Dean, R. A., and Whitehead, E. V. (1961), *Tetrahedron Letters 21*, 768.
Downing, D. T., Kranz, Z. H., and Murray, K. E. (1960), *Australian J. Chem. 13*, 80.
Hallgren, B., and Larsson, S. (1963), *Acta Chem. Scand. 17*, 543.
Kochloefl, K., Schneider, P., Rericha, R., Horak, M., and Bazant, V. (1963), *Chem. Ind. (London)*, 692.
Lambertsen, G., and Holman, R. T. (1963), *Acta Chem. Scand. 17*, 281.
Levy, E. J., Doyle, R. R., Brown, R. A., and Melpolder, F. W. (1961), *Anal. Chem. 33*, 698.
Mold, J. D., Stevens, R. K., Means, R. E., and Ruth, J. M. (1963a), *Biochemistry 2*, 605.
Mold, J. D., Stevens, R. K., Means, R. E., and Ruth, J. M. (1963b), *Nature 199*, 283.
Sörensen, J. S., and Sörensen, N. A. (1949), *Acta Chem. Scand. 3*, 939.
Stevens, R. K., and Mold, J. D. (1963), *J. Chromatog. 10*, 398.
Waldron, J. D., Gowers, D. C., Chibnall, A. C., and Piper, S. H. (1961), *Biochem. J. 78*, 435.

End-Product Inhibition of the Conversion of Cholesterol to Pregnenolone in an Adrenal Extract*

SEYMOUR B. KORITZ AND PETER F. HALL

From the Departments of Biochemistry and Physiology, University of Pittsburgh, School of Medicine, Pittsburgh, Pa.

Received April 30, 1964

An extract prepared from acetone-dried powder of mitochondria from bovine adrenal cortex was used to study the conversion of 7α-[^{3}H]cholesterol to [^{3}H]pregnenolone. This sequence of reactions is inhibited by pregnenolone at the stage of conversion of 7α-[^{3}H]cholesterol to 20α-hydroxy[^{3}H]cholesterol, and therefore represents an example of feedback or end-product inhibition. It is suggested that intramitochondrial levels of pregnenolone may play an important role in the regulation of steroid biosynthesis by the adrenal cortex.

It is at present thought (Lynn *et al.*, 1954; Solomon *et al.*, 1956; Shimizu *et al.*, 1960, 1961; Constantopoulos *et al.*, 1962; Hall and Koritz, 1964a) that the conversion of cholesterol to pregnenolone takes place in endocrine tissue by the following sequence of reactions:

$$\text{cholesterol} \xrightarrow{[I]} 20\alpha\text{-hydroxycholesterol} \xrightarrow{[II]} 20\alpha,22\xi\text{-dihydroxycholesterol} \xrightarrow{[III]} \text{pregnenolone}^1$$

These reactions and the enzyme systems associated with them will be referred to here as step [I], enzyme [I], and so forth, although it is realized that each step involves a number of enzymes and cofactors not shown.

During the course of experiments designed to study these reactions in bovine adrenal cortex it was observed that pregnenolone inhibited the conversion of 7α-[^{3}H]-cholesterol to [^{3}H]pregnenolone by acetone powder of "mitochondria."[2] Interest in this observation arose from two circumstances. First, the demonstration that in the adrenal cortex the overall reaction (steps I–III) is confined to the "mitochondria" (Halkerston *et al.*, 1961) suggests that the inhibition of these reactions by pregnenolone may constitute an important regulatory mechanism, since the rate of the overall reaction may be influenced by the rate of removal of pregnenolone from mitochondria. Second, it was noticed that inhibition by pregnenolone was not associated with the accumulation of either 20α-hydroxy-[^{3}H]cholesterol or 20α,22ξ-dihydroxy[^{3}H]cholesterol, suggesting that this inhibition was not simply an example of product inhibition.

The present report concerns experiments designed to examine the nature of the inhibition of the above sequence of reactions by pregnenolone. It appears that pregnenolone produces feedback or end-product inhibition.

Experimental Procedure

Preparation of Tissue.—Bovine adrenal glands were obtained fresh from a slaughterhouse, the cortex was separated from the medulla, and an acetone-dried powder of cortical "mitochondria" was prepared according to the procedure described by Halkerson *et al.* (1961). Before use in each of the present experiments a clear supernatant extract the powder was prepared in 0.07 M potassium phosphate buffer, *p*H 6.8 (20 mg powder/ml buffer), as described by the same authors. This extract will be referred to hereafter as adrenal extract. The following experiments were performed on six separate preparations of acetone-dried powder. The powder was stored at −18° and no significant loss of activity as measured by conversion of 7α-[^{3}H]-cholesterol to [^{3}H]pregnenolone was detected during the course of these studies. All six powders gave the same results in the various experiments to be described.

Incubation Procedure.—Incubation was performed in 20-ml beakers in air with constant agitation at 37.5° in a final volume of 2 ml for a period of 10 minutes unless otherwise stated. Additions were made in the following order: KCl, 0.15 M, to make a final volume of 2 ml; TPN, 5×10^{-4} M; glucose-6-phosphate, 5×10^{-3} M; glucose-6-phosphate dehydrogenase (approximately

* This investigation was supported by two grants (AM 03676-04 END and AM 06459-02 END) from the National Institutes of Health, U. S. Public Health Service.

