pressure at 35–40°, and the residue was kept in a vacuum of 0.01 mm until its weight was constant. The *N*-hexahydrophthaloyl L-α-(dipalmitoyl)cephalin, a waxy material, weighed 2.24 g (43.6% of theory). It was found to be soluble at room temperature in methanol, ether, chloroform, or benzene, and insoluble in water.

Anal. Calcd. for $C_{45}H_{82}O_{10}NP$ (828.1): N 1.69, P 3.74. Found: N 1.81, P 3.66.

Dipalmitoyl DL-*α-glycerylphosphoryl-2′-hydroxyethyl hexahydrophthalimide (VIIR).*—This compound was obtained from the barium salt of DL-α-glycerylphosphoryl-2′-hydroxyethylhexahydrophthalimide(VIR) by virtually the same procedure as that reported above for the L isomer. Its solubility properties resemble closely those of the L isomer.

Anal. Calcd. for $C_{45}H_{82}O_{10}NP$ (828.1): N 1.69, P 3.74. Found: N 1.75, P 4.12.

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The Effect of Interstitial-Cell-stimulating Hormone on the Biosynthesis of Testicular Cholesterol from Acetate-1-C^{14}*

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The influence of interstitial-cell-stimulating hormone (ICSH) on the incorporation of acetate-1-C^{14} into cholesterol and testosterone by slices of rabbit testis was examined by the application of a method for rigorous purification of testicular cholesterol-C^{14}. When slices were incubated with ICSH, the cholesterol-C^{14} showed less radioactivity and lower specific activity than slices incubated without ICSH. On the other hand, slices incubated with ICSH showed more testosterone-C^{14} than those incubated in the absence of ICSH. When ICSH was administered *in vivo* and serial testicular biopsies were incubated *in vitro* with acetate-1-C^{14}, a fall in cholesterol-C^{14} and an increase in testosterone-C^{14} were observed. It is considered that these findings could be explained by assuming that cholesterol is an intermediate in the biosynthesis of testosterone, and that ICSH stimulates the biosynthetic pathway between acetate and testosterone at some point(s) after cholesterol.

It is generally accepted that cholesterol can be converted to steroid[1] hormones by those tissues which produce these hormones (Zaffaroni *et al.*, 1951; Werbin *et al.*, 1957; Ungar and Dorfman, 1953; and Bloch, 1945). However, it has not been possible to establish an *obligatory* role for cholesterol in the biosynthesis of steroid hormones (Samuels, 1960). Measurements of the specific activity of cholesterol-C^{14} formed from acetate-C^{14} during the biosynthesis of C^{14}-labeled steroids by endocrine tissue *in vitro* have revealed that the specific activities of the labeled steroids are higher than that of the cholesterol-C^{14} isolated from the same tissue (Hechter, *et al.*, 1953; Bligh, *et al.*, 1955). Brady (1951) observed increased incorporation of acetate-1-C^{14} into testosterone by slices of testis in the presence of human chorionic gonadotrophin without any demonstrable change in the radioactivity of the digitonin-precipitable material isolated from the same slices. Such findings have led to the suggestion that much of of the cholesterol in steroid-forming organs is excluded from the pathway to steroids. Moreover, in some cases meticulous purification of cholesterol-C^{14} to eliminate "high-counting companions" as described by Schwenck and Werthessen, (1952) has not been undertaken, with the result that the role of cholesterol in the biosynthesis of steroids remains uncertain.

It has been shown that interstitial-cell-stimulating hormone (ICSH) both *in vivo* and *in vitro* increases the incorporation of acetate-1-C^{14} into testosterone by slices of rabbit testis (Hall and Eik-Nes, 1962a). The present experiments were designed to examine the influence

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[1] Throughout this paper the word steroid is used for 19- and 21-carbon compounds as distinct from sterol, which is reserved for cholesterol and other 27-carbon compounds. Abbreviations used are: ICSH, interstitial-cell-stimulating hormone; PPO, 2,5-diphenyloxazole; POPOP, 1,4-2-(5-phenyloxazolyl)-benzene.

