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THE INFLUENCE OF GONADOTROPINS *IN VIVO* UPON THE BIOSYNTHESIS OF ANDROGENS BY HOMOGENATE OF RAT TESTIS

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SUMMARY

Treatments of rabbits with gonadotropins *in vivo* failed to produce demonstrable stimulation of the conversion of [4-^{14}C]cholesterol to [^{14}C]testosterone by homogenate of testis. Short-term treatment (1–12 h) of hypophysectomized rats with human chorionic gonadotropin or interstitial cell-stimulating hormone also failed to increase the conversion of cholesterol to androgens by homogenate of testis. However, 24 h after a single injection of human chorionic gonadotropin or interstitial cell-stimulating hormone an increase in the conversion of cholesterol to androgens by homogenate of testis from hypophysectomized rats was observed.

INTRODUCTION

Evidence has been presented by SAMUELS AND HELMREICH to show that HCG is capable of causing an increase in the activity of testicular 3β-hydroxysteroid dehydrogenase in hypophysectomized rats[1]. Although this response was seen within 24 h of a single injection of HCG, the authors were careful to point out that the effect described should probably be regarded as part of a non-specific response involving increased protein synthesis.

Previous studies in this laboratory indicated that the response of slices of rabbit testis to ICSH involves increased protein synthesis[2]. So far it has not been possible to demonstrate stimulation of an homogenate of testis by ICSH added *in vitro*[2] and, since it appears that enhanced permeability of testicular cells to essential substrates and cofactors does not play a role in the mechanism of action of ICSH on testis *in vitro*[3], the failure of an homogenate to respond to the tropic hormone might be explained by failure of protein synthesis on the part of the subcellular system used. Moreover, since earlier experiments with ACTH suggested that this hormone increased the conversion of cholesterol to pregnenolone[4] and since it has been possible to demonstrate the conversion of [4-^{14}C]cholesterol to [^{14}C]testosterone by homogenate of rabbit testis[2], the

Abbreviations: HCG, human chorionic gonadotropin; ICSH, interstitial cell-stimulating hormone; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2(5-phenyloxazolyl)-benzene.

present experiments were undertaken to explore the possible response of a homogenate of testis to previous gonadotropic stimulation *in vivo*.

MATERIALS AND METHODS

Adult male hypophysectomized rats (Sprague Dawley) were obtained from Hormone Assay Laboratories, Inc. Operation was performed at 50 days of age and the animals were fed as described by SAMUELS AND HELMREICH[1]. Experiments were performed between 4 and 16 days after operation. Preliminary experiments were performed on intact adult male rats of the same species in order to find the most suitable conditions of incubation. In some experiments, hypophysectomized rats received a single intraperitoneal injection of ICSH or HCG dissolved in saline while control animals received an equal volume of saline. The animals were killed 24 h later. In other cases, hypophysectomized rats were lightly anaesthetized with ether, the left testis removed, HCG, ICSH or saline administered intraperitoneally and the animals allowed to recover. The animals were killed 24 h later and the right testes were removed.

Experiments with rabbits were performed on adult males of the New Zealand white strain. Tropic hormones were administered as a single intravenous injection or subcutaneously for periods of 2–7 days. In each case one testis was removed before treatment and the second testis after treatment.

In both rats and rabbits homogenate of testis was prepared as previously described[2] and incubated in Krebs–Ringer phosphate buffer at 37.5° with a final pH of 7.4 and with continuous agitation. The final volume was 3.0 ml, containing the equivalent of 500 mg (wet weight) of testis per flask as a 50 % (w/v) homogenate. Cofactors were added as shown in the legends of the accompanying tables and figures. [7α-^{3}H]Cholesterol and [7α-^{3}H]pregnenolone were dissolved in 0.2 ml propylene glycol.

Following incubation, 100 μg each of testosterone, androstenedione and progesterone were added to each flask, the tissue was extracted three times with ether (three volumes) and the ether extract washed with water and evaporated to dryness.