[1] The following abbreviations and trivial names are used: corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; cortisol, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; dehydroepiandrosterone, 3β-hydroxy-5-androsten-17-one; DOC, 21-hydroxy-4-pregnene-3,20-dione; 17α-hydroxypregnenolone, 3β,17α-dihydroxy-5-pregnen-20-one; 17α-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; pregnenolone, 3β-hydroxy-5-pregnen-20-one; progesterone, 4-pregnene-3,20-dione; TPN, triphosphopyridine nucleotide; ACTH, adrenocorticotropic hormone.

[2] The expression "mitochondria" is used here to refer to large particles prepared by the procedure of Halkerston *et al.* (1961).

0.1 Kornberg units); phosphate buffer (0.07 M), pH 6.8 (0.5 ml); and steroids. Beakers were then placed in ice, adrenal extract (0.2 ml/beaker unless otherwise stated) was added, and the beakers were transferred to the incubator. At the end of incubation the beakers were placed in ice and methylene chloride (6 ml) was added to each beaker. Sterols and steroids were added either in an aqueous solution of Tween 80 (Atlas Powder Co.)[3] or in absolute ethanol (0.02 ml) as indicated beneath the accompanying tables and figures; the appropriate vehicle was added to control beakers.

Extraction and Isolation of Pregnenolone.—The contents of each beaker was extracted three times with three volumes of methylene chloride, washed, and taken to dryness as described elsewhere (Hall and Kortiz, 1964a). The extract was applied to paper in the system ligroin–propylene glycol (Brady, 1951) for 10 hours (chromatogram I, Fig. 1). In this system of paper chromatography, the solvent front reached the ends of the strips after 3.5 hours and the solvent which ran from each strip during the remaining 6.5 hours of chromatography was collected in a beaker and applied to paper in the same system until the solvent front was within 2 cm of the ends of the strips (chromatogram II, Fig. 1). Dry chromatograms were examined in a windowless strip-counter and the positions of pregnenolone and 20α-hydroxycholesterol were identified by running a mixture of the authentic compounds on adjacent strips of paper (Fig. 1). The authentic steroids were located by a color reaction—either the ethanol–sulfuric acid reaction (Oertel and Eik-Nes, 1959) or the antimony trichloride reaction (Rosenkrantz, 1953).

When 7α-[^{3}H]pregnenolone was added to a beaker containing buffer, cofactors, adrenal, extract, and the contents of the beaker immediately extracted by the foregoing method, recovery of 7α-[^{3}H]pregnenolone from chromatogram I was between 90 and 94%. Similar recovery was achieved when 7α-[^{3}H]pregnenolone was added to heated adrenal extract. Values in the accompanying data are not corrected for these losses.

Gas Chromatography.—The cholesterol content of the adrenal extracts used in these experiments was measured by methods previously reported (Hall and Koritz, 1964a).

Measurement of Radioactivity.—Radioactivity was measured as reported elsewhere (Hall, 1963). In order to measure radioactivity in substances isolated by paper chromatography, chromatograms were cut into pieces 2 × 1 cm and each piece again was cut into fragments. Fragments from each piece were extracted by standing for 3 hours at 40° in 10 ml of a mixture of methanol–methylene chloride (1:1, v/v). The solvent was removed and the fragments were washed twice with 2 ml of the same mixture. The pooled extracts were filtered and dried in a 20-ml counting vial. The expression "dpm" indicates that values were corrected for the prevailing efficiency for counting tritium (25–30%). When radioactivity was recovered from glassware, 10 ml of scintillation fluid was added to the vessel concerned and the inside was repeatedly washed with this fluid which was then transferred to a counting vial.

Recrystallization.—To recrystallize [^{3}H]pregnenolone the sample was eluted from chromatogram I by the method described, and, after addition of 20 mg of authentic pregnenolone, crystals were allowed to form from various solvents. The mother liquor was removed and the crystals were carefully washed with hexane. The crystals were then dissolved in chloroform, appropriate aliquots were removed, and the remaining chloroform was evaporated. After each recrystallization, samples were taken for measurement of radioactivity and mass. The mass of pregnenolone was measured by the ethanol–sulfuric acid reaction (Oertel and Eik-Nes, 1959).

[3] Dr. T. T. Tchen, Department of Chemistry, Wayne State University, Detroit; personal communication.

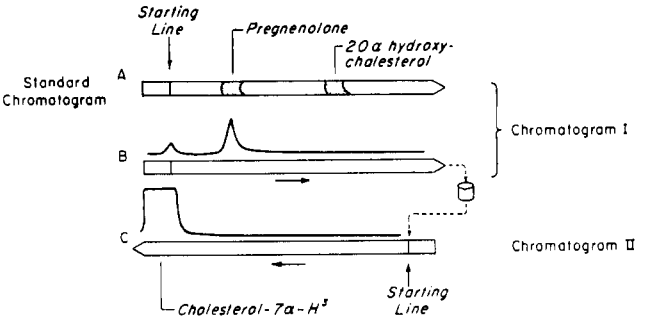

FIG. 1.—Diagram showing the distribution of radioactivity on typical chromatogram I and chromatogram II. (A) Standard chromatogram showing the positions of pregnenolone and 20α-hydroxycholesterol (100 μg each) detected by an ethanol–sulfuric acid reaction (Oertel and Eik-Nes 1959). (B) Tracing of radioactivity on a typical chromatogram I. Solvent running from the end of the strip was collected and applied to paper. (C) Chromatogram II (see Experimental Procedure). These chromatograms were developed in ligroin–propylene glycol (Brady, 1951).

