ON STEROID NUCLEOTIDES

II. SYNTHESIS OF Δ5-3β-HYDROXY-STEROID NUCLEOTIDES

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SUMMARY

5-androsten-3β-ol-17-one-ADP, 5-pregnen-3β-ol-20-one-ADP, and 5-pregnene-3β, 17α-diol-20-one-ADP have been prepared from Δ5-3β-hydroxy-steroid-phosphoric acid and the dicyclohexylguanidinium salt of AMP-phosphoramidate. The synthesis of 5-androsten-3β-ol-17-one-ATP was achieved by condensation of 5-androsten-3β-ol-17-one-phosphoric acid and ADP, using dicyclohexylcarbodiimide as condensing agent.

After purification by column chromatography, paper chromatography in two solvent systems and paper electrophoresis, the Δ5-3β-hydroxy-steroid nucleotides were found to be relatively unstable.

INTRODUCTION

The *in vivo* conversion of 5-pregnen-3β-ol-20-one (5-pregnenolone) and 5-pregnene-3β,17α-diol-20-one (17α-hydroxy-5-pregnenolone) to plasma 5-androsten-3β-ol-17-one (dehydroepiandrosterone) in the dog has been previously reported[1]. Approximately 70 % of the total dehydroepiandrosterone formed was conjugated in such a way that it could be liberated by incubation with commercial alkaline phosphatase. Furthermore, the isolation from peripheral human blood plasma of dehydroepiandrosterone conjugates containing phosphoric acid has already been described[2]. These data may reflect the possible existence of hitherto unknown steroid conjugates, such as steroid nucleotides. The preparation of dehydroepiandrosterone nucleotides has been reported in a preliminary communication[3]. The present paper contains a detailed description of the synthesis of dehydroepiandrosterone-ADP and dehydroepiandrosterone-ATP as well as preparation of 5-pregnenolone-ADP and 17α-hydroxy-5-pregnenolone-ADP.

RESULTS AND DISCUSSION

Steroid-ADP compounds were prepared from steroid phosphates and the dicyclohexylguanidinium salt of AMP in dry pyridine in analogy to the synthesis of phenyl-ADP and flavine-adenine dinucleotide[4]. Dehydroepiandrosterone-ATP was obtained

Abbreviations: AMP, adenosine-5'-phosphate; ADP, adenosine-5'-diphosphate; ATP, adenosine-5'-triphosphate.

by condensation of dehydroepiandrosterone-phosphoric acid[5] and ADP in 75% pyridine, employing dicyclohexylcarbodiimide as the condensing agent as in the preparation of amino acid adenylates[6]. The crude reaction products were submitted to a preliminary chromatography on Dowex 2 ion exchange resin (formate form)[7]. The steroid nucleotides were eluted by 0.001 *N* formic acid while the dicyclohexylguanidinium salt of AMP and AMP itself were removed by 0.01 *N* and 0.1 *N* formic acid respectively (Fig. 1). The compounds were purified further by chromatography on paper using the solvent systems *n*-butanol–1 *N* NH_4OH, a modified BUSH system[8] and isobutyric acid–0.1 *M* Versene–1 *N* NH_4OH[9]. The location of $\Delta 5$-3β-hydroxysteroid nucleotides on paper strips was determined by u.v. absorption, and either a color reaction with sulfuric acid–ethanol characteristic for the $\Delta 5$-3β-hydroxy-configuration of steroids[10] or the ZIMMERMANN reaction of 17-ketosteroids[11]. Table I

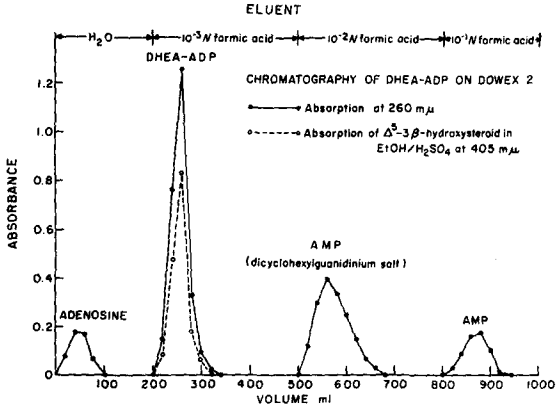

Fig. 1. Column chromatography of 5-androsten-3β-ol-17-one-ADP on Dowex-2 ion exchange resin (formate form).

