Further Studies on the Locus of Action of Interstitial Cell-Stimulating Hormone on the Biosynthesis of Progesterone by Bovine Corpus Luteum*

Seymour B. Koritz and Peter F. Hall†

ABSTRACT: The possibility that interstitial cell-stimulating hormone (ICSH) increases steroidogenesis in bovine corpus luteum by accelerating the conversion of acetate to cholesterol, as well as its observed effect in the conversion of cholesterol to progesterone, was examined by two approaches: (1) The influence of ICSH upon the conversion of [^{3}H]cholesterol to [^{3}H]progesterone was compared with the action of the hormone upon the production of progesterone from endogenous precursors, by measuring the specific activity of [^{3}H]-progesterone formed *de novo* during incubation with [^{3}H]cholesterol with and without ICSH. It was found that in some experiments the specific activity of the [^{3}H]progesterone formed in the presence of ICSH was considerably less than that in the absence of the hormone, while in other experiments the presence of ICSH did not change the specific activity. These data are open to several possible interpretations which are discussed. (2) Measurement of [^{14}C]cholesterol isolated from tissue incubated with [^{14}C]acetate with and without ICSH revealed that the cholesterol contained less radioactivity when incubation was performed in the presence of the hormone. In the light of previous observations these findings are interpreted to mean that the major, if not the only, site of action of ICSH in the corpus luteum is beyond cholesterol.

Although it is now apparent that trophic stimulation of steroidogenesis in adrenal (Yudaev and Razina, 1964; Karaboyas and Koritz, 1965), testis (Hall, 1963), and corpus luteum (Hall and Koritz, 1965) involves accelerated conversion of cholesterol to the respective steroid hormones of these glands, the possibility that interstitial cell-stimulating hormone[1] (ICSH) also stimulates some step(s) of the biosynthetic pathway from acetate to cholesterol has not been excluded. Indeed, certain experimental evidence has been interpreted in favor of a site of action of ICSH on the corpus luteum before cholesterol (Savard and Casey, 1964; Mason and Savard, 1964).

It was decided to examine the possibility that in bovine corpus luteum ICSH acts before cholesterol, in addition to its established action between cholesterol and progesterone. Two approaches were used: first the conversion of [^{14}C]acetate to [^{14}C]cholesterol and [^{14}C]progesterone by slices of corpora lutea incubated in the presence and absence of ICSH were compared. Second, the relative production of [^{3}H]progesterone from [^{3}H]cholesterol to that of progesterone from endogenous precursors was measured by determining the specific activity of [^{3}H]progesterone formed *de novo* during incubation, and again tissue incubated with ICSH was compared with that incubated in the absence of the hormone.

Experimental Procedure

Incubation of Tissue. Bovine corpora lutea were obtained from a slaughterhouse and slices were prepared by means of a Stadie–Riggs microtome. Corpora lutea were selected for these studies on the basis of morphological criteria discussed elsewhere (Hall and Koritz, 1965); the selection was intended to avoid corpora from the last days of the cycle. Corpora from pregnant cows were not used. Slices of corpora lutea (500 mg/beaker) were incubated in Krebs Ringer bicarbonate buffer containing glucose (2 mg/ml). Final volume was 3 ml and pH was 7.4. Incubation was performed at 37° in a Dubnoff metabolic shaker continuously gassed with a mixture of oxygen (95%) and carbon dioxide (5%). In certain experiments slices were removed from the medium, washed three times with buffer, and blotted; incubation was then continued in fresh medium. The period of incubation before the tissue was transferred will be referred to here as preincubation. Each experiment was performed with slices from one corpus luteum only. [7α-^{3}H]Cholesterol (25 μcuries, 2 μg/beaker) was suspended in Tween-80 before addition to the medium (Hall and Koritz, 1965).

Isolation and Measurement of [^{14}C]Cholesterol. Following incubation with [^{14}C]acetate (30 μcuries,

* From the Departments of Biochemistry and Physiology, University of Pittsburgh, Pittsburgh 13, Pennsylvania. *Received August 2, 1965; revised September 9, 1965.* This work has been supported by grants (AM 03676-05 END and AM 06459-03) from the National Institutes of Health, U. S. Public Health Service.

† Present address: Department of Biochemistry, University of Melbourne, Parkville N2 Victoria, Australia.

[1] Abbreviation used in this work: ICSH, interstitial cell-stimulating hormone.

