	0.0	—	—	
	6	Pregnant-mare's serum (20 i.u.)	7.2	1.5	0.20	83.4	16.6	3.2	12	
	5	Prolactin (10 $\mu\text{g.}$)	7.6	0.0	0.0	100.0	0.0	—	—	
	5	Prolactin (20 $\mu\text{g.}$)	7.2	2.8	0.38	60.0	0.0*	—	—	Mammary glands well developed
	4	Follicle-stimulating hormone (20 i.u.)	7.2	0.0	0.0	100.0	0.0	—	—	
	4	Human chorionic gonadotrophin (10 i.u.)	6.8	0.0	0.0	100.0	0.0	—	—	
	4	Luteinizing hormone (10 $\mu\text{g.}$)	7.2	0.0	0.0	100.0	0.0	—	—	
	6	Homogenate of two fresh pituitaries from adult female rats in 0.2 ml. of saline	7.6	0.0	0.0	100.0	0.0	—	—	
	6	Suspension of 2 mg. of acetone-dried powder of sheep pituitaries in 0.2 ml. of saline	7.2	0.0	0.0	100.0	0.0	—	—	
	10	Two pituitaries grafted under capsule of the left kidney, with no subsequent treatment	7.3	6.4	0.87	20.0	0.0*	6.8	45	Mammary glands well developed
	12	Pregnenolone (2000 $\mu\text{g.}$)	7.2	6.2	0.86	0.0	100.0	6.0	40	Mammary glands not well developed
	6	Progesterone (2000 $\mu\text{g.}$)	7.2	2.6	0.36	66.7	33.3	7.2	45	Pups died soon after birth
	6	Progesterone (2000 $\mu\text{g.}$) + oestradiol benzoate (0.5 $\mu\text{g.}$)	7.6	1.3	0.17	83.4	16.6	4.0	15	Pups died soon after birth
	6	Oestradiol-17 β (1.0 $\mu\text{g.}$)	7.2	6.8	0.94	0.0	0.0*	6.0	40	Mammary glands well developed

* Pups obtained by caesarean operation on day 21 of pregnancy.

Table 3. *Compensatory hypertrophy of the ovary of retinyl acetate- or retinoate-fed rats*

Weanling rats were raised to maturity on the vitamin A-deficient diet supplemented with 15–20 μg . of retinoic acid or retinyl acetate/rat/day. Further details are given in the text. Results are given as means $\pm$ s.d. where appropriate.

Vitamin A supplement	No. of rats	Body wt. at end of experiment	Treatment (dose/rat/day)	Wt. of hyper-trophied ovary (mg.)	Hyper-trophy (%)	Average no. of corpora lutea in hyper-trophied ovary	Wt. of other tissues (mg.)			
							Uterus	Adrenals	Thyroids	Pituitary
Retinyl acetate	15	165.2 $\pm$ 17.2*	None	58.4 $\pm$ 2.5	87.1	8.3 $\pm$ 1.0	242.5 $\pm$ 59.4	40.2 $\pm$ 4.2	7.5 $\pm$ 0.1	9.5 $\pm$ 0.4
Retinoate	15	162.2 $\pm$ 15.6	None	28.3 $\pm$ 4.6	36.2	2.8 $\pm$ 0.2	278.6 $\pm$ 82.9	42.8 $\pm$ 8.3	7.6 $\pm$ 0.1	8.0 $\pm$ 0.2
	6	152.2 $\pm$ 10.2	Saline (0.2 ml.)	30.6 $\pm$ 2.4	38.2	2.6 $\pm$ 0.2	260.2 $\pm$ 38.6	38.6 $\pm$ 5.6	7.2 $\pm$ 0.3	7.8 $\pm$ 0.2
	6	160.0 $\pm$ 8.6	Saline (1.0 ml.)	32.2 $\pm$ 4.0	37.0	2.6 $\pm$ 0.1	256.8 $\pm$ 30.0	40.4 $\pm$ 8.0	7.6 $\pm$ 0.4	8.0 $\pm$ 0.2
	6	123.6 $\pm$ 17.5	Homogenate of half a pituitary of adult female rat in 0.2 ml. of saline	33.0 $\pm$ 5.4	35.6	5.6 $\pm$ 0.8	221.0 $\pm$ 48.3	36.0 $\pm$ 7.0	7.2 $\pm$ 0.4	7.6 $\pm$ 0.4
	5	153.4 $\pm$ 10.2	Suspension of 0.5 mg. of acetone-dried powder of sheep pituitary in 0.2 ml. of saline	40.2 $\pm$ 8.6	44.2	6.0 $\pm$ 1.2	310.6 $\pm$ 80.4	50.6 $\pm$ 3.0	8.5 $\pm$ 0.2	8.0 $\pm$ 0.6
	5	156.7 $\pm$ 37.0	Thyroxine (5 μg .)	34.0 $\pm$ 3.1	32.0	2.0 $\pm$ 0.2	284.0 $\pm$ 68.0	35.0 $\pm$ 7.4	8.0 $\pm$ 0.2	8.4 $\pm$ 0.2
	5	165.8 $\pm$ 7.9	Corticosterone (5 μg .)	30.2 $\pm$ 2.6	38.9	3.0 $\pm$ 0.6	284.0 $\pm$ 60.4	38.0 $\pm$ 4.6	7.8 $\pm$ 0.6	7.6 $\pm$ 0.6
	5	160.2 $\pm$ 8.4	Corticosterone (5 μg .) + aldosterone (0.1 μg .)	38.0 $\pm$ 6.3	40.1	3.0 $\pm$ 0.6	250.2 $\pm$ 40.8	40.2 $\pm$ 2.6	7.0 $\pm$ 0.8	8.0 $\pm$ 0.4