Materials.—Glucose-6-phosphate, TPN, glucose-6-phosphate dehydrogenase, and pregnenolone were obtained from Sigma Chemical Co. The source and purity of cholesterol have been given elsewhere (Hall, 1963). The 20α-hydroxycholesterol used here was prepared by Dr. Marcel Gut of the Worcester Foundation. Methylene chloride was spectrograde (Fisher Scientific Co.). Other steroids were obtained from Sigma Chemical Co.

7α-[^{3}H]Cholesterol was obtained from New England Nuclear Corp. Before use in the present experiments 7α-[^{3}H]cholesterol was purified as described previously (Hall and Koritz, 1964b). When the purified material (70,000 dpm) was applied to paper in ligroin–propylene glycol for 10 hours and the areas corresponding to pregnenolone and 20α-hydroxycholesterol were examined for radioactivity, no radioactivity was detected (<100 dpm).

The 20α-hydroxy[^{3}H]cholesterol used in these experiments was generously provided by Dr. T. T. Tchen in whose laboratory the compound was prepared from 7α-[^{3}H]pregnenolone (Constantopoulos and Tchen, 1961). Before use in the present experiments this compound was purified by paper chromatography in ligroin–propylene glycol for 10 hours. An aliquot of the purified material behaved as a single peak of radioactivity on rechromatography in the same system. Examination of both chromatograms showed that no demonstrable [^{3}H]pregnenolone was present (<100 dpm in a sample containing 50,000 dpm).

The identity and radiochemical purity of [^{3}H]cholesterol remaining an unused substrate following incubation was examined by addition of cholesterol, digitonin precipitation, and repeated recrystallization as the dibromide. This procedure has been described in detail elsewhere (Hall, 1963).

RESULTS

The present experiments are based upon measurements of [^{3}H]pregnenolone formed during incubation of adrenal extract with 7α-[^{3}H]cholesterol and 20α-hydroxy[^{3}H]cholesterol. Evidence for the identity and radiochemical purity of the material measured as [^{3}H]-

TABLE I

RECRYSTALLIZATION OF [^{3}H]PREGNENOLONE ISOLATED FOLLOWING INCUBATION OF ADRENAL EXTRACT WITH 7α-[^{3}H]CHOLESTEROL

A[a] Recrystallization	Specific Activity (dpm/mg) Crystals	Mother Liquor	B[a] Recrystallization	Specific Activity (dpm/mg) Crystals	Mother Liquor
After addition of pregnenolone	6320		After addition of pregnenolone	4750	
(1st) Ligroin-acetone	6560	7350	(1st) Ligroin-acetone	4910	4400
(2nd) Hexane-chloroform	6410	6300	(2nd) Hexane-chloroform	5000	3930
(3rd) Pentane–ethyl acetate	6100	6000	(3rd) Pentane–ethyl acetate	4610	3100
(4th) Aqueous methanol	6000	6500	(4th) Aqueous methanol	4250	3500

[a] Samples A and B consist of material corresponding to chromatographic behavior to pregnenolone eluted from two chromatograms I of an experiment identical with that presented in Figure 2. Recrystallization and measurements of specific activity were performed as described under Experimental Procedure. In the case of sample A, no pregnenolone was added to the incubation medium; while in the case of sample B, 10 μg of pregnenolone was added.

TABLE II

RECRYSTALLIZATION OF [^{3}H]PREGNENOLONE ISOLATED FOLLOWING INCUBATION OF ADRENAL EXTRACT WITH 20α-HYDROXY[^{3}H]CHOLESTEROL

A[a] Recrystallization	Specific Activity (dpm/mg) Crystals	Mother Liquor	B[a] Recrystallization	Specific Activity (dpm/mg) Crystals	Mother Liquor
After addition of pregnenolone	15,900		After addition of pregnenolone	13,200	
(1st) Hexane-chloroform	16,800	13,400	(1st) Hexane-chloroform	12,700	11,900
(2nd) Pentane-acetone	16,600	15,000	(2nd) Pentane-acetone	12,900	12,700
(3rd) Hexane–ethyl acetate	16,100	17,000	(3rd) Hexane–ethyl acetate	12,400	12,200
(4th) Aqueous ethanol	16,700	16,800	(4th) Aqueous ethanol	12,200	12,000

[a] Samples A and B consist of material corresponding in chromatographic behavior to pregnenolone eluted from two chromatograms I of an experiment identical with that reported in Table VI. In the case of sample A, no pregnenolone was added to the incubation medium; while in the case of sample B, 10 μg of pregnenolone was added.

