Biosynthesis of Testosterone by Rabbit Testis: Homogenate V Slices.* (28800)

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Although it has been shown that interstitial cell-stimulating hormone (ICSH) increases the incorporation of acetate-1-C^{14} into testosterone-C^{14} by slices of rabbit testis (1), it has not been possible to demonstrate an increase in the amount of testosterone formed in the presence of ICSH *in vitro* nor to measure the specific activity of the testosterone-C^{14} produced. Brinck-Johnsen and Eik-Nes(2) showed that, when human chorionic gonadotrophin (HCG) was injected intravenously, there was an immediate increase in output of testosterone in the spermatic vein; and Mason and Samuels(3) found that, when acetate-1-C^{14} was infused into the spermatic artery of the dog, simultaneous infusion of human chorionic gonadotrophin increased the incorporation of C^{14} into testosterone even when the total output of the steroid hormone was not further increased because of a high initial output. It was accordingly considered of interest to measure the amount of testosterone produced by slices of testis *in vitro* in the presence of acetate-1-C^{14} with and without ICSH and, hence, to determine the specific activity of the testosterone-C^{14}.

A second problem related to the action of trophic hormones (including ICSH) has been failure to demonstrate stimulation of steroid biosynthesis under the influence of ICSH or ACTH in subcellular systems of testis(1) or adrenal(4), respectively. The present experiments were designed to compare the amount of testosterone produced by homogenate of rabbit testis with that produced by slices maximally stimulated with ICSH, basing the comparison upon tissue derived from one and the same rabbit in each experiment.

Experimental procedure. Slices of rabbit testis were incubated in Krebs-Ringer bicarbonate buffer (pH 7.4) at 37.5°(1) with and without ICSH. Homogenate of testis was incubated in phosphate buffer (pH 7.4) at 37.5°. Each flask contained sodium acetate-1-C^{14} (4.2 mg; 30 μc). Details of the preparation of tissue for incubation and the conditions of incubation have been reported(1).

In experiments designed to compare slices and homogenate from one rabbit, both testes were removed from the animal and sliced with a Stadie-Riggs microtome; from these slices 500 mg batches were carefully weighed and incubated (500 mg/flask). Homogenate of testis was prepared from a weight of slices equal to the total weight to be incubated as slices and divided equally between the same number of flasks as the slices. Protein content was determined on a small aliquot of the homogenate and on an aliquot removed at the end of incubation from each flask of slices after these were homogenized prior to extraction. The Folin-Ciocalteu method was used for measuring protein(5).

Following incubation, slices were homogenized in the incubation medium, saponified, extracted with ether and applied to a column of deactivated aluminum oxide as described elsewhere(6). Homogenate of testis was saponified and extracted in the same way.

The sterol fraction from the aluminum oxide column was submitted to digitonin precipitation and purified by bromination(6). The steroid fraction was applied to paper in the system ligroin/propylene glycol for 24 hours without addition of carrier testosterone. Authentic testosterone (100 μg) was applied to each of 2 strips of paper which were run on either side of the specimens. The standard strips were examined by means of a Haines' ultraviolet scanner to locate testosterone and the remaining strips were examined in a thin-window Geiger strip counter.

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TABLE I. Mass and Specific Activities of Cholesterol-C^{14} and Testosterone-C^{14} from Slices of Testis Incubated with Acetate-1-C^{14}.

		Cholesterol-C^{14}			Testosterone-C^{14}		
Exp	ICSH (μg/flask)	Mass (μg)	Radio-activity (dpm)	Specific activity (dpm/μg)	Mass (μg)	Radio-activity (dpm)	Specific activity (dpm/μg)
1	0	170	9,877	58.1	.1	150	—
	"	142	7,300	51.4			
	"	161	8,680	53.9			
	"	138	8,300	60.1			
	10	153	5,230	34.2	1.2	2,080	1,734
	"	157	5,500	35.0			
	"	162	4,860	30.0			
	"	184	5,540	30.1			
2	0	113	4,490	39.7	.1	108	—
	"	128	5,270	41.2			
	"	137	4,960	36.1			
	"	132	4,600	34.8			
	10	115	2,580	22.4	.8	1,538	1,923
	"	139	3,750	27.0			
	"	122	2,420	19.8			
	"	136	3,280	24.1			

Slices of rabbit testis (500 mg) were incubated in Krebs-Ringer bicarbonate buffer with acetate-1-C^{14} (4 mg/30 μc) at 37.5°. In Exp 1, incubation time was 3 hr and in Exp 2, 4 hr. Cholesterol-C^{14} was purified by bromination following digitonin precipitation. Testosterone-C^{14} was measured on extracts pooled from 4 flasks.