TABLE I: The Purification of [^{14}C]Cholesterol to Constant Specific Activity.[a]

Stage of Purification	Mass (μg)	Radio-activity (dpm)	Spec[b] Activity (dpm/μg)	[^{14}C]Cholesterol (corrected) Radio-activity (dpm)[c]	Spec Activity[d] (dpm/μg)
Before addition of cholesterol	210	118,000			
After addition of cholesterol	9,700	99,000	10.2		
After 1st bromination	4,600	23,000	5.0	57,800	275
After 2nd bromination	3,200	15,000	4.7		
After 3rd bromination	2,400	11,800	4.9		
Recrystallized from methanol	1,700	8,700	5.1		

[a] [^{14}C]Cholesterol isolated following incubation of slices of corpora lutea with [1-^{14}C]acetate as described under Methods and Materials was purified by digitonin precipitation, cleavage in pyridine, and repeated bromination as described in detail elsewhere (Hall, 1963). Following the third debromination, free cholesterol was recrystallized from methanol. Cholesterol was measured by gas chromatography. The table shows the purification of a single sample. [b] Specific activity = column 3/column 2. [c] Calculated on the basis of the fall in specific activity following bromination, *i.e.*, (118,000 × 5.0)/10.2. [d] Corrected specific activity = corrected radioactivity (column 5)/original mass (column 2), *i.e.*, 57,800/210 (dpm/μg).

45 μg/beaker), the tissue was homogenized in the incubation mixture and the homogenate extracted four times with diethyl ether (three volumes). The combined extracts were washed once with aqueous sodium acetate (1%, w/v) and three times with water. The extract was taken to dryness and partitioned between ligroin–(methanol–water, 90:10, v/v) (Savard and Casey, 1964). The ligroin fractions were pooled and the cholesterol content (mass and radioactivity) was measured after purification through digitonin precipitation and bromination. Mass was measured by gas chromatography and radioactivity by liquid scintillation spectrometry as reported in detail previously (Hall, 1963). Recovery of [^{14}C]cholesterol was measured by adding known amounts of [4-^{14}C]cholesterol to homogenized corpus luteum and isolating this substance as described above, up to and including digitonin precipitation. Recovery through bromination was measured by addition of carrier cholesterol to each sample as described previously (Hall, 1963).

Isolation and Measurement of Progesterone. A. [^{14}C]-PROGESTERONE. The aqueous methanol phase after partition against ligroin (see above) was taken to dryness and [^{14}C]progesterone was isolated by column and paper chromatography as reported elsewhere (Hall and Koritz, 1965). Mass of [^{14}C]progesterone was measured by absorbance at 240 mμ and radioactivity by liquid scintillation spectrometry. Recovery of progesterone was measured by adding known amounts of [1,2-^{3}H]-progesterone to homogenized corpus luteum and isolating this compound as described.

The identity and radiochemical purity of [^{14}C]-progesterone isolated by this method was demonstrated by adding authentic progesterone to samples of [^{14}C]-progesterone isolated as described above and purifying the mixture by recrystallization from various solvents and by countercurrent chromatography. Data from countercurrent chromatography were analyzed according to the method described by Baggett and Engel (1957). Details of these procedures have been reported elsewhere (Hall and Koritz, 1965).

B. [^{3}H]PROGESTERONE. The isolation and measurement of this compound and the methods of demonstrating radiochemical purity were those used for [^{14}C]progesterone except that ether extracts of the tissue were not washed with sodium acetate or water. The method of measuring cholesterol by gas chromatography and details of liquid scintillation spectrometry, including elution of samples from paper chromatograms, have been described in detail previously (Hall and Koritz, 1965; Hall, 1963).

Chemicals. Sodium [1-^{14}C]acetate (lot No. 93-245-3; 53 mcuries/mmole) and [7α-^{3}H]cholesterol (lot No. 66-129-99; 4.8 curies/mmole) were obtained from New England Nuclear Corp. [1,2-^{3}H]Progesterone (Tracer Lab. Lot No. 925-23-136) and interstitial cell-stimulating hormone (NIH-LH-S-8) were gifts from the Endocrine Study Section of the National Institutes of Health. Bovine ICSH, deactivated according to the method of Reichert (1961), was generously provided by Dr. L. E. Reichert, Emory University, Atlanta, Ga. This material (referred to here as deactivated ICSH) was found to produce no demonstrable response in slices of rabbit testis *in vitro* at levels of 100 μg/beaker in a system which has been shown elsewhere (Hall and Eik-Nes, 1962) to respond to less than 0.01 μg of untreated ICSH/beaker.