TABLE I

R_F VALUES OF STEROID NUCLEOTIDES, NUCLEOTIDES AND STARTING MATERIAL IN DIFFERENT SYSTEMS OF PAPER CHROMATOGRAPHY

Compound	*n-butanol–1 N NH_4OH*	*Isobutyric acid–0.1 M Versene–1 N NH_4OH (100:1.6:60 v/v/v)*
Steroid nucleotides		
5-androsten-3β-ol-17-one-ADP	0.40	0.79
5-pregnen-3β-ol-20-one-ADP	0.48	0.81
5-pregnene-3β,17α-diol-20-one-ADP	0.35	0.77
5-androsten-3β-ol-17-one-ATP	0.32	0.76
Starting materials		
5-androsten-3β-ol-17-one-phosphoric acid	0.72	1.00
5-pregnen-3β-ol-20-one-phosphoric acid	0.81	1.00
5-pregnene-3β,17α-diol-20-one-phosphoric acid	0.51	1.00
AMP	0.00	0.63
ADP	0.00	0.46
ATP	0.00	0.28
AMP-phosphoramidate, dicyclohexylguanidinium salt	0.27	0.70

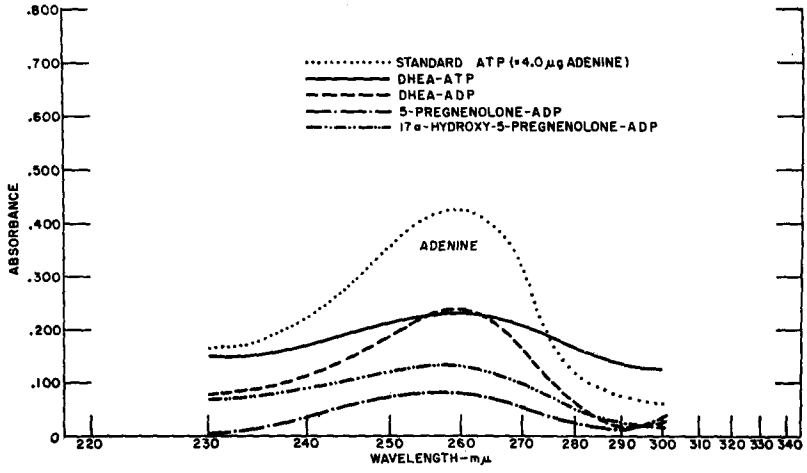

Fig. 2. Analysis of steroid nucleotides eluted from paper: absorption curves of adenine components (Table II).

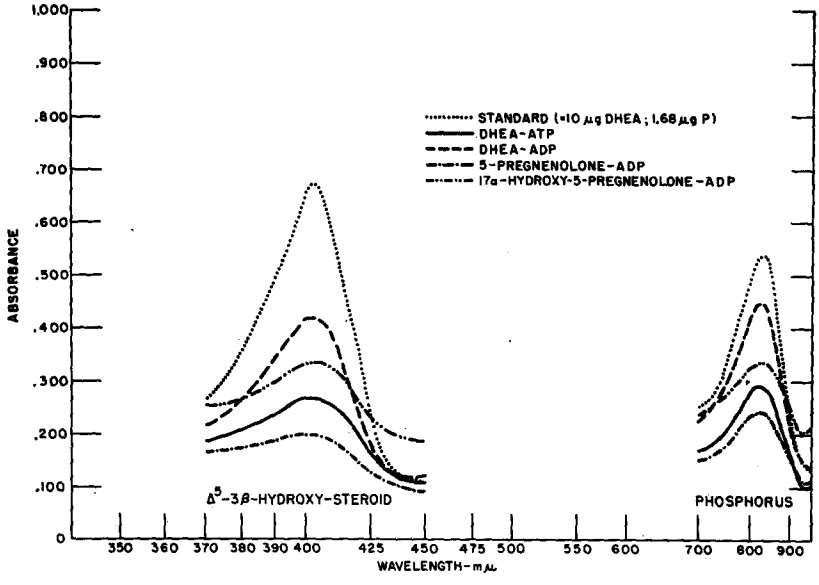

Fig. 3. Analysis of steroid nucleotides eluted from paper: absorption curves of Δ5-3β-hydroxy-steroids (in ethanol–sulfuric acid reagent[10]) and of phosphate (after color reaction with ammonium molybdate[12]) (Table II).

gives the R_F values of the steroid nucleotides as well as of nucleotides and starting material. Aliquots of 3β-hydroxy-steroid nucleotides were analysed for adenine using the u.v. absorption at 260 mμ and submitted to micro-analysis for phosphorus[12] and the sulfuric acid–ethanol reaction for Δ5-3β-hydroxy steroids (Figs. 2 and 3). Results of such experiments are seen in Table II.