TABLE I
THE PURIFICATION OF CHOLESTEROL-C^{14} TO CONSTANT SPECIFIC ACTIVITY[a]

Duration of Incubation (minutes)	ICSH (μg/flask)	Stage of Purification	Mass (μg)	Radioactivity: Aliquot Examined	Radioactivity: Total (dpm)	Specific Activity[b] (dpm/μg)	Cholesterol-C^{14}: Corrected Radioactivity[c] (dpm)	Cholesterol-C^{14}: Corrected Specific Activity[d] (dpm/μg)
		Before addition of cholesterol	250	1/10	60,900			
		After addition of cholesterol	19,900	1/25	48,700	2.45		
		After 1st bromination	9,000	1/10	8,900	0.99	24,600	97
30	0	After 2nd bromination	5,200	1/10	4,900	0.94		
		After 3rd bromination	3,800	1/10	3,600	0.95		
		Recrystallized from methanol	1,800	1/10	1,700	0.94		

[a] Slices of testis (500 mg) were incubated in buffer containing sodium acetate-1-C^{14} (180 μg, 30 μc). The cholesterol fraction from column chromatography was submitted to digitonin precipitation and cleavage in pyridine followed by the dibromide procedure described under Experimental Procedure. The table shows the purification of a single specimen only. [b] Specific activity = column 6/column 4. [c] Calculated on the basis of the fall in specific activity following bromination, 60,900 × 0.99/2.45 = 24,600 dpm. [d] Corrected specific activity = radioactivity (column 8)/original mass (column 4); e.g. 24,600/250 = 97 dpm/μg.

of ICSH upon the specific activity of cholesterol-C^{14} formed during the biosynthesis of testosterone-C^{14} from acetate-1-C^{14} by slices of rabbit testis using a method for purification of cholesterol-C^{14} to constant specific activity. Preliminary studies of the influence of gonadotrophic hormones upon the biosynthesis of cholesterol-C^{14} by slices of testis *in vitro* have already been reported (Hall and Eik-Nes, 1962b).

EXPERIMENTAL PROCEDURE

Incubation and Extraction.—Slices of rabbit testis (500 mg/flask) were prepared with a Stadie-Riggs microtome and incubated for various times in Krebs-Ringer bicarbonate buffer (*p*H 7.4) in an atmosphere of oxygen 95% and carbon dioxide 5%, with constant agitation at 37.5°. The final volume of incubation medium was 3 ml. ICSH was dissolved in buffer and the order of addition was: buffer, sodium acetate-C^{14}, ICSH, and finally testis. In each experiment tissue from a single rabbit was used. When slices were preincubated in acetate-1-C^{14}, the tissue was removed at the end of preincubation, blotted, immersed in Krebs-Ringer bicarbonate buffer (10 ml) for one minute, blotted, and transferred to fresh incubation medium (without acetate).

Following incubation, the tissue was homogenized in the incubation medium, saponified, and extracted with ether, and the ether extract was washed as described previously (Hall and Eik-Nes, 1962a). The ether extract was dried under nitrogen and applied, in hexane, to a column of deactivated aluminum oxide (10 g). Fractions were eluted by the serial application of the following solvents: (1) hexane, 50 ml; (2) hexane-benzene (1:1), 50 ml; (3) hexane-benzene (1:1), 150 ml; (4) benzene, 50 ml; (5) benzene, 50 ml; (6) ethyl acetate, 100 ml.