Results

Radiochemical Purity of [^{14}C]*Cholesterol.* Table I shows that the cholesterol isolated from corpus luteum

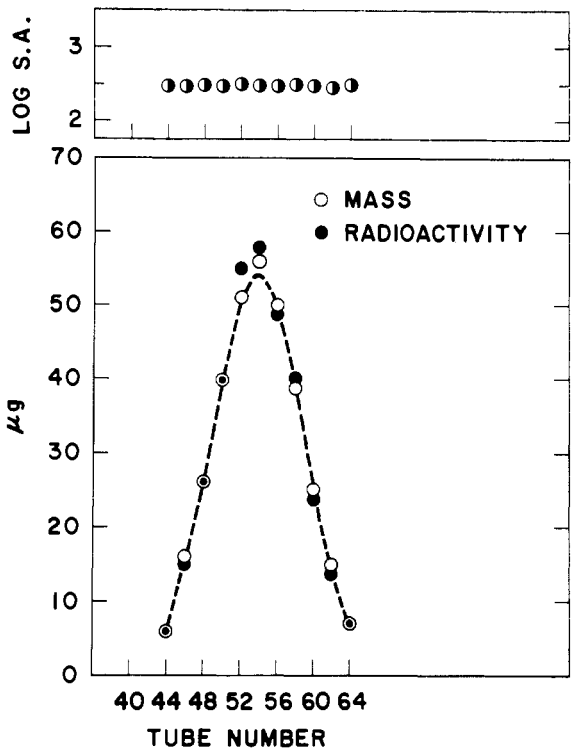

FIGURE 1: The 99-transfer countercurrent distribution of progesterone in (cyclohexane–ethyl acetate, 80:20, v/v)–(ethanol–water, 60:40, v/v). Values on the ordinate are mass (μg) and radioactivity divided by mean specific activity (μg). The theoretical normal distribution curve was constructed with the following parameters: $K = 1.2$, standard deviation ±4.96. Log specific activity of [^{14}C]progesterone in alternate tubes (dpm/μg) is plotted above the distribution curve. No significant regression is observed at the 5% level of significance (F 0.95 (1, 9) = 0.04) (Baggett and Engel, 1957).

following incubation with [1-^{14}C]acetate was accompanied by radioactive impurities ("high counting companions") (Schwenk and Werthesson, 1953) after digitonin precipitation, since the specific activity fell considerably following purification by bromination (10.2–5.0 dpm/μg). Moreover, the specific activity thereafter remained constant through two further cycles of bromination–debromination and recrystallization of cholesterol from methanol, in agreement with observations on the liver (Schwenk and Werthesson, 1952; Kabara and McLaughlin, 1961) and testis (Hall, 1963). In subsequent experiments [^{14}C]cholesterol was purified by a single bromination–debromination cycle. Recovery of [^{14}C]cholesterol through digitonin precipitation was 71–79% in ten estimations.

Radiochemical Purity of [^{14}C]Progesterone and [^{3}H]-Progesterone. The substance described here as [^{14}C]-progesterone was eluted from paper chromatograms following extraction, partition, and column chromatography (Savard and Casey, 1964). Proof that this compound was radiochemically pure is shown in Figure 1 and Table II (sample B). Figure 1 shows the result of countercurrent distribution of pooled samples of [^{14}C]-progesterone to which 500 μg of authentic progesterone was added. It will be seen that in the peak region mass and radioactivity coincide, and statistical analysis (Baggett and Engel, 1957) revealed that within the 5% level of significance there was no regression. Table II (sample B) shows the result of recrystallizing the sample shown in Figure 1 after pooling the fractions from the peak region of countercurrent distribution and adding progesterone (10 mg). It will be seen that the specific activity of the [^{14}C]progesterone remained constant through recrystallization from four different solvent systems.

