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Effects of hCG and of season on *in vitro* steroidogenesis by 18-day chick embryo gonads.

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Summary. Fragmented 18-day chick embryo gonads were explanted on synthetic media in the presence of hCG added to 24 h cultures at the beginning (0 h) and to 48 h cultures, only after 24 h or at 0 and 24 h. The production of seven steroids was assayed by RIA and compared to that of control cultures containing no hCG. The levels were expressed as *in vitro* production per gonad (after deduction of the non-explanted gonadal content). When 5 IU of hCG were added to the 24 h culture media at 0 h, the overall steroidogenesis of the left and right gonads of both sexes increased significantly, the DHA in both sexes showing the most increment while Δ^4-steroid (Prog. and Δ^4-dione) production was not stimulated. Moreover, female gonad DHA production, as in the controls, was always higher than that of the testis. The female gonads produced mainly more E_2, while testis T production was stimulated. Nevertheless, the discrepancy (observed earlier in the controls) between T and E_2 balances of the left and right female gonads, and of the female and male gonads, was maintained under stimulation with female gonads and even increased with testis. A higher dose of hCG (15 IU), in these conditions, did not however further stimulate overall steroidogenesis. The effects on this and DHA production, observed with 5 IU of hCG, were not seen when that gonadotropin was given *in vitro* only after 24 h. As compared to the 48 h control cultures, only E_2 was increased with both female gonads and T with left testis. On the other hand, when hCG was given at both 0 and 24 h or only after 24 h, Δ^4-androstene-dione decreased, while this decrement was not seen in the 24 h cultures when hCG was given at 0 h. There is probably a relation between the decrease of the Δ^4-steroid and the E_2 increment which might be explained by hCG action on the aromatase process. However, a significant seasonal difference was seen during *in vitro* steroidogenesis by gonads of both sexes. This discrepancy mainly concerned overall steroidogenesis by gonads of 10 to 15-day embryos but not qualitative sex differences. The observation could be related to season-dependent precursor accumulation during oogenesis.

Introduction.

The *in vitro* production of steroids and sex hormones from radioactive precursors by chick embryo gonads has been largely demonstrated in recent years by incubation techniques (Galli and Wassermann, 1972, 1973) and organotypic cultures (Cedard and Haffen, 1966 ; Weniger *et al.*, 1967 ; Guichard *et al.*, 1972).

The presence of steroids in the gonads and their *in vitro* secretion has been verified recently by radioimmunological assays (Guichard *et al.*, 1977 ; Teng and Teng, 1977). Our results have clearly defined the hormonal production abilities of both male and female gonads at different stages of embryological development. Female gonads produce estradiol while, owing to their restricted aromatizing capacity, the testes mainly secrete testosterone. A quantitative and qualitative difference in hormone secretion is observed between the left (ovary) and the right female gonads. Moreover, *in vitro* secretion can be enhanced by the addition of different steroid precursors of the sex hormones (Guichard *et al.*, 1979, in press).

On the other hand, the hypophysis and hCG influence the gonads of bird embryos. Estrogen biosynthesis from radioactive acetate and DHA is stimulated *in vitro* by hCG, mainly with ovary of older embryos (Cedard *et al.*, 1968). Hypophyseal *in vivo* control of the gonads is seen at the testicular androgen level in embryos from 13-14 days (Woods *et al.*, 1977). However, early hypophysectomy does not abolish the biological properties of 18-day male and female gonads (Akram and Weniger, 1969), and ovary is still able to produce *in vitro* estrogens from acetate (Akram *et al.*, 1973).

The purpose of the investigations reported in this paper was to improve the direct effects of hCG on the production of different steroids, according to the sex and the side of the chick embryo gonad and to the time of culture at which the gonadotropin was added *in vitro*. We also present the seasonal differences in steroid production observed during our previous radioimmunological assay (RIA) of control cultures.

Material and methods.