TABLE III

EFFECT OF VARIOUS STEROIDS UPON THE CONVERSION OF 7α-[^{3}H]CHOLESTEROL TO [^{3}H]PREGNENOLONE BY ACETONE POWDER OF ADRENAL MITOCHONDRIA

Compound Added[a] Name	(μg/beaker)	[^{3}H]Pregnenolone (dpm)	Compound Added[a] Name	(μg/beaker)	[^{3}H]Pregnenolone (dpm)
		197,000			188,000
		208,000			185,000
Pregnenolone	10	131,400	Pregnenolone	10	103,000
	10	136,800		10	101,000
Progesterone	10	197,500	Corticosterone	10	174,200
	10	195,000		10	178,300
DOC	10	181,000	Cortisol	10	184,100
	10	199,500		10	190,200
17α-Hydroxy-progesterone	10	171,900	Dehydroepi-androsterone	10	178,000
	10	191,800		10	182,000
17α-Hydroxy-pregnenolone	10	187,000	20β-Dihydro-progesterone	10	181,000
	10	195,100		10	175,200

[a] Steroids were added in absolute ethanol (0.02 ml/flask) and this solvent was added to control flasks. The conditions of incubation are those given in Figure 2 except for addition of the steroids shown.

pregnenolone is given in Tables I and II, where it will be seen that the radioactive material isolated following incubation with either substrate (7α-[^{3}H]cholesterol, Table I, or 20α-hydroxy[^{3}H]cholesterol, Table II) when mixed with authentic pregnenolone could be recrystallized from four different solvents to constant specific activity, and that even during the first recrystallization the specific activity was not significantly altered. It is therefore concluded that the radioactive compound isolated from the chromatograms was [^{3}H]-pregnenolone and that this was radiochemically pure after paper chromatography (chromatogram I). The conversion of these two substrates to [^{3}H]pregnenolone is in keeping with earlier reports from other laboratories (e.g., Shimizu *et al.*, 1960, 1961; Constantopoulos and Tchen, 1961).

Examination of the adrenal extracts showed that the amount added to each beaker for incubation contained less than 0.01 μg of cholesterol.

Inhibition by Pregnenolone. 7α-[^{3}H]CHOLESTEROL AS SUBSTRATE.—Fig. 2 shows the effect of various concentrations of pregnenolone upon the conversion of 7α-[^{3}H]cholesterol to [^{3}H]pregnenolone by adrenal extract. Pregnenolone inhibited this conversion and maximal inhibition was achieved by 10 μg per beaker, which reduced the conversion to approximately 55%

TABLE IV

INFLUENCE OF DURATION OF INCUBATION ON THE CONVERSION OF 7α-[^{3}H]CHOLESTEROL TO [^{3}H]PREGNENOLONE BY ADRENAL EXTRACT IN THE PRESENCE AND ABSENCE OF PREGNENOLONE[a]

Duration of Incubation (minutes)	Pregnenolone (μg/beaker)	[^{3}H]Pregnenolone (dpm)
10	0	474,000
	0	468,000
	6	188,000
	6	174,000
20	0	762,000
	0	739,000
	6	392,000
	6	389,000
60	0	1,080,000
	0	994,000
	6	503,000
	6	516,000

[a] Incubation was performed under the conditions described in Figure 2 except for the duration of incubation.

of the uninhibited level. The specificity of pregnenolone as an inhibitor in the present system is suggested by the findings reported in Table III. It is seen that of a number of steroids which occur in adrenal tissue, only pregnenolone shows inhibition of the conversion of cholesterol to pregnenolone at the concentration tested. Table IV shows that the inhibition produced by pregnenolone lasts for at least 60 minutes, and Fig. 3 indicates that the inhibition persists over a considerable range of tissue concentration.

Figure 4 shows that increasing concentrations of substrate (7α-[^{3}H]cholesterol) did not reverse the inhibition produced by pregnenolone. In this experiment the specific activity of 7α-[^{3}H]cholesterol was held constant and increasing amounts of the same specific activity were added to the various beakers. The inset of Figure 4 shows the same data presented as a Lineweaver-Burk plot, which further demonstrates that the inhibition produced by pregnenolone was not competitive with respect to cholesterol. In two other identical experiments this observation was repeated.

In order to determine whether inhibition by pregnenolone was associated with the accumulation of 20α-hydroxy[^{3}H]cholesterol, chromatograms I from two experiments identical with that shown in Figure 2 were eluted from a point 2 cm distal to the area occupied by [^{3}H]pregnenolone up to and including the ends of the chromatograms. To the eluate 100 μg 20α-hydroxycholesterol was added and the mixture was dried under nitrogen, an aliquot was taken for measurement of radioactivity, and the remainder was applied to paper in the system ligroin–propylene glycol for 4 hours (chromatogram II). The dry chromatograms were examined by means of a strip-counter which revealed no demonstrable radioactivity. The strips were then cut longitudinally into halves and one half was examined for radioactivity by liquid scintillation spectrometry as described under Experimental Procedure. In no case was significant radioactivity detected (i.e., none >100 dpm) in any part of the chromatogram. The remaining halves of the chromatograms were divided longitudinally and each half was submitted to a different color reaction (antimony trichloride and ethanol–sulfuric acid). The 20α-hydroxycholesterol added as carrier was clearly demonstrated by each of these reactions. The aliquots of eluates removed before chromatography revealed that the entire eluate contained less than 400 dpm in each sample. In no case was significant radioactivity found in the portion of the chromatogram occupied by carrier 20α-hydroxycholesterol in extracts from control beakers (no pregnenolone added), in the presence of maximal inhibition by pregnenolone or in the presence of less than maximal inhibition by smaller concentrations of pregnenolone. In fifteen experiments in which the inhibitory action of pregnenolone was observed, examination of the areas of chromatogram I corresponding to 20α-hydroxycholesterol revealed no significant radioactivity (<100 dpm).