Further evidence for the purity of the [^{14}C]progesterone isolated by the present methods is shown in

TABLE II: Recrystallization of [^{14}C]Progesterone Isolated Following Incubation of Slices of Corpus Luteum with [1-^{14}C]-Acetate.[a]

Sample A			Sample B		
	Spec Activity (dpm/mg)			Spec Activity (dpm/mg)	
Recrystallization	Crystals	Mother Liquor	Recrystallization	Crystals	Mother Liquor
After addition of carrier	10,400		After addition of carrier	12,100	
1st hexane–chloroform	10,500	10,000	1st hexane–chloroform	11,900	11,700
2nd heptane–benzene	10,100	10,200	2nd heptane–benzene	12,500	11,900
3rd ligroin–acetone	10,300	10,100	3rd ligroin–acetone	11,900	12,300
4th aqueous ethanol	10,000	10,300	4th aqueous ethanol	12,300	12,000

[a] Sample A represents pooled eluates from paper chromatograms. To the pooled eluates 10 mg of progesterone was added and crystals were allowed to form from the solvents shown. After each crystallization aliquots of crystals and mother liquor were taken for measurement of mass by absorbance at 240 mμ and of radioactivity by liquid scintillation spectrometry. Sample B consists of pooled fractions from the peak region of countercurrent chromatography (see Figure 1); the sample was treated as described for sample A.

TABLE III: The Influence of ICSH upon the Biosynthesis of [^{14}C]Cholesterol and [^{14}C]Progesterone from [1-^{14}C]Acetate by Slices of Bovine Corpora Lutea.[a]

Expt	ICSH (μg/flask)	[^{14}C]Cholesterol Mass (μg)	Radio-activity (dpm)	Spec Activity (dpm/μg)	[^{14}C]Progesterone Mass (μg)	Radio-activity (dpm)	Spec Activity (dpm/μg)
1	0	910	183,000	201	43.1	42,000	970
		943	201,000	213	36.3	45,000	1,240
	200	817	89,000	109	82.0	106,000	1,290
		902	93,000	103	86.0	107,000	1,240
2	0	1,108	329,000	297	8.2	7,000	850
		1,263	304,000	241	7.1	9,000	1,270
	200	1,413	165,000	117	14.3	23,000	1,610
		1,020	123,000	121	19.8	25,000	1,260
3	0	832	158,000	190	11.1	15,000	1,350
		924	170,000	184	9.0	10,000	1,100
	200	782	63,000	81	26.3	60,000	2,280
		714	52,000	73	21.4	55,000	2,570
4	0	972	304,000	313	13.8	20,000	1,450
		1,081	402,000	372	12.0	22,000	1,830
	200	1,012	183,000	181	34.7	54,000	1,560
		1,106	218,000	197	48.2	61,000	1,270
5	0	792	483,000	610	18.3	40,000	2,190
		814	557,000	684	13.1	31,000	2,370
	200	693	211,000	304	62.4	128,000	2,100
		743	283,000	381	71.3	164,000	2,300
6	0	480	80,000	169	32.2	22,000	683
		500	87,000	174	35.0	24,000	685
	200	360	36,000	100	46.5	33,000	709
		520	48,000	92	50.3	39,000	775

[a] Slices of corpus luteum were incubated for 2 hr in Krebs–Ringer bicarbonate buffer with [1-^{14}C]acetate as described under Experimental Section. The mass and radioactivity of cholesterol were measured after purification through digitonin precipitation and bromination.

Table II (sample A). In this case recrystallization from four solvent systems after addition of progesterone (10 mg) was performed on pooled samples not previously submitted to countercurrent chromatography. Again it will be seen that specific activity remained constant and, since no significant fall in specific activity occurred during the first recrystallization, it appears that the [^{14}C]progesterone measured in the experiments to be reported was radiochemically pure after paper chromatography.

The radiochemical purity of [^{3}H]progesterone isolated following incubation of corpus luteum with [7α-^{3}H]-cholesterol was demonstrated by the same methods. However, since evidence for the purity of [^{3}H]progesterone isolated from similar experiments has been published in detail elsewhere (Hall and Koritz, 1965), these data are not presented here.

Recovery of [^{3}H]progesterone by the methods used up to and including elution from paper chromatograms was 58–66% in eight estimations. Data presented here are not corrected for losses incurred during the isolation of progesterone.

Biosynthesis of [^{14}C]*Cholesterol.* Table III shows the results of six experiments in which slices of corpus luteum were incubated with [1-^{14}C]acetate with and without ICSH. It will be seen that in every experiment the cholesterol contains less ^{14}C when ICSH was present during incubation and that the specific activity of [^{14}C]cholesterol was lower in beakers to which ICSH was added (column 5). The table also shows that the specific activity of [^{14}C]progesterone is always considerably higher than that of [^{14}C]cholesterol from the same beaker (columns 5 and 8). In confirmation of the findings reported by Savard and Casey (1964), the specific activity of [^{14}C]progesterone does not show any consistent difference when tissues incubated with and without ICSH are compared.