Pieces of 18-day chick embryo gonads (5 gonads per Falcon dish) were explanted into organotypic cultures, and their secretion was tested by RIA as described earlier (Guichard *et al.*, 1977). Synthetic media (Parker 199 without serum) and short-time cultures were used in order to avoid or limit *in vitro* the modifying effects on the explants of added or secreted products (Erickson, 1974 ; Carlon and Erickson, 1978). Moreover, media with embryo extracts do allow feminization by estrogens or androgens (Weniger and Zeis, 1973), while synthetic media do not (Stenger-Haffen, 1957). Five or 15 IU of human gonadotropin (hCG) per Falcon dish (0.8 ml) were added to the media in different conditions : at time 0 for 24 h cultures (5 series), and at 0 and 24 h (2 series) or only after 24 h (2 series) for 48 h cultures (media changed after 24 h). The results were compared to controls explanted in the same conditions but without hCG.

The RIA's were performed simultaneously on several different steroids : progesterone (Prog.), Δ^4-androstene-dione (Δ^4-dione), dehydroepiandrosterone (DHA), testosterone (T), dihydrotesterone (DHT), estrone (E_1) and estradiol (E_2). The results express the *in vitro* production per gonad in 24 or 48 h ; they were obtained by subtracting the level of gonads removed at the same time, but not explanted, from the *in vitro* steroid content (explants + media).

Results.

a) *Addition of* 5 *or* 15 IU *of* hCG *at the beginning of* 24 *h culture* (fig. 1, table 1).

The total production of the seven steroids assayed (fig. 1) was significantly enhanced ($P < 0.05$) in the presence of 5 IU of the gonadotropin in the left as well as in the right gonad of both sexes (ovary : 24 p. 100 ; right female gonad : 38.7 p. 100 ;

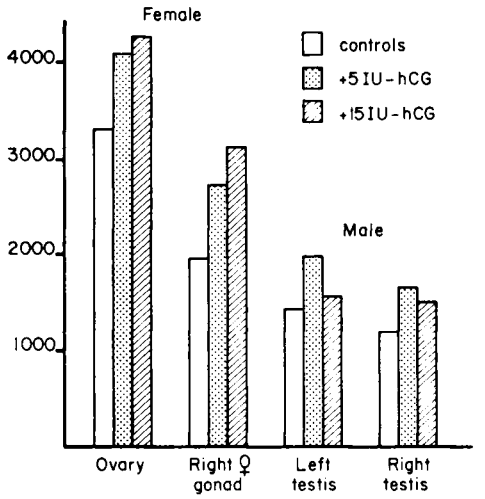

FIG. 1. — *Compared* in vitro *effects of* hCG (5 or 15 IU) *on steroidogenesis by* 18*-day chick embryo gonads.* The data express in pg/gonad/24 h the total production of the seven steroids assayed.

TABLE 1

In vitro *production of different steroids by* 18*-day chick embryo gonads explanted* 24 *hrs when* 5 *or* 15 IU *of* hCG *are added at the onset of culture.* Data are expressed in pg/gonad/24 h

			DHA	Prog	Δ^4-dione	E_1	E_2	T	DHT	Total
♀	Left (ovary)	Control	666	679	304	282	906	315	145	3 297
		+ 5 IU	1 374	435	320	274	1 155	441	103	4 102
		+ 15 IU	885	501	468	414	1 449	373	191	4 281
	Right gonad	Control	378	365	283	148	304	414	63	1 965
		+ 5 IU	1 290	129	202	161	371	458	115	2 726
		+ 15 IU	860	351	340	191	736	587	58	3 123
♂	Left testis	Control	305	340	227	69	30	319	133	1 423
		+ 5 IU	653	290	199	46	32	698	78	1 996
		+ 15 IU	369	273	259	84	57	424	92	1 558
	Right testis	Control	281	153	223	54	19	348	111	1 189
		+ 5 IU	779	98	141	36	14	669	66	1 662
		+ 15 IU	337	233	343	65	13	448	67	1 506

left or right testis : about 40 p. 100). However, a threefold dose of hCG (15 IU) did not, in these conditions, further stimulate steroid synthesis at the same rate (ovary : 30 p. 100; right female gonad : 50 p. 100) ; testicular steroid synthesis showed a lesser increase. For this reason, we centered our observations mainly on the results obtained with 5 IU of hCG.