The extract was dissolved in hexane and applied to a column of deactivated aluminum oxide[5] and eluted with hexane 50 ml, benzene–hexane (50:50) 100 ml, benzene 100 ml and ethyl acetate 100 ml. Trial columns revealed that the ethyl acetate fraction contained more than 98 % of added [4-^{14}C]progesterone and [7α-^{3}H]pregnenolone and less than 0.1 % of added [4-^{14}C]cholesterol. The ethyl acetate fraction was applied to paper in the system hexane–benzene–formamide until the front had moved 25 cm (see ref. 6). The chromatogram was then transferred, without drying, to the system hexane–benzene–formamide and allowed to run half as long again as the time required for the mobile phase to reach the end of the paper strips (first chromatogram).

The dry chromatograms were examined with a Haines' ultraviolet scanner[7] to locate the carrier steroids and then counted in a windowless Geiger strip-counter. The areas occupied by the added testosterone and androstenedione were separately eluted, acetylated and run in hexane–formamide (second chromatogram). These chromatograms effectively separated testosterone acetate from 17α-hydroxyprogesterone in one case and dehydroepiandrosterone acetate from androstenedione in the other.

The identity of the [^{3}H]androstenedione and [^{3}H]testosterone formed in these experiments from tritiated precursors was established by the preparation of deriva-

tives with repeated determinations of specific activity. At each step radioactivity was measured by submitting an aliquot to counting by liquid scintillation after dissolving the specimen in 10 ml of scintillation fluid and mass was determined by dissolving an appropriate aliquot in vacuum distilled methanol and measuring the absorbancy of this solution between 220 and 260 mμ. Measurement of the mass of steroids was based upon ALLEN's correction[8] of the specimen after comparison with the ultraviolet absorbancy of standard solutions of the steroid under examination. After examination of absorbancy the methanolic solution was added to the remainder of the specimen and the next derivative prepared. The derivatives were prepared and examined as follows:

[^3H]Androstenedione

After determination of specific activity of [^{3}H]androstenedione on the second chromatogram this compound was reduced to [^{3}H]testosterone by sodium borohydride[9], extracted with warm benzene and applied to paper in the system hexane–benzene–formamide (third chromatogram). [^{3}H]Testosterone was located by the Haines' scanner and by strip-counting, eluted and submitted to determination of specific activity by measurement of ultraviolet absorbancy and by liquid scintillation counting. The [^{3}H]testosterone was next acetylated and applied to paper in hexane–formamide (fourth chromatogram), the acetate located by the Haines' scanner and by strip-counting and specific activity determined as before.

[^3H]Testosterone acetate

After determination of specific activity of [^{3}H]testosterone acetate on the second chromatogram this compound was saponified by refluxing for 1 h in 1 ml ethanol and 0.2 ml of 4 % (w/v) aqueous sodium hydroxide. The mixture was extracted with ether, washed with water and applied to paper in the system hexane–benzene–formamide (third chromatogram). The free testosterone was oxidized to androstenedione by chromic oxide, extracted with ether, washed with water and applied to paper in hexane–benzene–formamide (fourth chromatogram).

Further support for the identity of these steroids was achieved by pooling material from several flasks incubated in an earlier experiment and recrystallizing to constant specific activity by adding authentic "cold" steroid and weighing crystals after each recrystallization. Radioactivity was determined by liquid scintillation counting of an aliquot of a solution of the crystals (Table II). Scintillation fluid was prepared by dissolving PPO (4 g) and POPOP (40 mg) in 1 l of toluene.

Measurements of [^{3}H]androstenedione and [^{3}H]testosterone were expressed as disintegrations/min/30 mg testicular protein since each flask contained between 15 and 25 mg of protein.

Cholesterol was measured by submitting appropriate fractions (the benzene–hexane (1:1) and 100 % benzene fractions from the aluminum oxide column) to digitonin precipitation, hydrolysis in pyridine and ether extraction[10]. An aliquot of the ether extract was applied to gas–liquid chromatography on SE 30 and the peak corresponding to authentic cholesterol was measured by triangulation[11].

HCG was a gift of Ayerst Laboratories and ICSH (NIH-LH-S-1) was kindly provided by the Endocrine Study Section of the U.S. Public Health Service. Radioactive steroids were obtained from New England Nuclear Corporation and the co-

factors TPN, glucose 6-phosphate, glucose-6-phosphate dehydrogenase from Sigma Chemical Company.