Table IV shows two control studies. For expt 7 a corpus luteum was chosen which appeared "old," *i.e.*, from last days of the cycle. It will be seen that slices

TABLE IV: The Conversion of [1-^{14}C]Acetate to [^{14}C]Cholesterol and [^{14}C]Progesterone by Corpora Lutea (Control Experiments).[a]

		[^{14}C]Cholesterol			[^{14}C]Progesterone		
Expt	ICSH (μg/beaker)	Mass (μg)	Radio-activity (dpm)	Spec Activity (dpm/μg)	Mass (μg)	Radio-activity (dpm)	Spec. Activity (dpm/μg)
7	0	1,980	5,900	3.0	17.0	162	9.5
	0	2,430	5,100	2.1	14.6	246	16.9
	200	1,660	5,300	3.2	11.0	119	10.8
	200	1,450	7,200	5.0	19.2	280	14.6
8	0	903	168,000	190	27.0	28,000	1,040
	0	1,220	195,000	160	21.5	33,000	1,540
	200[b]	898	161,000	180	23.3	24,000	1,030
	200[b]	985	197,000	200	19.4	29,000	1,490
	200	892	101,000	110	51.0	48,000	940

[a] Slices of corpus luteum were incubated with [1-^{14}C]acetate as described under Experimental Section. In expt 7 the corpus luteum used was selected on the grounds that it showed morphological characteristics expected of corpora during the last days of the cycle. The nature of these characteristics is discussed elsewhere (Hall and Koritz, 1965). In two beakers of expt 8 deactivated ICSH was used (see Experimental Section). [b] Deactivated ICSH.

TABLE V: The Influence of ICSH upon the Specific Activity of [^{3}H]Progesterone Formed from [7α-^{3}H]Cholesterol by Slices of Bovine Corpus Luteum.[a]

		[^{3}H]Progesterone			
		Mass (μg)		Radio-activity (dpm)	Spec Activity (dpm/μg)
Expt	Conditions	Total	Synthesis *de novo*		
9	Zero time	8.0			
	No ICSH	7.8		138,000	
	ICSH	17.5	9.5	153,000	16,100
10	Zero time	13.7			
	No ICSH	20.1	6.4	144,000	22,500
	ICSH	34.6	20.9	225,000	10,800
11	Zero time	8.7			
	No ICSH	12.2	3.5	126,000	36,000
	ICSH	29.8	21.1	159,000	7,500
12	Zero time	15.2			
	No ICSH	25.5	10.3	120,000	11,700
	ICSH	30.2	15.0	179,000	11,900
13	Zero time	16.2			
	No ICSH	25.3	9.1	168,000	18,500
	ICSH	43.3	27.1	504,000	18,600

[a] Slices of corpora lutea were incubated with [7α-^{3}H]cholesterol, and [^{3}H]progesterone was measured as described under Experimental Section. The contents of one beaker of each experiment was extracted without incubation (zero time); one was incubated with and one without ICSH. Synthesis *de novo* indicates the difference between total progesterone at the end of incubation and the amount present at zero time.

of this corpus did not respond to ICSH either in terms of progesterone biosynthesis from endogenous precursors (mass) or from [^{14}C]acetate (radioactivity). Moreover, the [^{14}C]cholesterol was of low specific activity, and incubation of slices with ICSH has produced no demonstrable change in either ^{14}C content or specific activity of the cholesterol. Experiment 8 shows that deactivated ICSH (see Experimental Section) is without effect upon either the biosynthesis of progesterone or that of [^{14}C]cholesterol, while the same tissue responded to untreated ICSH both in terms of [^{14}C]progesterone and [^{14}C]cholesterol synthesis.

Conversion of [7α-^{3}H]Cholesterol to [^{3}H]Progesterone. In each of five experiments shown in Table V tissue from one corpus luteum was added to each of three beakers containing [7α-^{3}H]cholesterol. The contents of one beaker were extracted immediately (zero time) and the remaining flasks were incubated for 2 hr (one with and one without ICSH). The specific activity of [^{3}H]progesterone was measured as described under Experimental Section. In each experiment ICSH increased the conversion of [^{3}H]cholesterol to [^{3}H]progesterone, in confirmation of a previous report from this laboratory (Hall and Koritz, 1965). In expt 9 no biosynthesis of progesterone *de novo* was observed in the absence of ICSH, and hence it was not possible to compare specific activities of [^{3}H]progesterone formed in the presence and absence of ICSH. In expt 10 and 11 the specific activity of [^{3}H]progesterone in beakers containing ICSH was considerably less than in beakers without ICSH. In expt 12 and 13 the specific activities were approximately equal in beakers with and without ICSH.