Table V presents the results of one experiment representative of five in which the inhibitory action of pregnenolone was demonstrated and an attempt was made to account for all the tritium added to the incubation medium as 7α-[^{3}H]cholesterol. It will be seen that within the limits of experimental error most of the radioactivity added as 7α-[^{3}H]cholesterol has been recovered (89–94%) as [^{3}H]pregnenolone, 7α-[^{3}H]cho-

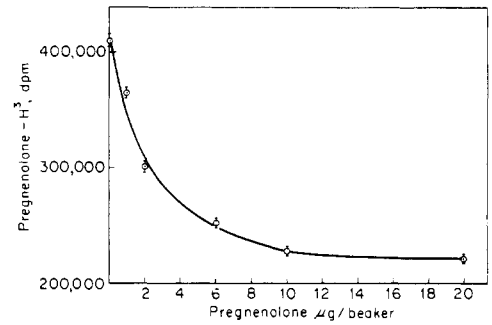

FIG. 2.—The effect of pregnenolone concentration on the conversion of 7α-[^{3}H]cholesterol to [^{3}H]pregnenolone. Adrenal extract was incubated with 7α-[^{3}H]cholesterol (3 μg; 3,000,000 dpm/beaker) at the concentrations of pregnenolone indicated; the other additions are given under Experimental Procedure. Pregnenolone was added in ethanol. Each point represents the mean of duplicate beakers and I shows the range.

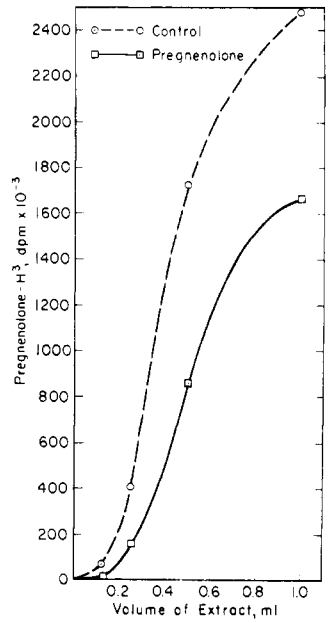

FIG. 3.—The influence of enzyme concentration upon the conversion of 7α-[^{3}H]cholesterol (3 μg; 3,000,000 dpm/beaker) to [^{3}H]pregnenolone in the presence and absence of pregnenolone. Adrenal extract was incubated under the conditions given under Experimental Procedure. The concentration of pregnenolone used was 10 μg/beaker. The expression dpm × 10^{-3} indicates that the true values are obtained by multiplying the figures shown by 10^3.

TABLE V

RECOVERY OF RADIOACTIVITY FOLLOWING INCUBATION OF ADRENAL EXTRACT WITH 7α-[^{3}H]CHOLESTEROL WITH AND WITHOUT PREGNENOLONE[a]

Conditions	Radioactivity at Origin (Chromatogram I) (dpm)	[^{3}H]Pregnenolone (dpm)	7α-[^{3}H]-Cholesterol (dpm)	Radioactivity Recovered from Glassware (dpm)	Total Radioactivity Recovered (dpm)	Total Radioactivity Recovered Per Cent Radioactivity Added
No Pregnenolone	69,500	510,000	2,675,000	29,000	3,284,000	94
	71,000	500,000	2,450,000	55,000	3,076,000	89
Pregnenolone (6 μg)	59,500	317,500	2,825,000	55,000	3,257,000	93
(6 μg)	59,500	315,500	2,775,000	98,000	3,248,000	93
TPN omitted	42,000	63,100	3,025,000	54,000	3,184,000	91
Boiled enzyme	34,000	43,000	3,030,000	45,000	3,152,000	90

[a] Adrenal extract was incubated with 7α-[^{3}H]cholesterol (3 μg; 3,500,000 dpm per beaker added as a suspension in Tween 80) with TPN and a reducing system as indicated under Experimental Procedure. Pregnenolone was added in ethanol. Following incubation [^{3}H]pregnenolone was extracted from chromatogram I and measured as described under Experimental Procedure. The origins of these chromatograms were eluted and radioactivity of eluates was counted by liquid-scintillation spectrometry. The origin included the are acorresponding to 20α,22-dihydroxycholesterol (see Results). The remainder of the chromatograms were eluted and no significant radioactivity was detected when radioactivity of these eluates was counted by liquid-scintillation spectrometry. The solvent which ran from the strips of chromatogram I was collected and applied to paper in ligroin–propylene glycol until the solvent front approached the end of the paper strips (chromatogram II). The strip-counter revealed a single peak at the front of each chromatogram. An aliquot of this material was assayed by liquid scintillation spectrometry. The remainder of chromatogram II was found to be free of significant radioactivity. The nature of the compound at the front of the chromatogram II was shown to be [^{3}H]cholesterol by purification as the dibromide (see Experimental Procedure); during this procedure the specific activity of the [^{3}H]cholesterol remained constant within the limits ± 8%.