The areas of the chromatograms corresponding to testosterone showed a single peak of radioactivity. These areas were eluted, aliquots were counted by liquid scintillation and the remainder pooled as shown in the accompanying tables. The pooled eluates were dried under nitrogen and submitted to gas-liquid chromatography, using a Barber-Coleman model 10 chromatograph, according to the method of VandenHeuvel *et al*(7). This method employs a phase of SE-30 and at 225° testosterone showed a retention time of 7 minutes. The mass of testosterone was determined by integration of the area under the curve corresponding to authentic testosterone. When standards of authentic testosterone were examined by the present method, it was found that the smallest amount of testosterone which could be detected was 0.1 μg. While 17α-hydroxyprogesterone moves in the same region as testosterone in the paper chromatographic system used, it separates sharply in the gas system, and was not found by this latter method.

Results and discussion. Table I shows that ICSH increased the incorporation of acetate-1-C^{14} into testosterone-C^{14} as previously reported(1). The radiochemical purity of testosterone-C^{14} isolated under the present conditions has been established(1). In addition, it will be seen that the absolute amount of testosterone produced was greater in the presence of ICSH. Although it is not possible to calculate the specific activity of testosterone-C^{14} formed in the absence of ICSH, if a *maximal* mass of testosterone is assumed (i.e., 0.1 μg, the minimal amount detectable by the present method) it is possible to make an approximate calculation of the *minimal* specific activity. In Experiment 1, for instance, the specific activity of testosterone-C^{14} in flasks to which ICSH was not added must be more than 1500 dpm/μg. The true specific activity in unstimulated slices may, however, be higher than this and possibly higher than that in stimulated slices. This could occur if ICSH stimulated the production of testosterone from unlabeled intermediates not on the direct pathway from acetate.

Table II shows that homogenate of testis (without ICSH) is capable of producing approximately as much testosterone as stimulated slices, weight for weight. In 6 rabbits used as controls, no detectable testosterone (*i.e.*, less than 0.1 μg) was recovered from

TABLE II. Biosynthesis of Testosterone by Slices and Homogenate of Testis.

Exp	Flask	Testis	Protein (mg/flask)*	Testosterone (μg)
3	1	Slices	27.6	1.2
	2	"	22.8	
	3	"	24.3	
	4	"	25.1	
	5	Homogenate	9.4	1.1
	6	"	"	
	7	"	"	
	8	"	"	
4	1	Slices	27.3	.8
	2	"	29.7	
	3	"	28.2	
	4	"	28.4	
	5	Homogenate	7.3	1.0
	6	"	"	
	7	"	"	
	8	"	"	

Slices of rabbit testis (500 mg/flask) and homogenate (corresponding to 500 mg/flask) were incubated as described elsewhere(1). In Exp 3 incubation was for 3 hr and in Exp 4 for 4 hr.

* The lower protein content of the homogenate is due to the fact that following homogenization the tissue was centrifuged at 1250 × *g* for 15 min at 0°, and the supernatant layer was decanted for use in the present experiments.

either homogenate or slices of testis extracted without incubation. Table II also shows that incubation for 4 hours did not increase the production of testosterone beyond that produced in 3 hours (compare Exp. 3 and 4).

In Table III evidence based upon 3 experiments is presented to show that 10 μg of ICSH produced a maximal response when added to 500 mg of slices under the conditions reported. Since only 2 measurements (each based upon material pooled from 4 flasks) can be made on 1 rabbit, the method of comparison shown in Table III was necessary. Without extensive investigations the exact level at which ICSH begins to show maximal stimulation cannot be determined and may well very from rabbit to rabbit. In view of the response shown to 50 μg of ICSH it seems likely, however, that within the limitations of the present methods 10 μg of ICSH is above the level required for maximal stimulation. It is therefore considered that the production of testosterone by slices under the conditions reported in Table II represents a maximal response to ICSH.