Table VI shows five experiments in each of which three flasks were preincubated with [7α-^{3}H]cholesterol for 1 hr and the contents carefully washed, blotted, and transferred to fresh buffer. Extraction was performed immediately in the case of one beaker, while the other two were incubated for 2 hr in buffer (one with ICSH and one without) but without exogenous substrate. In the absence of synthesis of progesterone *de novo* when ICSH was not added to the incubation medium (Table VI, columns 3 and 5), no comparison of specific activities of [^{3}H]progesterone with and without the hormone is possible. However, it will be seen that, when increase in mass and radioactivity in the presence of the hormone are expressed as a percentage of zero time values (columns 4 and 6), ICSH has produced increases in the conversion of [^{3}H]cholesterol to [^{3}H]progesterone (radioactivity) and of endogenous precursors to progesterone (mass) of approximately the same magnitude.

TABLE VI: The Effect of ICSH upon Slices of Corpora Lutea Preincubated with [7α-^{3}H]Cholesterol.[a]

		[^{3}H]Progesterone			
		Mass		Radioactivity	
Expt	Conditions	(μg)	Increase over Zero Time (%)	(dpm)	Increase over Zero Time (%)
14	Zero time	38.0		51,000	
	No ICSH	40.0		51,000	
	ICSH	42.2	11	58,000	14
15	Zero time	55.5		42,000	
	No ICSH	55.8		39,000	
	ICSH	63.6	15	51,000	21
16	Zero time	46.6		45,000	
	No ICSH	43.7		45,000	
	ICSH	67.0	46	61,000	36
17	Zero time	31.9		66,000	
	No ICSH	30.3		63,000	
	ICSH	52.3	63	126,000	91
18	Zero time	31.0		51,000	
	No ICSH	36.7		48,000	
	ICSH	65.0	110	107,000	110

[a] The experiments were performed like those reported in Table V except that the tissue was preincubated for 1 hr with [7α-^{3}H]cholesterol, washed three times with buffer, and either extracted (zero time), incubated without, or incubated with ICSH.

Discussion

The present findings are based upon two distinct approaches to the problem of whether or not ICSH acts before cholesterol. One approach (incubation with [^{3}H]cholesterol) provided equivocal results open to more than one interpretation. The second (biosynthesis of [^{14}C]cholesterol in the presence and absence of ICSH) can best be interpreted in favor of at least the major action of ICSH in the system under study, taking place beyond cholesterol.

If [^{3}H]cholesterol readily penetrated luteal cells and rapidly equilibrated with that portion of the intracellular cholesterol which acts as a precursor of progesterone, one would expect the specific activity of the [^{3}H]progesterone formed during incubation to be the same in the presence and absence of ICSH, provided that cholesterol is the source of the progesterone formed *de novo* and that ICSH acts only beyond cholesterol. If, however, intermediates between cholesterol and progesterone are present in the tissue in large amounts, if sterols other than cholesterol can act as precursors of progesterone without first forming cholesterol, or if under the influence of ICSH cholesterol is made available from some source other than synthesis *de novo* (*e.g.*, esterified cholesterol or extramitochondrial cholesterol transported into mitochondria), if equilibration between exogenous [^{3}H]cholesterol and endogenous cholesterol is slow, or if ICSH acts before cholesterol, this expectation no longer holds. The present data indicate that the specific activity of [^{3}H]progesterone was never greater in the presence of ICSH than in its absence and was in some experiments considerably

lower in the presence of the hormone (Table V). In one case (expt 9) no demonstrable synthesis of progesterone *de novo* was observed in the absence of ICSH, while in two experiments (12 and 13) no difference in specific activity of [^{3}H]progesterone with and without ICSH could be detected and in the remaining two experiments (10 and 11) this specific activity was lower in the presence of ICSH (Table V). Clearly these findings are open to several interpretations.

The present experiments do not indicate which of the possible explanations mentioned best account(s) for the variations observed in the specific activity of [^{3}H]-progesterone formed in the presence and in the absence of ICSH. However, data in Table V suggest that mixing of [7α-^{3}H]cholesterol and endogenous cholesterol may be variable and complex. For example, in expt 9 almost as much [^{3}H]cholesterol was converted to [^{3}H]-progesterone in the absence of ICSH as in its presence, but significant synthesis *de novo* from endogenous precursors occurred only in the presence of the hormone. In expt 13, on the other hand, radioactivity and mass changed proportionately, giving similar specific activities.