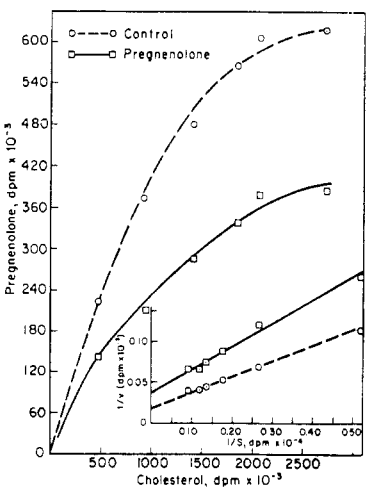

FIG. 4.—The effect of substrate concentration (7α-[^{3}H]-cholesterol) upon the inhibition of the conversion of 7α-[^{3}H]cholesterol produced by pregnenolone. The experiment was conducted under the conditions described under Experimental Procedure, except that the amount of 7α-[^{3}H]cholesterol added in Tween 80 was varied from beaker to beaker as shown. The specific activity of the 7α-[^{3}H]-cholesterol was the same in every beaker, namely, 1,000,000 dpm/μg. The inset shows the same data presented in the form of a Lineweaver-Burk plot. The concentration of pregnenolone used was 10 μg/beaker (added in ethanol), and 0.02 ml of ethanol was added to control beakers. The expression dpm × 10^{-3} (10^{-4}) indicates that the true values are obtained by multiplying the figures shown by 10^3 (10^4).

lesterol, radioactivity at the origins of chromatogram I, and radioactivity recovered from glassware; the term "origin" refers to the portion of the chromatogram from the starting line to the proximal margin of the pregnenolone area and includes that part of the chromatogram in which 20α,22ξ-dihydroxycholesterol would be expected. No 20α-hydroxy[^{3}H]cholesterol was found; and although the nature of the compound(s) at the origin of these chromatograms is (are) unknown, it is clear that inhibition by pregnenolone is not accompanied by increase in the amount of radioactivity found at the origin. Moreover, it will be seen that the decrease in tritium found as [^{3}H]pregnenolone in the presence of exogenous pregnenolone is accounted for, within the limits of experimental error, by increased recovery of 7α-[^{3}H]cholesterol (compare columns 3 and 4, lines 1 and 2, with lines 3 and 4 in Table V). This experiment (and four like it) demonstrates that inhibition by pregnenolone does not involve accumulation of 20α-hydroxy[^{3}H]cholesterol or 20α,22ξ-dihydroxy-[^{3}H]cholesterol, but that decreased production of [^{3}H]pregnenolone can be accounted for by increased recovery of unused substrate (7α-[^{3}H]cholesterol).

TABLE VI

CONVERSION OF 20α-HYDROXY[^{3}H]CHOLESTEROL TO [^{3}H]-PREGNENOLONE BY ADRENAL EXTRACT AND THE EFFECT OF PREGNENOLONE, PROGESTERONE, AND CHOLESTEROL ON THIS CONVERSION[a]

Addition	[^{3}H]Pregnenolone (dpm)	Relative Conversion of 20α-Hydroxy-[^{3}H]cholesterol
0	210,500	
0	253,500	100
Pregnenolone 10 μg	240,600	
	242,400	104
20 μg	267,000	
	257,100	113
0	248,500	
0	247,000	100
Progesterone 20 μg	231,600	
	232,400	94
Cholesterol 20 μg	248,600	
	233,300	97

[a] Adrenal extract was incubated with 20α-hydroxy-[^{3}H]cholesterol (560,000 dpm/beaker) added in an aqueous suspension of Tween 80. Pregnenolone, progesterone, and cholesterol were added in ethanol.

20α-HYDROXY[^{3}H]CHOLESTEROL AS SUBSTRATE.—Table VI shows that 20α-hydroxy[^{3}H]cholesterol was readily converted to [^{3}H]pregnenolone by adrenal extract (approximately 40% conversion under the present

conditions), in keeping with previous findings by other workers (e.g., Constantopoulos and Tchen, 1961; Schimizu *et al.*, 1961). Moreover, this conversion was not significantly inhibited by pregnenolone at concentrations (10 and 20 μg/beaker) which caused marked inhibition of the conversion of 7α-[^{3}H]cholesterol to [^{3}H]pregnenolone (Fig. 2). Similar concentrations of cholesterol and progesterone were also without demonstrable effect upon the conversion of 20α-hydroxy[^{3}H]-cholesterol to [^{3}H]pregnenolone.

Discussion

Special interest in the conversion of cholesterol to pregnenolone arises from the suggestions that this series of reactions includes the slow step in steroid biosynthesis and that these reactions may include a step specifically stimulated by trophic hormones (Stone and Hechter, 1954; Halkerston *et al.*, 1961; Koritz, 1962; Ichii *et al.*, 1963). The present findings indicate that pregnenolone inhibits the conversion of 7α-[^{3}H]cholesterol to [^{3}H]pregnenolone by the adrenal extract used in these experiments and that this inhibition takes place at the first of these reactions (the conversion of 7α-[^{3}H]cholesterol to 20α-hydroxy[^{3}H]cholesterol). This conclusion is based upon the following findings: (1) Under a variety of conditions pregnenolone inhibition was demonstrated without accumulation of either 20α-hydroxy[^{3}H]cholesterol or 20α,22ξ-dihydroxy[^{3}H]-cholesterol in detectable amounts (Table V and Results). (2) The decrease in formation of [^{3}H]pregnenolone from 7α-[^{3}H]cholesterol in the presence of pregnenolone could be accounted for, within the limits of experimental error, as unconverted 7α-[^{3}H]cholesterol (Table V). (3) Pregnenolone did not inhibit the conversion of 20α-hydroxy[^{3}H]cholesterol to [^{3}H]-pregnenolone (Table VI). The conclusion based on these observations is also in agreement with the data presented in Figure 4 which indicate that the inhibition is not a simple product inhibition.