RESULTS

Experiments in the intact rabbit failed to demonstrate enhanced steroid biosynthesis from [4-^{14}C]cholesterol by a homogenate of testes removed 1 h following injection of HCG or ICSH *in vivo*. In these experiments the testis removed after stimulation was always compared with the testis removed before stimulation from the same animal. One such experiment is reported in Table I. In addition, a number of studies were conducted in which duration (1 h to 7 days) and extent of stimulation *in vivo*, nature and concentration of cofactors and duration of incubation were varied; always without success. In these experiments, [^{14}C]testosterone was measured by liquid scintillation counting on material from the first chromatogram. Proof of the identity of this material has been given previously[2]. When hypophysectomized rats were given a single intraperitoneal injection of HCG, no significant alteration in the conversion of cholesterol to androgens by testicular homogenate was observed 1 h or 12 h after the injection.

TABLE I

CONVERSION OF [4-^{14}C]CHOLESTEROL TO [^{14}C]TESTOSTERONE (MEASURED AS ACETATE) BY HOMOGENATE OF RABBIT TESTIS BEFORE AND AFTER ICSH

One testis was removed under nembutal anaesthesia and incubated. The rabbit was then given 1 mg ICSH intravenously and 1 h later the second testis was removed and incubated. Incubation was for 2 h in each case and the substrate was [4-^{14}C]cholesterol (3.2 μC, 56 μg) in each flask. When cofactors were used the following additions were made: TPN $1.5 \cdot 10^{-3}$ M, glucose-6-phosphate dehydrogenase 50 μg/flask (specific activity 90 K units/mg protein), and glucose 6-phosphate $3 \cdot 10^{-3}$ M.

Flask	*Testosterone acetate (disintegrations/min/flask)*					
	Unstimulated			*Following ICSH (1 mg)*		
	1	*2*	*3*	*1*	*2*	*3*
Without cofactors	1500	1712	1680	1320	1485	1600
TPN and reducing system	3847	3927	3780	4060	3412	3530

TABLE II

RECRYSTALLIZATION OF [^{14}C]TESTOSTERONE ACETATE AND [^{14}C]ANDROSTENEDIONE

The conditions of incubation were those given under Table I. In this experiment [4-^{14}C]cholesterol was used instead of the tritiated compound which is available in higher specific activity.

*[^{14}C]Testosterone acetate**			*[^{14}C]Androstenedione**		
Recrystallization	*Solvent*	*Specific activity (disintegrations/min/mg)*	*Recrystallization*	*Solvent*	*Specific activity (disintegrations/min/mg)*
Original mixture	Methanol	365	Original mixture	Methanol	423
1st	Acetone	358	1st	Benzene–methanol	417
2nd	Ethanol	354	2nd	Acetone	419
3rd	Ligroin	360	3rd	Ligroin	413
4th	Methanol	357	4th	Ethanol	420

* In each case the mixture recrystallized consisted of 50 mg of the authentic steroid and the radioactive material pooled from 3 flasks in which homogenate of testis from a hypophysectomized rat had been incubated with [4-^{14}C]cholesterol (11.2 μg, 0.64 μC).

On the other hand, Fig. 1 shows that considerable increase in the conversion of [7α-^{3}H]cholesterol to [^{3}H]androstenedione by homogenate of testis from hypophysectomized rats was seen 24 h after a single intraperitoneal injection of ICSH. In one rat the response is less marked and since in this case the prestimulation level of conversion was high, this difference could be partly explained by incomplete hypophysectomy. A similar but less striking response is seen in the conversion of [7α-^{3}H]-

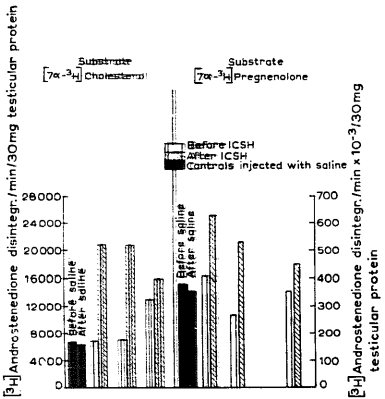

Fig. 1. The conversion of [7α-^{3}H]cholesterol and [7α-^{3}H]pregnenolone to [^{3}H]androstenedione by homogenate of testis from 4 hypophysectomized rats. Conditions of incubation are given under Table IV.