that growth, maturation and endocrine functions of gonads are regulated by the hypothalamus-hypophysis-gonad complex. When female rats are unilaterally ovariectomized, the remaining ovary assumes the functions of both the ovaries and hypertrophies with consequent increases in size and weight (Carmichael & Marshall, 1908; Biggers, Finn & McLaren, 1962; Edgren, Parlow, Peterson & Jones, 1965). Although the exact mechanism that evokes this response of the contralateral ovary is still obscure, the corollary that the correct response of the ovary would reflect proper functioning of the hypothalamus-hypophysis-gonad axis seems to be valid. Therefore the functioning of the hypothalamus-hypophysis-gonad axis was investigated by inducing compensatory hypertrophy of the ovary of the retinoate-fed rats.

Regularly cycling adult female rats, of comparable age and receiving the supplements of retinoate or retinyl acetate, were unilaterally ovariectomized (preferably at the early proestrus phase of their oestrous cycle), and were allowed to continue on their respective dietary regimen for 1 month. They were then autopsied (at the proestrus phase) and the remaining ovary was dissected out, freed of its bursa and adherent fats, after which its weight and number of corpora lutea were recorded. The uterus, adrenals, thyroids and pituitary glands were also removed and their weights recorded.

The results (Table 3) show that the weight of the ovary and the number of corpora lutea of the retinoate-treated rats were significantly less compared with the corresponding group receiving retinyl acetate, but that no such difference in the weights of the other tissues was observed. Histological examination further revealed that the corpora lutea of the retinoate-treated rats were poorly developed (H. S. Juneja, N. R. Moudgal & J. Ganguly, unpublished work).

Daily administration of thyroxine, corticosterone or corticosterone plus aldosterone did not promote the response of the affected ovary, whereas daily

administration of 0.2 ml. of saline homogenate of half a pituitary of an adult female rat or 0.2 ml. of saline suspension of 0.5 mg. of acetone-dried powder of sheep pituitary, although it did not increase the weight of the hypertrophied ovary, resulted in an increase in the number of corpora lutea.

Changes in Δ^5 - 3β -hydroxy steroid dehydrogenase activity in the compensating ovary and in the ovaries of pregnant rats. The marked differences in the weight and in the number of corpora lutea of the compensating ovary of the rats from the two different groups suggested that a decrease in the synthesis or secretion of steroid hormones might be taking place in such ovaries. This reasoning assumed importance because, during pregnancy, when large amounts of such hormones are required for sustaining the rapidly growing foetus, a slight lowering in the rate of their synthesis might be expected to lead to the foetal resorption of the retinoate-fed rats.

Δ^5 - 3β -Hydroxy steroid dehydrogenase is a key enzyme in the biosynthesis of almost all the biologically active steroid hormones, and it is present in the steroid-producing glands of most vertebrates (Samuels, 1960). The changes in the activity of this enzyme in the ovaries of the rats of different physiological states are recorded in Table 4. Although no significant difference in activity was noticed when the ovaries of the rats receiving either form of the vitamin were compared, the activity was markedly less when the retinoate-fed rats were subjected to stress conditions such as unilateral ovariectomy or pregnancy. It should be noted that, although this enzyme showed no difference in its activity in the ovaries of the non-pregnant rats of the two groups, a marked difference became obvious on day 13 of pregnancy; day 13 was particularly chosen for comparing the enzyme activity because this day precedes the start of the signs of resorption in the retinoate-treated rats.

Total gonadotrophic potency in the pituitary and plasma of female rats raised on the vitamin A-deficient

Table 4. *Changes in Δ^5 - 3β -hydroxy steroid dehydrogenase activity in the ovary of retinyl acetate- or retinoate-fed rats during compensatory hypertrophy or pregnancy*

Rearing of the rats was as described in Table 3. The preparation and assay of the enzyme system were as described by Rubin, Deane & Hamilton (1963). The results are given as means $\pm$ S.D. for six rats in each group.