Preliminary experiments showed that more than 99% of cholesterol was removed in fractions 3 and 4, and more than 99% each of progesterone and testosterone were eluted in fraction 6. The combined fractions 3 and 4 were dried under nitrogen, dissolved in ethanol (1 ml), and submitted to digitonin precipitation followed by cleavage of the digitonide in pyridine according to the method of Sperry and Webb (1950). Aliquots of the free cholesterol were then examined by gas chromatography and by liquid scintillation spectrometry. Cholesterol (20 mg) was added to the remainder of each sample. The mixture was then converted to the dibromide and free cholesterol was regenerated as described by Schwenck and Werthessen (1952). The process of bromination and regeneration of free cholesterol was repeated twice and the cholesterol remaining after the last bromination was recrystallized from methanol. The tissue extracts (without addition of carrier cholesterol) contained too little cholesterol for bromination by present micromethods; and although the procedure of Schwenck and Werthessen, when scaled for 20 mg, gives poor yields, it proved satisfactory since in the present experiments the state of purification of the cholesterol was more important than the yield (see calculation under Table I). For this reason the crystals of dibromide were repeatedly washed (at least four times) with ice-cold methanol until the specimen was perfectly white.

Following each regeneration of free cholesterol, the sample was dissolved in 10 ml benzene in a volumetric flask and aliquots were examined by liquid scintillation spectrometry and by gas chromatography or by the Liebermann-Burchard reaction (see Results).

Fraction 6 from the aluminum oxide column was dried under nitrogen and testosterone (100 μg) was added. The sample was applied to paper in the system ligroin–propylene glycol for 24 hours (Brady, 1951) and the dry chromatograms were examined by means of a Haines ultraviolet scanner (Haines and Drake, 1950) and then by a thin-window Geiger strip-counter. The area of the chromatogram corresponding to the added testosterone was eluted in methanol, dried under nitrogen, acetylated at room temperature for 24 hours in pyridine–acetic anhydride (5:1), and applied to paper in the system hexane–formamide until the mobile phase reached the ends of the paper strips (Zaffaroni *et al.*, 1950). The dry chromatograms were examined by means of a Haines scanner and the areas occupied by testosterone acetate were cut out and counted by liquid scintillation. Evidence for the radiochemical purity of testosterone-C^{14} acetate isolated by the present method has been given elsewhere (Hall and Eik-Nes, 1962a).

Recovery of cholesterol-4-C^{14} added to slices of testis and extracted by the above method varied between 72% and 80%, and that of testosterone-4-C^{14} between 71% and 76%.[2] The data presented are not corrected for losses incurred during recovery.

Testicular Biopsy and Stimulation in vivo.— In order to study stimulation by ICSH *in vivo* using each rabbit as its own control, a technique was devised for removing

TABLE II
THE INFLUENCE OF ICSH *in vitro* UPON THE SPECIFIC ACTIVITY OF CHOLESTEROL-C^{14} [a]

Expt. No.	Duration of Incubation (minutes)	ICSH (μg/flask)	Cholesterol-C^{14} Mass (μg)	Cholesterol-C^{14} Radioactivity (dpm)	Cholesterol-C^{14} Specific Activity (dpm/μg)	Testosterone C^{14} (dpm)
1	60	0	210	79,200	375	280
	120	0	230	131,000	560	820
	180	0	160	113,000	725	1,700
	60	10	280	76,400	278	3,100
	120	10	210	70,300	329	6,500
	180	10	350	113,000	321	10,300
2	60	0	220	75,600	344	190
	120	0	240	131,000	546	670
	180	0	220	153,000	695	1,500
	60	10	220	49,600	225	2,700
	120	10	200	62,800	314	5,100
	180	10	210	65,900	314	9,200
3	60	0	210	56,300	268	160
	120	0	230	96,900	421	710
	180	0	210	123,000	586	1,500
	60	10	200	32,600	163	1,800
	120	10	220	52,100	237	4,200
	180	10	260	63,700	245	8,400

[a] Slices of rabbit testis were incubated in the presence of acetate-1-C^{14} (180 μg, 30 μc) with and without ICSH for the periods shown, and the cholesterol fraction was purified as described under Experimental Procedure.

one-half a testis without impairing the blood supply to the remainder of the organ. The rabbit was anesthetized by means of intravenous nembutal, the lower half of one testis was removed with sharp scissors and two small blood vessels cut in the process were clamped. ICSH (0.5 mg total dose) was administered intravenously and, at various intervals, the remaining testicular tissue was removed (half of one testis at a time).