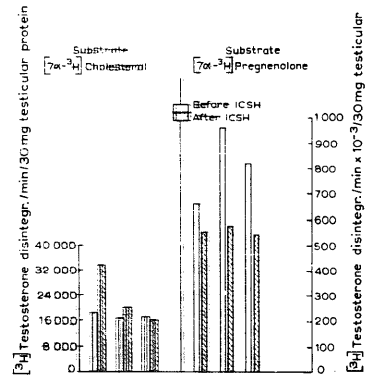

Fig. 2. The conversion of [7α-^{3}H]cholesterol and [7α-^{3}H]pregnenolone to [^{3}H]testosterone by homogenate of testis from 3 hypophysectomized rats. Conditions of incubation are those given under Table IV and the rats are from the experiment reported in Fig. 1.

pregnenolone to [^{3}H]androstenedione by hypophysectomized rats similarly treated. In the same rats the production of [^{3}H]testosterone was examined (Fig. 2) and with [7α-^{3}H]cholesterol as substrate, a less pronounced stimulation is evident in two rats while a slight fall was seen in the rat which showed least change in the production of [^{3}H]androstenedione (Fig. 1). Moreover, the production of [^{3}H]testosterone from [7α-^{3}H]pregnenolone shows a fall following stimulation, so that the conversion of pregnenolone to testosterone plus androstenedione is scarcely affected by stimulation (Table III). Saline-treated controls showed no gross change in the production of androstenedione from either [7α-^{3}H]cholesterol or from [7α-^{3}H]pregnenolone (Fig. 1).

Fig. 3 illustrates an experiment in which three hypophysectomized rats, 10 days after operation, were given a single intraperitoneal injection of HCG while three

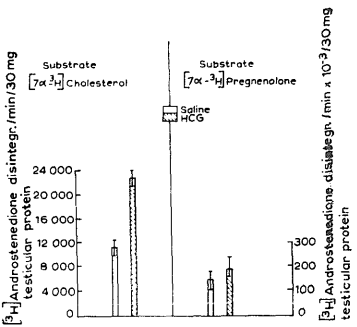

Fig. 3. The influence of HCG on the conversion of [7α-^{3}H]cholesterol and [7α-^{3}H]pregnenolone to [^{3}H]androstenedione by homogenate of testis from hypophysectomized rats. Conditions of incubation are given under Table IV. Rats receiving HCG were given a single intraperitoneal injection of 2000 I.U. in 2 ml saline 24 h before sacrifice, at which time control animals were given an equal volume of saline. The bars represent the mean of two determinations on each of 3 rats and I indicates the range.

TABLE III

THE CONVERSION OF [7α-^{3}H]CHOLESTEROL AND [7α-^{3}H]PREGNENOLONE TO TESTICULAR ANDROGENS (SUM OF [^{3}H]TESTOSTERONE AND [^{3}H]ANDROSTENEDIONE) BY HOMOGENATE OF TESTIS FROM HYPOPHYSECTOMIZED RATS BEFORE AND AFTER A SINGLE INJECTION OF ICSH

The data are taken from Figs. 1 and 2 where the experimental conditions are shown.

Substrate	*Sum [^{3}H]testosterone and [^{3}H]-androstenedione (disintegrations/min/30 mg testicular protein)*	
	Before ICSH	*After ICSH*
[7α-^{3}H]Cholesterol	26 040	56 280
	25 200	43 456
	30 996	32 928
[7α-^{3}H]Pregnenolone	1076 992	1196 888
	1231 440	1114 848
	1177 176	1000 440

TABLE IV

THE CONVERSION OF [7α-^{3}H]CHOLESTEROL AND [7α-^{3}H]PREGNENOLONE TO [^{3}H]ANDROSTENEDIONE BY HOMOGENATE OF TESTIS FROM HYPOPHYSECTOMIZED RATS

The substrates ([7α-^{3}H]cholesterol 2.5 μC, 43 μg or [7α-^{3}H]pregnenolone 2.0 μC, 94 μg) were dissolved in 0.2 ml propylene glycol. Homogenate was prepared as previously described (each flask containing the equivalent of 500 mg wet weight of testis) and the following cofactors added: TPN $1.5 \cdot 10^{-3}$ M, glucose 6-phosphate $3 \cdot 10^{-3}$ M and glucose-6-phosphate dehydrogenase 50 μg/flask (specific activity 90 K units/mg protein). Final volume was 3.0 ml and incubation was for 2 h. No [^{3}H]androstenedione or [^{3}H]testosterone were detected in zero-time controls nor when the homogenate was heated for 30 min at 50° before incubation.