If we now consider the production of the different steroids assayed (table 1), it appears that with gonads of both sexes and sides, the DHA was mainly increased ($P < 0.02$), while the Δ^4-steroids (Prog., Δ^4-dione) were not. The DHA level rose 106 p. 100 with left female gonad and 241 p. 100 with right female gonad, and 114 p. 100 with left testis and 177 p. 100 with right testis.

Concerning sex hormone production, there was an increase in female gonad estradiol (E_2) (ovary : 27 p. 100 ; right gonad : 22 p. 100), while the estrogens (E_2 and E_1) were not increased in testes. The testosterone production was more enhanced with both testes (left : 118 p. 100 ; right : 92 p. 100) than with female gonads (ovary : 40 p. 100 ; right gonad : 10 p. 100).

Moreover, the discrepancy observed earlier between the hormonal balances (E_2 and T) (Guichard *et al.*, 1977) of the left and right female gonads, and of the female and male gonads, was maintained during *in vitro* stimulation. Compared to female gonads, the T/E_2 ratio was high in male gonads, and even increased, when stimulated by hCG, from 10.6 to 21.8 for left testis and from 18.3 to 47.7 for right testis. On the other hand, the E_2/T ratio in female gonads was maintained during stimulation : 2.87 and 2.61, respectively, for control and stimulated ovaries, 0.73 and 0.81 for control and stimulated right gonads. Once more, it appeared that the rudimentary right gonads produced a rather high level of testosterone when compared to their estrogen production and to that of ovarian testosterone.

b) *Addition of* hCG *in* 48 *h cultures* (table 2).

TABLE 2

Effects of hCG *on steroidogenesis by* 18*-day chick embryo gonads explanted* 48 *h.* The gonadotropin (5 IU) is added *in vitro* only after 24 h (B) or at 0 and 24 h (C) ; the data are expressed in pg/gonad/48 h

			DHA	Prog.	Δ^4-dione	E_1	E_2	T	DHT	Total
♀	Left (ovary)	Control (A)	2 064	846	710	532	645	423	432	5 652
		+ hCG (B)	707	623	699	562	2 976	395	265	6 227
		+ hCG (C)	2 534	965	247	1 374	4 755	1 062	129	10 866
	Right gonad	Control (A)	1 742	466	2 774	309	533	368	322	6 514
		+ hCG (B)	403	141	588	337	1 311	398	175	3 053
		+ hCG (C)	2 396	488	37	615	2 644	472	163	6 815
♂	Left testis	Control (A)	1 820	387	1 437	310	32	304	249	4 539
		+ hCG (B)	173	153	551	90	0	428	0	1 395
		+ hCG (C)	1 805	290	82	134	144	1 057	416	3 928
	Right testis	Control (A)	1 422	342	844	142	23	521	327	3 621
		+ hCG (B)	84	211	504	110	0	502	397	1 908
		+ hCG (C)	1 668	85	12	87	18	675	51	2 596

In the second experimental group we tested the *in vitro* effects of the gonadotropin on cultures lasting 48 h (medium changed after 24 h). In two experiments, 5 IU of hCG were added only after 24 h (B) ; while in the other two it was added both at 0 and 24 h (C). The data were compared to the control explants cultured in the same conditions without hCG (A).

When hCG was supplied only 24 h after the onset of the culture (B), the levels of the global steroid production decreased by about 50-60 p. 100 of that of the controls, except for ovary. However, with this delayed hCG action, E_2 mainly increased with both female gonads, while testicular estrogens (E_1 and E_2) dropped. In these conditions, all the other steroids (mainly DHA) tended to decrease ; testosterone production was somewhat enhanced (40 p. 100) only with left testis.