Gas Chromatography.—Samples were examined by gas chromatography as the trimethylsilyl ethers on a phase consisting of 0.5% Hi-Eff-8B (cyclohexanedimethanol polysuccinate)[3] supported by gas-chrom-P, acid- and base-washed and silicone-treated (80–100 mesh) (Wells and Makita, 1962). The column was 8 feet by 1 inch in an F & M Scientific Corp. Model 609 chromatograph with a hydrogen flame detector. Column temperature was 220°, flash temperature, was 280°, and detector temperature was 250° with argon as the carrier gas. Under these conditions cholesterol showed a retention time of 14 minutes. Measurement of the mass of cholesterol was performed by integration of the area under the appropriate curve with the aid of a planimeter. In every case determinations were performed in duplicate and before and after each series of determinations, duplicate standards of cholesterol were examined.

Measurement of Radioactivity.—Suitable aliquots of samples in which C^{14} was to be measured were evaporated to dryness under nitrogen in 20-ml vials and dissolved in 10 ml of scintillation fluid prepared by dissolving 5 g of 2,5-diphenyloxazole (PPO) and 300 mg of 1,4-2-(5-phenyloxazolyl)-benzene (POPOP) in one liter of reagent grade toluene. The measurement of testosterone-C^{14} acetate on paper chromatograms was performed by cutting the paper in strips 1 cm wide and standing each strip in a vial to which 15 ml of scintillation fluid was added. Samples were counted in a Packard Tri-Carb Model 314 liquid scintillation spectrometer. Sufficient counts were allowed to accumulate to give a probable error of less than 5%. The addition of a standard solution of toluene-C^{14} showed that no significant quenching occurred either with strips of paper or with evaporated samples.

Chemicals.—Cholesterol was purchased from Pfanstiehl Laboratories, Inc., Illinois, and was recrystallized three times from methanol. The crystals were dried in an Abderhalden oven and the melting point was measured in a Hershberg apparatus. The final melting point was 146.5–148°. Acetate-1-C^{14} was obtained from New England Nuclear Corporation. Interstitial-cell-stimulating hormone was provided by the Endocrine Study Section of the National Institutes of Health in the form of the preparation NIH-LH-S-4.

RESULTS

Purification and Measurement of Cholesterol-C^{14}.—The sterol fraction extracted from testicular tissue contained between 160 μg and 414 μg of cholesterol per 500 mg testis. This was identified and measured by gas chromatography as the trimethylsilyl ether (Wells and Makita, 1962). Associated with the cholesterol was a small peak corresponding in retention time to Δ^7-cholesterol (Wells and Makita, 1962). This and other "high-counting companions" were removed by addition of carrier cholesterol and repeated crystallization of the dibromide, and finally by crystallization of free cholesterol. It will be seen from Table I that the specific activity of cholesterol-C^{14} was constant after the first bromination, in agreement with the experience of other workers with other tissues (Schwenck and Werthessen, 1952; Kabara and McLaughlin, 1961). In view of these findings which were repeated in two further experiments, the procedure was modified in two ways, namely, by measuring specific activity after a single bromination-debromination cycle and by using the Liebermann-Burchard reaction to estimate the mass of cholesterol *after* addition of cold cholesterol (10 mg). In this way the original mass of cholesterol was measured by submitting an aliquot to gas chromatography and the C^{14} associated with this cholesterol was measured by calculating the specific activity before and

[2] Recovery of cholesterol is calculated after digitonin precipitation and does not include the process of bromination. The recovery of testosterone includes the final measment as testosterone acetate.

[3] Applied Science Corporation, State College, Pennsylvania.