			Androstenedione (2nd chromatogram)		*Testosterone (3rd chomatogram)*		*Testosterone acetate (4th chromatogram)*	
Rat	*Conditions*	*Substrate*	*Radioactivity (disintegrations/min)*	*Specific activity (disintegrations/min/mmole)*	*Radioactivity (disintegrations/min)*	*Specific activity (disintegrations/min/mmole)*	*Radioactivity (disintegrations/min)*	*Specific activity (disintegrations/min/mmole)*
A	Before ICSH	[7α-^{3}H]Cholesterol	21 643	62.6	7 056	56.5	3 264	63.4
	After ICSH	[7α-^{3}H]Cholesterol	62 798	206.2	21 672	207.9	6 528	239.3
B	Before ICSH	[7α-^{3}H]Cholesterol	17 623	60.1	7 896	48.1	3 319	57.4
	After ICSH	[7α-^{3}H]Cholesterol	43 904	144.4	21 504	131.3	5 406	148.8
C	Before ICSH	[7α-^{3}H]Cholesterol	17 057	62.6	5 020	74.9	3 621	74.1
	After ICSH	[7α-^{3}H]Cholesterol	40 690	161.6	15 960	153.2	7 752	172.8
D	Before ICSH	[7α-^{3}H]Pregnenolone	784 096	2669	407 232	2606	124 236	2701
	After ICSH	[7α-^{3}H]Pregnenolone	1028 916	4483	642 768	4307	117 832	4287
E	Before ICSH	[7α-^{3}H]Pregnenolone	475 412	1510	264 264	1596	66 300	1579
	After ICSH	[7α-^{3}H]Pregnenolone	795 480	2428	536 592	3091	192 576	2986
F	Before ICSH	[7α-^{3}H]Pregnenolone	1 372	2978	353 472	2547	101 592	2624
	After ICSH	[7α-^{3}H]Pregnenolone	838 261		451 080	3093	120 770	2913

TABLE V

THE CONVERSION OF [7α-^{3}H]CHOLESTEROL AND [7α-^{3}H]PREGNENOLONE TO [^{3}H]TESTOSTERONE BY HOMOGENATES OF TESTIS FROM HYPOPHYSECTOMIZED RATS

This table is based upon studies in the same experiment as that reported in Table IV.

Rat	Conditions	Substrate	Testosterone (2nd chromatogram)		Testosterone acetate (3rd chromatogram)		Androstenedione (4th chromatogram)	
			Radioactivity (disintegrations/min)	Specific activity (disintegrations/min/mmole)	Radioactivity (disintegrations/min)	Specific activity (disintegrations/min/mmole)	Radioactivity disintegrations/min)	Specific activity (disintegrations/min/mmole)
A	Before ICSH	[7α-^{3}H]Cholesterol	20 670	87	18 984	78	12 741	77
	After ICSH	[7α-^{3}H]Cholesterol	92 257	305	34 608	300	21 412	297
B	Before ICSH	[7α-^{3}H]Cholesterol	59 758	150	17 304	60	10 847	63
	After ICSH	[7α-^{3}H]Cholesterol	52 550	137	21 952	74	14 321	75
C	Before ICSH	[7α-^{3}H]Cholesterol	21 287	57	17 976	52	12 488	53
	After ICSH	[7α-^{3}H]Cholesterol	39 536	123	16 968	78	10 120	74
D	Before ICSH	[7α-^{3}H]Pregnenolone	2 174 424	3 322	669 760	2692	412 610	2710
	After ICSH	[7α-^{3}H]Pregnenolone	859 370	3 376	554 120	2572	388 719	2603
E	Before ICSH	[7α-^{3}H]Pregnenolone	1 485 989	3 714	967 176	3350	624 213	3309
	After ICSH	[7α-^{3}H]Pregnenolone	811 440	3 479	578 256	2272	384 100	2140
F	Before ICSH	[7α-^{3}H]Pregnenolone	1 662 328	3 048	823 704	2362	620 198	2354
	After ICSH	[7α-^{3}H]Pregnenolone	891 845	3 267	549 360	1976	413 012	2071