After paper electrophoresis in acetate buffer and repeated paper chromatography in the solvent systems already described, the purified Δ5-3β-hydroxy-steroid nucleotides were found to be uniform, although the pure compounds were relatively unstable.

TABLE II

ANALYSIS OF STEROID NUCLEOTIDES ELUTED FROM PAPER (Figs 2 and 3)

Compound	*Adenine* μg	*Phosphoric acid* μg	*$\Delta 5$-3β-hydroxy-steroid* μg	*Molecular ratio*
5-androsten-3β-ol-17-one-ADP	2.6	1.27	5.5	1.00:2.08:0.97
5-pregnen-3β-ol-20-one-ADP	1.1	0.53	2.6	1.00:2.10:1.01
5-pregnene-3β,17α-diol-20-one-ADP	1.1	0.51	2.5	1.00:2.02:0.94
5-androsten-3β-ol-17-one-ATP	1.0	0.68	2.2	1.00:3.09:1.03

TABLE III

ENZYMIC HYDROLYSIS OF 5-PREGNEN-3β-OL-20-ONE-ADP BY PHOSPHODIESTERASE

Substrate μg	*Enzyme*	*Units*	*Found after*			
			Enzymic hydrolysis			*Subsequent acid hydrolysis*
			Steroid μg	*Phosphorus* μg	*Steroid phosphate* μg	*Steroid* μg
34.5	Crude (A)	0.0055	15.0	1.44	18.8 (A_1)	0.0 (A_2)
34.5	—	—	0.0	0.0	0.0	15.2
34.5	Purified (B)	0.0063	5.5	0.69	6.9 (B_1)	7.6 (B_2)
34.5	—	—	0.0	0.0	0.0	15.1
20.5	Crude (A)	0.0055	8.6	Not determined	10.8 (A_1)	0.0 (A_2)
20.5	—	—	0.2	Not determined	0.3	8.5
20.5	Purified (B)	0.0063	3.4	Not determined	4.3 (B_1)	4.9 (B_2)
20.5	—	—	0.4	Not determined	0.5	8.3

Storage at room temperature and chromatography in the alkaline solvent system *n*-butanol–1 *N* NH_4OH resulted in partial decomposition of the steroid nucleotides into steroid phosphates and AMP as identified by paper chromatography.

After incubation of 5-pregnenolone-ADP with crude phosphodiesterase* (approx. $1.5 \cdot 10^{-4}$ units/μg substrate) complete hydrolysis of the steroid nucleotide was observed, whereas only 37 % of steroid substrate was hydrolyzed by a comparable amount of the purified enzyme preparation (approx. $1.8 \cdot 10^{-4}$ units/μg substrate). The degree of hydrolysis was determined by the amount of steroid phosphate formed.

EXPERIMENTAL

5-androsten-3β-ol-17-one-phosphoric acid

The synthesis of this compound has been described in a previous publication[5].

5-pregnen-3β-ol-20-one-phosphoric acid

316 mg (1 mmole) 5-pregnen-3β-ol-20-one were dissolved in 15 ml dry pyridine and 100 ml dry ether. At —15°, 0.084 ml (0.9 mmole) phosphorus oxychloride in 25 ml ether were added over the course of 1 h with continuous stirring. The reaction mixture was kept for 2 h below 0° and subsequently at room temperature for an

* Obtained through the courtesy of Dr. S. A. DICKMAN, University of Utah, Salt Lake City, Utah (U.S.A.).

additional 2 h before 25 ml ice water were added slowly. After 2 h at room temperature, the two phases were separated and the aqueous layer extracted 3 times, each with 100 ml ether. The combined ether extracts were treated once with 50 ml 1 *N* sodium hydroxide. The combined alkaline solutions were acidified with 5 % sulfuric acid. The precipitate, consisting of 5-pregnen-3β-ol-20-one-phosphoric acid was separated by centrifugation and, after the supernatant had been decanted, was washed with 10 ml water. For further purification, the precipitate was dissolved in a minimal volume of 1 *N* sodium hydroxide, the undissolved material removed by filtration and the steroid phosphoric acid precipitated with 5 % sulfuric acid. 5-pregnen-3β-ol-20-one phosphoric acid was recrystallized from aqueous methanol or ethyl acetatehexane. The melting point of the dry material was 142–144°, the yield 163 mg (41 %).

Calculated for $C_{21}H_{33}O_5P.\ H_2O$ (414.47): C, 60.84; H, 8.51; P, 7.47 %. Found: C, 60.53; H, 8.40; P, 7.52 %. Molecular ratio: Δ53-β-hydroxy-steroid: phosphoric acid = 1.00:0.98.