TABLE III

THE INFLUENCE OF ICSH *in vitro* UPON THE SPECIFIC ACTIVITY OF CHOLESTEROL-C^{14} DURING INCUBATION FOR SHORT PERIODS AND THE EFFECT OF SUBSTRATE CONCENTRATION ON THE PRODUCTION OF CHOLESTEROL-C^{14} AND TESTOSTERONE-C^{14}[a]

Expt. No.	Duration of Incubation (minutes)	ICSH (μg/flask)	Sodium Acetate-1-C^{14} μg	Sodium Acetate-1-C^{14} μc	Cholesterol-C^{14} Mass (μg)	Cholesterol-C^{14} Radioactivity (dpm)	Cholesterol-C^{14} Specific Activity (dpm/μg)	Testosterone-C^{14} (dpm)
4	10	0	180	30	190	12,600	66	73
	10	10	180	30	250	16,300	65	850
	15	0	180	30	210	16,900	80	98
	15	10	180	30	190	15,700	83	990
	20	0	180	30	160	18,900	118	120
	20	10	180	30	210	24,300	116	1,100
5	20	0	180	30	200	24,400	122	90
	20	10	180	30	190	22,900	121	1,200
	20	0	360	60	190	21,800	115	80
	20	10	360	60	190	23,700	125	1,200
	20	0	720	120	240	26,800	112	80
	20	10	720	120	210	24,200	115	1,000
6	120	0	180	30	310	108,000	348	1,100
	120	10	180	30	290	84,300	291	12,300
	120	0	720	120	230	111,000	483	1,800
	120	10	720	120	240	81,400	339	11,900

[a] The conditions of incubation were those given under Experimental Procedure.

TABLE IV

THE INFLUENCE OF ICSH *in vitro* UPON THE PRODUCTION OF CHOLESTEROL-C^{14} AND TESTOSTERONE-C^{14} BY SLICES OF TESTIS PREINCUBATED WITH ACETATE-1-C^{14} [a]

Experiment 7		Cholesterol-C^{14}			
Duration of Incubation (minutes)	ICSH (μg/flask)	Mass (μg)	Radioactivity (dpm)	Specific Activity (dpm/μg)	Testosterone-C^{14} (dpm)
30	0	220	16,200	74	610
60	0	210	26,500	126	1,600
90	0	210	32,200	153	2,200
30	10	220	11,500	52	9,200
60	10	220	17,300	79	11,900
90	10	230	21,500	93	16,100

[a] Slices of rabbit testis were preincubated in the presence of acetate-1-C^{14} (180 μg, 30 μc) for thirty minutes, removed, blotted, and immersed in fresh buffer for one minute, blotted, and transferred to Krebs-Ringer bicarbonate buffer (without acetate) and incubated for the period shown, with and without ICSH.

after debromination in the presence of added cholesterol as shown under Table I. This table also shows that less than one-half the radioactivity present in the digitonide proved to be cholesterol-C^{14}.

Effect of ICSH in vitro upon the Specific Activity of Cholesterol-C^{14}.—Table II shows the effect of ICSH upon the specific activity of cholesterol-C^{14} extracted from slices of testis following incubation with acetate-1-C^{14}. It will be seen that in each experiment the specific activity and the total radioactivity present as cholesterol-C^{14} is less in the presence of ICSH, that this difference increases with the duration of incubation, and that ICSH causes an increase in the conversion of acetate-1-C^{14} to testosterone.

When slices of testis were incubated in the presence of acetate-1-C^{14} with and without ICSH for short periods (20 minutes or less), the amount of cholesterol-C^{14} was not significantly different in the presence of ICSH (Table III). Moreover, the system was saturated with acetate-1-C^{14}, since a 4-fold increase in the concentration of this substrate did not result in greater production of cholesterol-C^{14} or testosterone-C^{14} (Table III).

When testis was preincubated in acetate-1-C^{14} for 30 minutes, washed, and transferred to fresh buffer without acetate, ICSH produced the same changes in cholesterol-C^{14} (Table IV).