Vitamin A supplement	Enzyme activity (μ g. of androstenedione formed/min./mg. of protein)					
	Compensating			Pregnant		
	Before hypertrophy	After hypertrophy	Change in activity (%)	Day 0	Day 13	Change in activity (%)
Retinyl acetate	1.26 $\pm$ 0.18	2.06 $\pm$ 0.08	63.5	1.02 $\pm$ 0.06	7.20 $\pm$ 0.34	605.8
Retinoate	1.22 $\pm$ 0.14	1.54 $\pm$ 0.12	26.2	1.10 $\pm$ 0.10	4.92 $\pm$ 0.28	347.3
			$P < 0.01$			$P < 0.01$

diet supplemented with retinyl acetate or sodium retinoate. In general, the experiments on ovarian compensatory hypertrophy indicated that the ovaries of the retinoate-treated rats do not respond to gonadotrophin stimulation. But it was not clear whether the poor hypertrophy of the ovaries observed in these rats was due to any changes in the gonadotrophin contents of the pituitary and plasma or, alternatively, whether it was due to insensitivity of the ovary to the gonadotrophins circulating in blood. This aspect was investigated by determining the total gonadotrophin contents of the pituitary and plasma of rats.

The results (Table 5) show that the total gonadotrophic potency of the pituitary and plasma, as reflected by the uterine weight of the mouse, did not differ significantly in the rats that were supplemented with retinoate or retinyl acetate. In contrast, the total gonadotrophin contents of the pituitary of the vitamin A-deficient rats were significantly increased, and the plasma values showed a corresponding fall.

Effect of intrauterine instillation of retinoate in the pregnant rats. It was also possible that the foetal resorption in the retinoate-fed rats might be due either to an insufficient availability of the acid to the developing foetus or to some toxic effect of retinoate on the foetal development. These

points were investigated by intrauterine administration of sodium retinoate to groups of pregnant rats maintained on the vitamin A-deficient diet supplemented with sodium retinoate or retinyl acetate.

On day 8 of pregnancy a catheter (Adams intradermic polyethylene tubing, internal diam. 0.011 in., external diam. 0.024 in.; Medical Formulation, PHF) was introduced dorsally into the uterus from the ovarian end and was held in position by securing knots with threads along its base. In those cases where the implantation sites were noticed close to the ovary, the catheter was introduced laterally into the blank space immediately after the foetal sites and additional knots were provided at the ovarian end to hold the catheter in position. The free end of the catheter was exposed to the outside through the body wall and the wound was sutured. Sodium retinoate (5 µg.) was injected daily from day 8 to day 19 of pregnancy with a hypodermic syringe (26G needle), and the free end of the catheter was clipped after each injection to prevent backflow of the retinoate. Care was exercised to prevent infection by using aseptic conditions, by keeping the cages clean and by frequently transferring the animals to fresh cages. In none of the controls was the pregnancy interrupted by the catheter or by the vehicle used

Table 5. *Contents of total gonadotrophins in the pituitary and plasma of retinoate-fed, retinyl acetate-fed or vitamin A-deficient rats*

The assay procedure was as described in the text. Results are given as means $\pm$ S.D. The values in the groups receiving the pituitary or blood plasma from rats treated with retinyl acetate or retinoate are significantly not different ($P > 0.01$), whereas those in the groups given pituitary or blood plasma from the rats supplemented with no vitamin A are statistically significant ($P < 0.01$), when compared with those that were treated with the pituitary or plasma of rats receiving retinyl acetate or retinoate.

Treatment	Uterine wt. (mg./100 g. body wt. of mouse)
Saline	62.6 $\pm$ 10.6
Macerated pituitary from retinyl acetate-treated rats	369.3 $\pm$ 106.2
Macerated pituitary from retinoate-treated rats	316.6 $\pm$ 53.5
Macerated pituitary from vitamin A-deficient rats	512.2 $\pm$ 40.8
Plasma from retinyl acetate-fed rats	103 $\pm$ 16.0
Plasma from retinoate-fed rats	98 $\pm$ 21.2
Plasma from vitamin A-deficient rats	70 $\pm$ 14.6

Table 6. *Effect of intrauterine instillation of retinoate on foetal development in pregnant rats*

Sodium retinoate (5 µg.) was administered daily into the uterus of pregnant rats from day 8 to day 19 of pregnancy. The procedure was as described in the text. Litter sizes and litter weights are given as means $\pm$ S.D.

Vitamin A supplement	No. of rats	Compound instilled	No. of rats resorbing foetuses	Average litter size	Average litter wt. (g.)
Retinyl acetate	4	Vehicle only	0	8.2 $\pm$ 1.2	46 $\pm$ 4.2
Retinyl acetate	6	Retinoate	0	7.0 $\pm$ 2	50 $\pm$ 6.0
Retinoate	8	Retinoate	8	—	—

for dissolving the retinoate. It was consistently found that retinoate was not toxic to the foetus of the rats receiving retinyl acetate, nor did it prevent foetal resorption in the retinoate-fed pregnant rats (Table 6).