others were given an equal volume of saline. Homogenate of testis from each rat was incubated with [7α-^{3}H]cholesterol and with [7α-^{3}H]pregnenolone and it can be seen that the conversion of cholesterol to androstenedione is almost twice as great in HCG-treated rats. The conversion of pregnenolone to androstenedione, however, is about the same in both groups.

Tables IV and V show the specific activities of [^{3}H]androstenedione and [^{3}H]-testosterone from the experiment illustrated in Figs. 1 and 2. The radiochemical purity of these compounds strongly supports the idea that homogenate of testes from hypophysectomized rats is capable of converting cholesterol to androgens.

In order to determine the extent to which [7α-^{3}H]cholesterol would be diluted by "cold" cholesterol, the cholesterol content of homogenate of testes from five hypophysectomized rats was measured and the following amounts (expressed as μg/30 mg testicular protein) were found: 184, 204, 210, 197 and 192.

DISCUSSION

The present findings demonstrate that cholesterol can be converted to androgens by homogenate of testes from hypophysectomized rats and that the conversion of cholesterol to androstenedione by such an homogenate is increased by gonadotropic hormones administered *in vivo*. When pregnenolone was used as substrate, increase in the production of androstenedione was observed but a concomitant fall in the production of testosterone makes the significance of this observation uncertain. These responses to gonadotropic hormones were evident 24 h after a single intraperitoneal injection but the failure to demonstrate similar changes 1 or 12 h after such an injection makes it probable that as in the case of the increase in 3β-hydroxysteroid dehydrogenase described by SAMUELS AND HELMREICH[1], the changes seen in the present studies are part of a non-specific response to tropic stimulation and cannot be regarded as indicating a primary effect of ICSH on testicular tissue.

The findings of SAMUELS AND HELMREICH are based upon experiments performed 40 days after hypophysectomy[1], while the present experiments were conducted 10 days after operation. This difference may account for the equivocal response when pregnenolone was used as substrate. The specific activity of the [7α-^{3}H]cholesterol added as substrate in these experiments was approx. 3.5 times as great as that of the [7α-^{3}H]-pregnenolone (2.5 μC (43 μg) as compared with 2.0 μC (94 μg)) but the testis contained approx. 200 μg of "cold" cholesterol per 30 mg testicular protein. If all this cold cholesterol were free to dilute the [7α-^{3}H]cholesterol and if the amount of "cold" pregnenolone in the homogenate is ignored, it will be seen that the specific activity of the cholesterol would be about one half that of pregnenolone. The amount of [^{3}H]androstenedione produced from [7α-^{3}H]pregnenolone in these experiments was approx. 30 times as great as that from [7α-^{3}H]cholesterol and in the case of testosterone the difference was 15 to 20 fold. It therefore appears that even allowing for the diluting influence of "cold" cholesterol, the conversion of cholesterol to androgens in the present system is considerably lower than that of pregnenolone. Moreover it is unlikely that all the cold testicular cholesterol is free to dilute the added radioactive substrate.

Table III shows that conversion of [7α-^{3}H]cholesterol to C_{19} steroids was increased by HCG in all cases and that no consistent change is seen in the case of

[7α-^{3}H]pregnenolone. With [7α-^{3}H]pregnenolone there appears to be a consistent fall in the production of [^{3}H]testosterone after treatment with HCG. The present findings cannot explain this fall although the fact that less testosterone is produced makes enzyme saturation unlikely and suggests some change in the equilibrium testosterone ⇌ androstenedione. Since in the present experiment on rat testicular homogenate reduced TPN was added in concentrations which, at least in the rabbit, were above the optimal level required for maximal steroid biosynthesis[2], it seems unlikely that a relative depletion of reduced TPN could account for this change.

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