Effect of ICSH in vivo upon the Biosynthesis of Cholesterol-C^{14}.—Table V shows the effect of ICSH (total dose 0.5 mg) administered intravenously as a single injection upon the biosynthesis of cholesterol-C^{14} and testosterone-C^{14}. In contrast to the appreciable fall in the specific activity of cholesterol-C^{14} when ICSH is added *in vitro*, the specific activity is not altered following administration of ICSH, although there is a small but consistent fall in the total radioactivity of cholesterol-C^{14} following stimulation. This fall in radioactivity of cholesterol-C^{14} increases with the duration of stimulation *in vivo* and is *approximately* equal to the increase in radioactivity found in testosterone-C^{14} (Table V). In the control animal no fall in cholesterol-C^{14} is seen.

In all these experiments, it will be seen that the mass of cholesterol shows no consistent change in the stimulated slices (Tables I–V).

DISCUSSION

At first glance, a fall in the specific activity and the radioactivity of cholesterol-C^{14} in association with increased production of testosterone-C^{14} from acetate-1-C^{14} may seem anomalous. However, four possible explanations for this finding suggest themselves.

(1) The effect of ICSH upon the biosynthesis of testosterone is exerted at some step in the pathway from acetate after cholesterol, and the bulk of testicular cholesterol is not engaged in steroid biosynthesis.

TABLE V

THE INFLUENCE OF ICSH *in vivo* UPON THE BIOSYNTHESIS OF CHOLESTEROL-C^{14} AND TESTOSTERONE-C^{14} FROM ACETATE-1-C^{14} [a]

Expt. No.	Time of Biopsy after ICSH Injection (minutes)	Cholesterol-C^{14} Mass (μg)	Cholesterol-C^{14} Radioactivity (dpm)	Cholesterol-C^{14} Specific Activity (dpm/μg)	Testosterone-C^{14} (dpm)
8 (control)[b]	0	230	114,000	496	610
	30	240	117,000	488	730
	60	280	120,000	429	490
	90	270	119,000	441	520
9	0	410	177,000	432	1,100
	30	310	169,000	545	9,900
	60	340	161,000	474	14,500
	90	340	161,000	474	18,200
10	0	220	127,000	577	980
	30	230	117,000	508	12,300
	60	210	112,000	533	16,300
	90	220	111,000	505	18,600
11	0	220	121,000	550	460
	30	220	117,000	531	3,200
	60	220	113,000	514	7,400
	90	250	111,000	444	9,100

[a] Slices of rabbit testis were prepared from testicular biopsies taken before and at the stated times following a single intravenous injection of ICSH (0.5 mg). The slices were incubated for 2 hours in the presence of sodium acetate-1-C^{14} (180 μg, 30 μc) and the cholesterol-C^{14} fraction was purified as described under Experimental Procedure. [b] In experiment 8, 2 ml of normal saline was injected instead of ICSH.

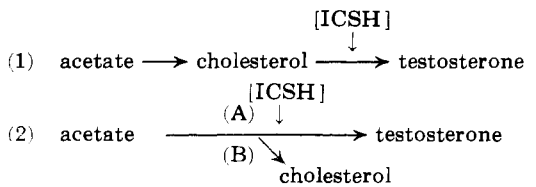
Upon stimulation by ICSH, the rate at which cholesterol-C^{14} is converted to testosterone-C^{14} is greater than the rate at which acetate-C^{14} is converted to cholesterol-C^{14}. In such a heterogeneous tissue as testis, it is likely that much of the cholesterol (e.g., that of the germinal epithelium) is not engaged in the production of steroids. It is, therefore, possible that a pool of cholesterol-C^{14} of high specific activity is being depleted to form testosterone-C^{14} under the influence of ICSH more rapidly than it is replenished from acetate-1-C^{14}.

(2) Cholesterol is not on the biosynthetic pathway to testosterone and ICSH increases the conversion of acetate-1-C^{14} to testosterone-C^{14} at the expense of its conversion to cholesterol.