DISCUSSION

In a preliminary communication (Juneja *et al.* 1964) we reported that, when rats are orally supplemented with aqueous dispersions of 5 μ g. of retinoic acid/rat/day after they were brought to the weight-plateau stage of vitamin A deficiency, they do not conceive on being allowed to mate with normal males. But in our present experiments, where the rats were daily given 15–20 μ g. of retinoic acid mixed with the diet, conception took place. It therefore seems probable that in our previous trials the amounts of retinoic acid given were probably inadequate, and also, since it was given in one single dose, that it could not sustain its effects for the following 24 hr., whereas in the present experiments it proved more effective because larger amounts of it were given mixed with the diet and the animals thus had a continuous supply of it.

During the present investigations attempts were made to prevent foetal resorption by injecting different hormones. Of the several hormones tested only pregnenolone and oestradiol-17 β were fully effective; the others were only partially effective or completely ineffective. It is surprising that injections of progesterone, at the dosage used, were without any appreciable effect in preventing resorption in most of the rats, because we have shown that, compared with the controls, the activity of Δ^5 -3 β -hydroxy steroid dehydrogenase is markedly lower in the ovaries of the retinoate-fed pregnant rats (day 13 of pregnancy), and it is difficult to explain this anomaly. However, it is possible that, in spite of a decrease in the activity of the enzyme, progesterone is formed in amounts that are sufficient to sustain the pregnancy.

Coward *et al.* (1966) made similar attempts to prevent foetal resorption in retinoate-treated rats by injections of oestrone (1 μ g./rat/day) or progesterone (4 mg./rat/day), but their attempts were unsuccessful. Our findings with progesterone are in full agreement with theirs. However, our attempts with oestradiol-17 β were successful whereas their trials with oestrone were unsuccessful. It should be possible to explain this difference on the basis of the relative activities of oestrone and oestradiol-17 β and the mode of their administration. Thus oestradiol-17 β is several times more effective than oestrone; also, whereas we injected 1 μ g. of this hormone in divided doses, Coward *et al.* (1966) gave the same amount of oestrone in a single dose. However, although we were able to prevent re-

sorption in the retinoate-fed rats by injections of oestradiol-17 β or pregnenolone, we were not able to induce normal littering and lactation in these rats by such treatments.

The hypertrophy of the ovary in a unilaterally ovariectomized rat is a function of the hypophysis-gonad axis, and therefore the poor response of the ovary of the retinoate-treated rats to compensatory hypertrophy, as observed here, suggested that its hypophysis-gonad axis was not functioning normally. Moreover, the response of the compensating ovary of the retinoate-fed rats could not be increased by the administration either of saline homogenates of fresh pituitary or of saline suspensions of acetone-dried sheep pituitary; at the same time the gonadotrophin contents of the plasma and pituitary of the two groups of rats receiving the two forms of vitamin A did not show any marked difference. It is thus clear that the ovaries of the retinoate-treated rats are insensitive to gonadotrophin stimulus.

Although these experiments showed that the ovaries of the retinoate-fed rats are not responsive to exogenous stimulation by follicle-stimulating hormone and luteinizing hormone, the possibility that the foetal resorption could be due to inadequate supply of some of the steroid hormones as well as of prolactin could not be ruled out. Thus, although a copious endogenous production of prolactin by homografts of pituitary under the kidney capsules (Everett, 1956) supported gestation in 80% of the rats, exogenous administration of pregnant-mare's serum and prolactin were effective, in varying degrees, in supporting the gestation, but not in promoting littering.

Howell, Thompson & Pitt (1964) could find no histological changes in the ovaries of the rats that received either retinoic acid or retinyl acetate; even on day 13 of pregnancy they failed to find any change. But we have demonstrated here a significantly diminished compensatory hypertrophy of the ovaries of the rats given retinoate, compared with the corresponding group receiving retinyl acetate. There was a simultaneous increase in Δ^5 -3 β -hydroxy steroid dehydrogenase activity in the compensating ovary of both groups, but the increase was markedly higher in the retinyl acetate-treated group. It has also been shown here that, whereas the specific activity of this enzyme is increased in the ovaries of the pregnant rats compared with that in the corresponding non-pregnant ones, the percentage increase is lower in the retinoate-fed rats compared with the corresponding retinyl acetate-treated ones. It therefore seems probable there are small differences in the functioning of these ovaries that are not perceptible under normal conditions, but become significant under stress conditions such as pregnancy and compensatory hypertrophy. These aspects have been fully discussed by Juneja (1968).

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