It therefore appears that the production of testosterone by slices of testis *in vitro* was increased by ICSH and that homogenate of testis, which does not respond to ICSH under conditions so far tested(1), produced approximately the same amount of testosterone as stimulated slices. This observation serves to recall the hypothesis of Hechter(8), namely that adrenal homogenate behaves as though it were stimulated and that the effect of ACTH upon intact adrenal cells may involve redistribution of some intermediate in steroid biosynthesis (*e.g.*, cholesterol) within the cell. This hypothesis was based upon the observation that slices of rat adrenal produced 10 μg of corticosterone/g adrenal/hour of incubation and 40 μg/g/hour in the presence of ACTH, while homogenate produced 30 μg/g/hour and in the latter case, production of corticosterone was not increased by ACTH. This comparison was based upon observations by 2 groups of workers working respectively with homogenate(4) and slices (9). The present findings were based upon observations made with a single rabbit in each experiment. Although the production of corticosterone per gram of adrenal tissue is considerably greater than that of testosterone by testis, it must be remembered that the cells responsible for the biosynthesis of testosterone constitute only a small part of the testis.

Comparison between the biosynthetic activities of slices of adrenals in the presence of ACTH and homogenate of adrenals from

TABLE III. Response of Slices of Testis to Various Concentrations of ICSH.

Exp	ICSH (μg/flask)	Testosterone (μg)
5	10^{-1}	.4
	1.0	.6
6	1.0	1.0
	10	1.0
7	10	1.0
	50	.8

Slices of rabbit testis were incubated in Krebs-Ringer bicarbonate buffer. Each experiment was performed on tissue from a single rabbit and each measurement of testosterone was based upon extracts pooled from 4 flasks each originally containing 500 mg of slices and the dose of ICSH indicated.

rat and hog was also reported in abstract form by Schonbaum(10) who studied homogenate of adrenals in both species and compared his findings with those reported by other workers using slices. Again the comparison showed that homogenate of adrenal was capable of producing approximately the same amount of corticosterone as stimulated slices. Schonbaum suggested that these findings could be explained if homogenization causes the dilution of some inhibitor of steroid biosynthesis or if ACTH stimulates the adrenal cortex by removing such an inhibitor. The fact that the response to ACTH *in vitro* is enhanced by preincubation of slices(11) would support both suggestions. In contrast, preincubation under a number of conditions does not increase the response of testis to ICSH(1). Again, dilution of testicular homogenate does not increase the conversion of cholesterol-4-C^{14} to testosterone-C^{14}.† It is therefore concluded that the present findings are in keeping with the original hypothesis of Hechter(8).

Summary. ICSH *in vitro* has been shown to increase the production of testosterone by slices of rabbit testis. Homogenate of testis produces approximately the same amount of testosterone as slices stimulated maximally by ICSH when the comparison is made on the same weight of tissue from the same rabbit incubated for the same time. It is considered that these observations support a hypothesis originally proposed for ACTH and its action on the adrenal, namely that a homogenate behaves as though it were stimulated.

† Unpublished observations by the authors.

1. Hall, P. F., Eik-Nes, K. B., *Biochim. Biophys. Acta,* 1962, v63, 411.
2. Brinck-Johnsen, T., Eik-Nes, K. B., *Endocrinology,* 1957, v61, 676.
3. Mason, N., Samuels, L. T., *ibid.,* 1961, v68, 899.
4. Macchi, A., Hechter, O., *Arch. Biochem. Biophys.,* 1954, v53, 305.
5. Colowick, S. P., Kaplan, N. O., *Methods in Enzymology,* Acad. Press, N. Y., 1957, vIII, 448.
6. Hall, P. F., *Biochemistry,* 1963, in press.
7. VandenHeuvel, W. J., Sweeley, C. C., Horning, E. C., *Biochem. Biophys. Res. Comm.,* 1960, v3, 33.
8. Hechter, O., *Vitamins & Hormones,* 1955, v XIII, 293.
9. Haynes, R., Savard, K., Dorfman, R. I., *J. Biol. Chem.,* 1954, v207, 925.
10. Schonbaum, E., *Rev. Canad. de Biol.,* 1954, v13, 495.
11. Saffran, M., Bayliss, M. J., *Endocrinology,* 1953, v52, 140.

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The Bentonite Flocculation Test for Detection of Plant Viruses and Titration of Antibody. (28801)

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This report describes the application of the bentonite flocculation test to detection of plant viruses in both crude plant saps and purified preparations. In previous studies, a bentonite suspension sensitized with antigen has been used for detection of antibodies(1, 2,3,4). The data presented here demonstrate that the system can be employed in reverse for detection of antigen without decreasing the sensitivity or reliability of the test. A method for titrating antisera with bentonite particles coated with virus antigen is also described.

Serological techniques for detection and identification of plant viruses have only recently been widely applied. Most of these techniques are limited either by lack of sensitivity or because of nonspecific reactions due to contaminating plant components. The complement fixation test, although extremely