(3) ICSH, in addition to increasing the conversion of acetate to testosterone, also increases the conversion of this substrate to some product which is neither steroid nor sterol, e.g, fatty acids or carbon dioxide. This possibility is unlikely in view of the findings shown in Table III, namely, that short-term incubation in the presence of ISCH is not associated with any significant change in the radioactivity of cholesterol-C^{14}, so that before appreciable amounts of testosterone-C^{14} appeared the stimulated slices contained as much cholesterol-C^{14} as the unstimulated. This suggests that the later changes reflect removal of cholesterol-C^{14} rather than prior diversion to another pathway. These changes are seen when the system is saturated with substrate (Table III) and when exposure to substrate is limited to a period of preincubation in the absence of ICSH (Table IV).

(4) Stimulation by ICSH increases the production of unlabeled cholesterol from some precursor, e.g., a polar ester such as cholesterol sulfate, which might escape hydrolysis by the mild conditions used in these experiments (Hall and Eik-Nes, 1962a). This possibility is excluded by the fact that no increase in the mass of cholesterol is seen in the presence of ICSH and the lower specific activity seen under these conditions is due to less radioactivity. Moreover, vigorous acid hydrolysis in methanolic hydrochloric acid failed to reveal a more polar ester escaping the present extraction and hydrolysis.[4]

The first two alternatives may be depicted as follows:



The first alternative proposes that all the C^{14} of the testosterone-C^{14} has previously existed as cholesterol-C^{14}. The amount of cholesterol-C^{14} formed in the presence or absence of ICSH would be the same and the effect of stimulation is to convert more of this cholesterol-C^{14} to testosterone-C^{14}. The second alternative postulates that some step common to the two pathways (A) and (B) is rate-limiting for the biosynthesis of both cholesterol and testosterone from acetate, so that the effect of ICSH is to cause more C^{14} to enter (A) and proportionately less to enter (B).

Table II shows that the lower specific activity of cholesterol-C^{14} in the stimulated slices is due to the presence of less radioactivity, not to any consistent change in the mass of cholesterol. Moreover, at short intervals before testosterone-C^{14} has appeared in significant quantities, the radioactivity of cholesterol-C^{14} does not differ in the slices incubated with ICSH from that in unstimulated slices (Table III). These findings suggest that stimulation by ICSH has the effect of increasing the production of testosterone-C^{14} from previously synthesized cholesterol-C^{14} and thereby favor the first of the two alternatives above. This conclusion is further supported by Table II which clearly shows that the difference in the content of cholesterol-C^{14} between stimulated and unstimulated slices becomes more pronounced as the production of testosterone-C^{14} continues to increase.

These findings in experiments with stimulation *in vitro* are confirmed by the results of stimulation *in vivo*

[4] Unpublished observation by the author.

(Table V). Here the fall in radioactivity of cholesterol-C^{14}, although relatively less than that seen with stimulation *in vitro*, is consistent and is not found in the control experiment (experiment 8). Moreover, it is clear that the fall in radioactivity in cholesterol-C^{14} in experiments 9–11 is *approximately* equal to the increase in testosterone-C^{14} resulting from the administration of ICSH. This lends some support to the first alternative, although in experiments in which ICSH was added *in vitro* the fall in the radioactivity of cholesterol-C^{14} is much greater than can be accounted for by the testosterone produced.

Although the present studies do not provide unequivocal support for the role of cholesterol as a precursor of steroids, they are compatible with such a role and with a site of action of ICSH at some stage of the biosynthetic pathway to steroids after cholesterol. Such a conclusion is in keeping with evidence already presented in preliminary form (Hall and Eik-Nes, 1962b) that the site of action of gonadotrophic stimulation is before pregn-5-en-3β-ol-20-one (pregnenolone).[5] Moreover, the methods of studying sterol biosynthesis in the testis reported here, suggest a way of pursuing this problem; experiments are at present being undertaken with the aid of inhibitors of cholesterol biosynthesis. In the meantime, some caution is necessary in accepting cholesterol as an obligatory intermediate in steroid biosynthesis in view of the findings of Goodman *et al.* (1962).

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[5] In preparation for publication in full.

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