In order to limit the influence of rate of mixing between exogenous and endogenous cholesterol, the experiments shown in Table VI were performed by removing the tissue from the medium containing [7α-^{3}H]-cholesterol before adding ICSH. It will be seen that in no case (with the possible exception of expt 18) was synthesis *de novo* of progesterone, from either [7α-^{3}H]-cholesterol or from endogenous precursors, observed after a period of preincubation, when the following incubation was performed without ICSH. No comparison of specific activities with and without the hormone is therefore possible. However, when the increased production of [^{3}H]progesterone resulting from incubation with ICSH is expressed as a percentage of zero time values, the increases in mass and radioactivity are *approximately* equal (Table VI). It would appear that these observations do not permit unequivocal interpretation. It is clear, however, that an earlier consideration by the present authors (Hall and Koritz, 1965), based upon the specific activity of *total* [^{3}H]-progesterone, can no longer be regarded as tenable.

The results of experiments in which the biosynthesis of [^{14}C]cholesterol was measured show that when ICSH stimulates the production of [^{14}C]progesterone less [^{14}C]cholesterol is isolated from the tissue than that present in the unstimulated slices. In control studies (deactivated ICSH and unresponsive corpus luteum) this difference was not observed (Table IV). Not only was there less radioactivity present in [^{14}C]cholesterol in responsive slices (Table III), but also the mass of cholesterol showed no difference in the presence or absence of ICSH and accordingly the specific activity of [^{14}C]cholesterol was lower in flasks containing ICSH. Moreover, in keeping with observations by Armstrong *et al.* (1964) in the rat, the specific activity of [^{14}C]-cholesterol was always considerably less than that of the [^{14}C]progesterone isolated from the same tissue, both in the presence and absence of ICSH (Table III), indicating that only a fraction of luteal cholesterol acts as a precursor of progesterone.

These findings with respect to [^{14}C]cholesterol are in complete agreement with observations already reported on the action of ICSH on the testis, where possible interpretations were discussed in detail (Hall, 1963). It was concluded that if cholesterol is a major intermediate in the conversion of acetate to steroid hormones, the findings point to a site of action of ICSH beyond cholesterol. In view of the unequivocal stimulation by ICSH of the conversion of [^{3}H]cholesterol to [^{3}H]progesterone by slices of bovine corpus luteum previously reported (Hall and Koritz 1965), it seems unnecessary to postulate a second site of action before cholesterol. Moreover, the fact that less ^{14}C was always found in highly purified cholesterol isolated after incubation with [^{14}C]acetate and ICSH than in the absence of ICSH is difficult to reconcile with a significant effect of the hormone before cholesterol, in the present system. These observations are in accordance with analogous findings in the case of the adrenocortical response to ACTH (Karaboyas and Koritz, 1965).

In conclusion it should be pointed out that this interpretation of the present observations applies only to the short term action of ICSH *in vitro*. The findings do not exclude other effects of ICSH *in vivo*, especially in the case of chronic exposure to the hormone. For example, increased demethylation of lanosterol in testicular tissue was recently reported by Ying *et al.* (1965), following treatment with human chorionic gonadotrophin *in vivo*.

Acknowledgments

The authors are grateful to Dr. Irving Welicky, Department of Biochemistry, University of Pittsburgh, for the use of the countercurrent apparatus, and to Miss June Fry and Miss Virginia Gates for excellent technical assistance.

References

Armstrong, D. T., O'Brien, J., and Greep, R. O. (1964), *Endocrinology 75*, 488.

Baggett, B., and Engel, L. L. (1957), *J. Biol. Chem. 229*, 443.

Hall, P. F. (1963), *Biochemistry 2*, 1232.

Hall, P. F., and Eik-Nes, K. B. (1962), *Biochim. Biophys. Acta 63*, 411.

Hall, P. F., and Koritz, S. B. (1965), *Biochemistry 4*, 1037.

Kabara, J. J., and McLaughlin, J. T. (1961), *J. Lipid Res. 2*, 283.

Karaboyas, G., and Koritz, S. B. (1965), *Biochemistry 4*, 462.

Mason, N. R., and Savard, K. (1964), *Endocrinology 75*, 215.