These findings differ from those of Ichii *et al.* (1963), who found that pregnenolone inhibition of the conversion of cholesterol to pregnenolone by acetone-powder of homogenate of bovine *corpus luteum* was accompanied by accumulation of both 20α-hydroxycholesterol and 20α,22ξ-dihydroxycholesterol. This difference could not be ascribed to the method of extraction used by the Worcester group, since the findings reported here were repeated when the method described by Ichii *et al.* (1963) was used.[4] Since the methods used by the two groups were in other respects similar, it is presumed that these different results must arise from the use of different tissues (*corpus luteum* and adrenal cortex) or of different preparations (homogenate and mitochondria).

The data presented here indicate that the inhibition produced by pregnenolone in the adrenal extract represents an example of feed-back or end-product inhibition, since the inhibitory effect is exerted by the product of a series of reactions acting upon the first of these reactions. Pregnenolone may be regarded as the product of a series of reactions (although it is also a precursor of adrenal steroid hormones), since it has been shown by Halkerston *et al.* (1961) that the conversion of cholesterol to pregnenolone by bovine adrenal cortex is confined to mitochondria. The reactions by which pregnenolone is subsequently converted to other steroids, with the exception of 11β-hydroxylation, take place outside the mitochondria (Samuels, 1960). Furthermore, the preferred pathway to steroid hormones involves hydroxylation at C_{17} and C_{21} before 11β-hydroxylation. It is thus apparent that pregnenolone must leave the mitochondria in order to undergo these enzymatic transformations. In effect this means that the conversion of cholesterol to pregnenolone in mitochondria constitutes a confined segment of a biosynthetic pathway and that inhibition of step [I] by pregnenolone can be regarded as an example of feed-back inhibition (Monod *et al.*, 1963).

Although the exact nature of the precursors of adrenal pregnenolone and the possible pathways berween these precursors and the hormones of the adrenal cortex are not entirely settled, it has been shown that endogenous precursors in mitochondria from rat adrenal are converted to pregnenolone *in vitro* (Koritz, 1962) and that cholesterol is at least one such precursor (Péron and Koritz, 1960; Hall and Koritz, 1964b). The concept that adrenal steroids are derived from cholesterol by way of pregnenolone may be an oversimplification, and the role of pregnenolone as an obligatory intermediate was challenged by a recent report based upon finding in an adrenal tumor (Gual *et al.*, 1962). In addition, evidence for the role of sulfated intermediates in this sequence of reactions has recently been presented (Roberts *et al.*, 1964). However, it is probable that this pathway (mitochondrial cholesterol → pregnenolone → other steroids), if not the only one, is at least a major route to steroid hormones.

A further consequence of the location of the present series of reactions within the mitochondria concerns the removal of the inhibitor (pregnenolone) from the site of the inhibited reaction (the mitochondria). It is clear that removal of pregnenolone from mitochondria will release step [I] from inhibition and that the rate of removal of pregnenolone from mitochondria may be a critical factor in the overall rate of biosynthesis of steroids. These observations are in keeping with the view that the conversion of cholesterol to pregnenolone includes the slow step in steroid biosynthesis. It should also be borne in mind that the concentration of pregnenolone in mitochondria may be considerably greater than the concentration of this steroid in the whole cell.

It becomes apparent, therefore, that any factor which promotes the removal of pregnenolone from the mitochondria will release step [I] from inhibition. One such possible factor would be increased transport of pregnenolone out of mitochondria. In this connection it is interesting to recall the view presented by Stone and Hechter (1954) that ACTH increases the conversion of cholesterol to pregnenolone. A mechanism by which ACTH could stimulate this conversion, namely, by increasing the rate of removal of pregnenolone from mitochondria, is suggested by the present observations.

[4] Unpublished observations by the authors.

References

Brady, R. O. (1951), *J. Biol. Chem. 193*, 145.

Constantopoulos, G., Satoh, P. S., and Tchen, T. T. (1962), *Biochem. Biophys. Res. Commun. 8*, 50.

Constantopoulos, G., and Tchen, T. T. (1961), *J. Biol. Chem. 236*, 65.

Gual, C., Lemus, A. E., Kline, I. T., Gut, M., and Dorfman, R. (1962), *J. Clin. Endocrinol. 22*, 1193.

Halkerston, I. D. K., Eichhorn, J., and Hechter, O. (1961), *J. Biol. Chem. 236*, 374.

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

Hall, P. F., and Koritz, S. B. (1964a), *Biochemistry 3*, 129.

Hall, P. F., and Koritz, S. B. (1964b), *Endocrinology 75*, 135.

Ichii, S., Forchielli, E., and Dorfman, R. I. (1963), *Steroids 2*, 631.

Koritz, S. B. (1962), *Biochim. Biophys. Acta 56*, 63.