When hCG was added both at 0 and 24 h (C), the total production of the seven steroids assayed was significantly increased ($P < 0.05$) only with ovary (108 p. 100). However, the levels of some hormones rose with the different gonads. This observation concerned chiefly E_2 with ovary (637 p. 100) and right gonad (396 p. 100) and even left testis (350 p. 100), but in the latter the levels remained low in comparison to that of female gonads. Testosterone was also increased mainly with left testis (247 p. 100) and ovary (151 p. 100), but less with right female (28 p. 100) and male (41 p. 100) gonads. In these conditions, DHA did not drop as above (B), and progesterone levels were not significantly modified while that of Δ^4-dione decreased drastically.

c) *Seasonal influence on hormone production* (fig. 2).

During our numerous investigations using RIA on *in vitro* steroidogenesis by chick embryo gonads, spread over more than two years, we noticed large quantitative variations in the data which depended on the season at which the gonads were explanted. So, we decided to examine this point more closely and to classify the results into two groups, one covering the short daylight season (winter gonads : 15 September-15 March) and the other the longer photoperiod season (summer gonads : 15 March-15 September).

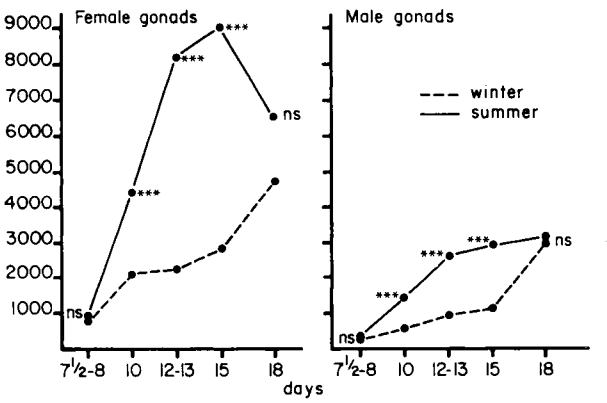

FIG. 2. — *Compared seasonal differences in steroidogenesis* by gonads of female and male embryos (7 1/2 to 18 days). The data express total *in vitro* production in pg/gonads (left + right) for 24 h cultures.

It appeared in both sexes that the total steroid production by summer gonads was much higher than that of winter gonads (fig. 2). This difference was highly significant ($P < 0.05$) for gonads of 10, 12 and 15-day embryos, and it was not observed at the beginning of sexual differentiation (7 1/2 days) or towards the end of incubation.

Female gonads of 10 to 18 days always produced more estrogens in summer than in winter, and much more at any season than the testes. However, season and sex variations in testosterone levels were not significantly different : they were higher in summer for 15 and 18-day female gonads and in winter for 8 to 12-day testes.

Discussion.

The present results demonstrate that hCG can stimulate *in vitro* the spontaneous steroidogenesis of 18-day chick embryo gonads. The gonadotropic action was more effective when the gonadotropin was added at the beginning of 24 or 48 h cultures than only after a delay of 24 h : the total steroid production was increased, and mainly that of DHA for both sexes.

For female gonads the stimulation concerned mainly E_2 production. This result confirmed those obtained in the presence of radioactive precursors (Cedard *et al.*, 1968), and has also been observed in short *in vitro* incubations with 8 to 18-day chick embryo gonads (Teng and Teng, 1977). Testosterone production increased most with the testes.

hCG acts on pregnenolone formation from esterified cholesterol. This was confirmed for post-hatching 7-day testis in which esterified cholesterol was significantly reduced with hCG (Massa and Aoki, 1976) ; in the embryo the interstitial cells of male and female gonads also contain such esterified cholesterol (Scheib, 1959). Our results suggest that hCG has an effect on cholesterol utilization which could be caused by cholesterol-esterase or 20-22 cholesterol hydroxylase stimulation, leading to an increased endogenous pregnenolone production. It has also been demonstrated that hCG can stimulate *in vitro* cholesterol formation from acetate by chick embryo gonads (Haffen *et al.*, 1969).

Moreover, our different data do not indicate an immediate and direct effect of hCG on the Δ^5-3β HSDH enzymatic system, and some results obtained with rat testis show that *in vivo* hCG needs some time to act on the Δ^5-3β oxydoreductase (Wiebe, 1978). In the chick embryo, the gonads are under hypophysal control from 14 days on, as shown by the level of Δ^5-3β HSDH activity in ovaries (Woods and Weeks, 1969) and by that of testicular androgens (Woods *et al.*, 1977) which decrease after early hypophysectomy.