Reichert, L. E., Jr. (1961), *Endocrinology 69*, 398.

Savard, K., and Casey, P. J. (1964), *Endocrinology 74*, 599.

Schwenk, E., and Werthesson, N. T. (1952), *Arch. Biochem. Biophys. 40*, 334.
Schwenk, E., and Werthesson, N. T. (1953), *Arch. Biochem. Biophys. 42*, 91.
Ying, B. P., Chang, Y., and Gaylor, J. L. (1965), *Biochim. Biophys. Acta 100*, 256.
Yudaev, N. A., and Razina, L. G. (1964), *Vopr. Med. Khim. 9, 597* (in *Federation Proc. 23, T981*, 1964).

Catalysis of the H_2–HTO Exchange by Hydrogenase. A New Assay for Hydrogenase*

S. R. Anand and Alvin I. Krasna

ABSTRACT: A method is described for the assay of hydrogenase activity by its ability to catalyze the reaction $H_2 + HTO \rightleftharpoons HT + H_2O$. The assay measures the rate of appearance of HT in the gas phase by determining its radioactivity in an ionization chamber. The effects of sample size, filling gas pressure, and enzyme concentration are studied.

Optimum conditions are suggested for a routine rapid and reproducible assay. The kinetics of the tritium exchange are compared with that of a similar reaction using deuterium as a tracer. The isotope effect manifests itself in the reaction of the proton of water with the activated hydrogen and not in the splitting of the hydrogen molecule itself.

The enzyme hydrogenase, which activates molecular hydrogen, can be assayed by three general methods. The most commonly used procedure measures the rate of reduction of some acceptor, organic or inorganic, by hydrogen gas. The reduction is followed by measuring the rate of hydrogen uptake or the change in concentration of the acceptor. Some of the acceptors often used are: methylene blue (Stephenson and Stickland, 1931), benzyl viologen (Krasna and Rittenberg, 1956), oxygen (Wilson *et al.*, 1942), nitrate (Krasna and Rittenberg, 1954a), ferricyanide (Hyndman *et al.*, 1953), and fumarate (Farkas and Fischer, 1947). A second method measures the rate of hydrogen evolution from compounds which have a lower oxidation–reduction potential than hydrogen, such as reduced methyl viologen (Tamiya *et al.*, 1955), reduced benzyl viologen (Krasna and Rittenberg, 1956), and malate (Gest *et al.*, 1962). Both these methods measure the resultant of at least two consecutive reactions, *i.e.*, the activation of molecular hydrogen and the transfer of the activated hydrogen to the electron acceptor. Therefore, the rate of reduction of an acceptor is not necessarily a direct measure of the activation of hydrogen, since other enzyme systems or electron carriers may be involved in the utilization of the activated hydrogen for the chemical reduction. This has been clearly demonstrated by many authors (Krasna and Rittenberg, 1956; Hoberman and Rittenberg, 1943; Curtis and Ordal, 1954; Whitely and Ordal, 1955).

A third assay method for hydrogenase which we have used extensively depends on its ability to catalyze the exchange reactions between hydrogen and heavy water, *i.e.*

$$H_2 + HDO \rightleftharpoons HD + H_2O \qquad (1)$$

This reaction was first used by Farkas *et al.* (1934) and studied later in great detail by Hoberman and Rittenberg (1943). Hydrogenase was also shown (Krasna and Rittenberg, 1954b) to catalyze the conversion of para- to orthohydrogen and, on the basis of this and the deuterium exchange, the mechanism of activation of hydrogen by hydrogenase was postulated to be a heterolytic split of hydrogen to a hydride and a proton, *i.e.*

$$E + H_2 \rightleftharpoons EH^- + H^+ \qquad (2)$$

where E represents the enzyme. These two exchange reactions do not require the addition of any electron acceptors or cofactors and, therefore, directly measure the rate of reaction of hydrogen with the enzyme.

The deuterium exchange assay is very rapid and accurate but requires the use of a mass spectrometer which is not always available. The parahydrogen assay is slow and not very sensitive. We therefore investigated the use of tritium exchange as an assay for hydrogenase. Recently (Goldsby, 1961; Gingras *et al.*, 1963) hydrogenase has been assayed by studying the

* From the Department of Biochemistry, Columbia University, College of Physicians and Surgeons, New York, N. Y. 10032. *Received August 4, 1965.* This investigation was supported by a Public Health Service research grant (GM 12198) from the National Institute of General Medical Sciences.