Lynn, W. S., Jr., Staple, E., and Gurin, S. (1954), *J. Am. Chem. Soc. 76*, 4048.
Monod, J., Changeux, J., and Jacob, F. (1963), *J. Mol. Biol. 6*, 306.
Oertel, G. W., and Eik-Nes, K. B. (1959), *Anal. Chem. 31*, 98.
Péron, F. G., and Koritz, S. B. (1960), *J. Biol. Chem. 235*, 1625.
Roberts, K. D., Bandi, L., Drucker. W. D., and Lieberman, S. (1964), *J. Am. Chem. Soc. 86*, 958.
Rosenkrantz, H. (1953), *Arch. Biochem. Biophys. 44*, 1.
Samuels, L. T. (1960), *Metab. Pathways 2*, 431.
Shimizu, J., Dorfman, R. I., and Gut, M. (1960), *J. Biol. Chem. 235*, PC25.
Shimizu, J., Hayano, M., Gut, M., and Dorfman, R. I. (1961), *J. Biol. Chem. 236*, 695.
Solomon, S., Levitan, P., and Lieberman, S. (1956), *Rev. Can. Biol. 15*, 282.
Stone, D., and Hechter, O. (1954), *Arch. Biochem. Biophys. 51*, 457.

Fatty Acid Distribution in Bacterial Phospholipids. The Specificity of the Cyclopropane Synthetase Reaction*

JOHN G. HILDEBRAND† AND JOHN H. LAW

From the James Bryant Conant Laboratory, Harvard University, Cambridge, Mass.

Received May 11, 1964

The distribution of fatty acids in purified phospholipids isolated from several different bacteria has been examined by use of the specific phospholipase A of snake venom. In general the distribution is in accord with that found elsewhere in nature; the unsaturated acids are found in the β position and the saturated acids in the γ position. The distribution of cyclopropane fatty acids follows closely that of the unsaturated fatty acids. A notable exception was encountered with the phosphatidylethanolamine of *Clostridium butyricum*, in which unsaturated and cyclopropane fatty acids were found in more abundance in the γ position. The specificity of the *C. butyricum* cyclopropane synthetase reaction has been examined by employing as substrate a phosphatidylethanolamine with a known distribution of unsaturated fatty acids and analyzing the distribution of cyclopropane fatty acids in the phospholipid produced by the enzyme reaction. These experiments indicate that the enzyme has a definite, but not absolute, specificity for an unsaturated fatty acid in the γ position.

Natural phosphoglycerides commonly show a nonrandom distribution of fatty acids such that the γ (or α') position is usually occupied by a saturated fatty acid, while unsaturated fatty acids are usually found in the β position. This fact finds its explanation, at least in part, in the specificity of certain enzymes for catalyzing acylation of lysophosphatides. Thus the studies of Lands (Lands and Merkl, 1963; Merkl and Lands, 1963) with liver enzymes show clearly that the acylating enzymes will discriminate between saturated and unsaturated fatty acid coenzyme A esters in determining which acid will occupy the β or γ position.

Nearly all studies of the distribution of fatty acids in phospholipids have employed the highly specific snake venom phospholipase A. The specificity of this enzyme has been in question during recent years, but because of the elegant work of Van Deenen and De Haas (1963), there is now no doubt that this enzyme cleaves a fatty acid esterified to a hydroxyl group on a carbon adjacent to a carbon bearing a phosphate ester. The steric relationship of this *vic*-glycol system is also important, so that in the natural phosphoglycerides only the β-ester in the L-α-glycerophosphate type would be subject to hydrolysis. The products from a natural phosphoglyceride are a free fatty acid arising from the β-ester and a lysophosphatide which contains the γ-ester. These products may be separated quite easily and examined.

The presence in several species of bacteria of fatty acids containing a cyclopropane ring (O'Leary, 1962), as well as the high proportion of phospholipid in these organisms (see, for example, Kaneshiro and Marr, 1962) lends particular interest to an examination of phospholipids from this source. Furthermore, recent studies of the synthesis of cyclopropane fatty acids in *in vitro* systems (Zalkin *et al.*, 1963; Chung and Law, 1964) have shown that this reaction involves olefinic fatty acids already esterified in intact phospholipids. On the basis of this finding, one could predict that if this reaction is an important one *in vivo* and if there is no subsequent redistribution of fatty acids, the distribution of cyclopropane fatty acids and olefinic fatty acids should be similar. Also, the techniques for examining the distribution of fatty acids enable one to investigate the question of whether the cyclopropane synthetase system *in vitro* has a specificity for an olefinic fatty acid in the β or γ position of the phospholipid substrate.

EXPERIMENTAL

Materials

DEAE-cellulose for column chromatography was obtained from Carl Schleicher and Schuell Co., Keene, N.H., and was washed as suggested by Rouser *et al.* (1961). Silica gel for thin-layer chromatography was obtained from Brinkmann Instruments, Westbury, N.Y., or from Research Specialties Co., Richmond, Calif. These materials occasionally contained volatile impurities which later interfered with gas-chromatographic analysis of the fatty acid esters. This problem was eliminated by extraction of the silica gel with solvents before use, or by use of Adsorbosil-1 (Applied Science Laboratories, Inc., State College, Pa.), which was found to be relatively free of impurities.

Standard fatty acids were purchased from Applied

* Supported by a research grant (GB952) from the National Science Foundation and, in part, by a Public Health Service Research Career Program award (8521).
† Present address: The Rockefeller Institute, New York 21, York, N. Y.