The very significant increase of DHA and testosterone, chiefly by male embryo gonads, confirms results obtained with 2-day old chick testis, in which testosterone biosynthesis from acetate goes through DHA and is stimulated by hCG (Connel *et al.*, 1966).

The increased estrogen production with a drop of the Δ^4-androstene-dione level suggests that hCG acts on aromatizing enzymes ; this has also been observed previously with radioactive DHA (Cedard *et al.*, 1968).

The seasonal differences in the hormone production capacities of chick gonads from embryos not submitted to photoperiod or maternal influence at first glance seem

rather surprising. As these differences are greatest at 12-15 days, and not at 18 days, they are probably not due to embryonic hypophysis activity. The higher metabolic rate of the gonads may be linked to an egg precursor supply which is determined in the hen during ovogenesis ; the yolk has been shown to contain different enzymes transforming the precursors of steroidogenesis (Delrio *et al.*, 1968). Moreover, estrogens can be transferred *in vivo* to the eggs in laying ring doves and lead to feminization of the embryos (Riddle and Dunham, 1942) ; also, injected radioactive estrogens in laying hens are transferred to their eggs (Arcos, 1972). Season may as well influence the relative proportion of active and inactive theca cells in the duck ovary (Deray and Gomot, 1978).

The technique of the organotypic culture of gonads on synthetic media, combined with radioimmunoassay, is a reliable experimental model for *in vitro* studies of steroid biosynthesis capacities and for investigating the direct effects of various factors.

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Résumé. Des gonades d'embryons de Poulet de 18 jours ont été cultivées sur milieux synthétiques, en présence de HCG ajouté dans différentes conditions. La production de 7 stéroïdes a été estimée par dosages radioimmunologiques et comparée à celle de gonades explantées en absence de HCG. Les valeurs expriment pour 24 h ou 48 h, la production *in vitro* (explants + milieux) en pg/gonade (après déduction des teneurs en stéroïdes de gonades non cultivées).

L'adjonction de 5 UI de HCG au début de cultures de 24 h stimule la production globale des stéroïdes, dans les gonades gauches et droites des deux sexes. L'augmentation la plus forte se situe au niveau de la synthèse de la DHA dont les taux sont toujours plus élevés pour les gonades femelles que pour les testicules ; les Δ^4-stéroïdes (Prog. et Δ^4-dione) ne sont, par contre, pas concernés par cette stimulation. Au niveau de la secrétion hormonale, les gonades femelles produisent surtout plus de E_2 et les testicules plus de T. L'équilibre entre ces deux stéroïdes (E_2/T) n'est pas modifié pour les gonades femelles et reste toujours plus élevé dans l'ovaire que dans la gonade droite. Pour les testicules, le rapport élevé T/E_2 est encore augmenté après stimulation par HCG. L'adjonction d'une dose triple de HCG (15 UI) n'augmente cependant pas en rapport la production hormonale.

Les effets de HCG (5 UI) ajouté seulement après 24 h (cultures de 48 h) révèlent que la production globale augmente seulement pour l'ovaire et celle de E_2 uniquement pour les gonades femelles. A part le testicule gauche, la synthèse de T n'est pas stimulée dans ces conditions.

Par contre, si on fournit aux explants 5 UI de HCG à la fois au départ et après 24 h de culture, on observe surtout une chute de la Δ^4-dione parallèle à une augmentation des œstrogènes et surtout de E_2 pour les gonades femelles, ce qui indique pour ce sexe une stimulation de l'aromatisation par HCG.

Enfin, une différence saisonnière significative de la stéroïdogenèse *in vitro* par les gonades embryonnaires est observée pour les stades de 10 à 15 jours. Pour les deux sexes, la production globale est nettement plus élevée au printemps-été et il en est de même pour les œstrogènes par les gonades femelles. Ces résultats suggèrent l'existence de capacités endogènes des gonades, variables avec les saisons, et probablement transmises à l'œuf pendant l'ovogenèse.

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