5-pregnene-3β,17α-diol-20-one-phosphoric acid

This was prepared from 332 mg (1 mmole) 5-pregnene-3β,17α-diol-20-one and 0.084 ml. (0.9 mmole) phosphorus oxychloride in the same manner as for 5-pregnen-3β-ol-20-one-phosphoric acid. Recrystallization yielded 150 mg of colorless crystals with m.p. 162–163° (36 % yield).

Calculated for $C_{21}H_{33}O_6P$ (412.45): C, 61.09; H, 8.00; P, 7.51 %. Found: C, 61.31; H, 8.24; P, 7.28 %. Molecular ratio: Δ5-3β-hydroxy-steroid: phosphoric acid = 1.00:0.96.

5-androsten-3β-ol-17-one-ADP

15 mg (0.025 mmole) of the dicyclohexylguanidinium salt of AMP-phosphoramidate and 92 mg (0.25 mmole) of 5-androsten-3β-ol-17-one-phosphoric acid were dried separately for 6 h at 80° *in vacuo* and subsequently dissolved in 25 ml dry pyridine and kept for 3 days at 40° in a sealed tube. After evaporation of the solvent *in vacuo* 25 ml water were added. After acidification the solution was filtered and the filtrate extracted three times with 25 ml ethyl acetate–*n*-butanol (1:1, v/v) in order to remove unreacted steroid phosphoric acid. The aqueous solution was evaporated *in vacuo* and the residue washed 3 times each, with 25 ml absolute ethanol, the supernatant being removed after centrifugation. The residue was dried *in vacuo* and submitted to column chromatography on Dowex-2 ion exchange resin (formate form)[7] as will be described. Recrystallization from methanol–water gave 11 mg (62 %) of colorless crystals. The pure compound did not possess a definite melting point.

Calculated for $C_{29}H_{41}O_{11}N_5P_2$ (697.63): P, 8.88 %. Found: P, 8.65 %. Molecular ratio: adenine:phosphoric acid:Δ5-3β-hydroxy-steroid = 1.00:2.06:0.96.

5-pregnen-3β-ol-20-one-ADP

This was prepared from 15 mg (0.025 mmole) dicyclohexylguanidinium salt of AMP phosphoramidate and 103 mg (0.25 mmole) 5-pregnen-3β-ol-20-one-phosphoric acid. After column chromatography 8.5 mg (48 %) of a colorless powder without definite melting point were obtained.

Calculated for $C_{31}H_{45}O_{11}N_5P_2$ (725.69): P, 8.54 %. Found: P, 8,31 %. Molecular ratio: adenine:phosphoric acid:Δ5-3β-hydroxy-steroid = 1.00:2.03:0.95.

5-pregnene-3β,17α-diol-20-one-ADP

This was prepared from 15 mg (0.025 mmole) AMP phosphoramidate-dicyclohexylguanidinium salt and 103 mg (0.25 mmole) 5-pregnene-3β,17α-diol-20-one-phosphoric acid as described. 9.3 mg of colorless powder without definite melting point were obtained (yield: 49 %).

Calculated for $C_{31}H_{45}O_{12}N_5P_2$ (741.69): P, 8.35 %. Found: P, 8.27 %. Molecular ratio: adenine:phosphoric acid:Δ5-3β-hydroxy-steroid = 1.00:2.04:0.97.

5-androsten-3β-ol-17-one-ATP

85 mg (0.2 mmole) ADP and 72 mg (0.2 mmole) 5-androstene-3β-ol-17-one-phosphoric acid were dissolved in 10 ml 70 % pyridine. At 5° a solution of 1 g dicyclohexylcarbodiimide in 1 ml dry pyridine was added. After 24 h of continuous stirring at 5°, the reaction mixture was kept for an additional 24 h at room temperature. The precipitate was collected by filtration and washed with 5 ml 70 % pyridine, and the filtrate diluted with 50 ml water. This aqueous solution was kept at room temperature for 1 h, filtered and the filtrate evaporated to dryness *in vacuo*. The residue was treated twice with 20 ml acetone and twice with 20 ml absolute ethanol, dried *in vacuo* and purified by column chromatography. 17 mg (11 %) of a colorless powder without definite melting point were obtained.

Calculated for $C_{29}H_{42}O_{14}N_5P_3$ (777.59): P, 11.96. Found: P, 11.64. Molecular ratio: adenine:phosphoric acid:Δ5-3β-hydroxy-steroid = 1.00:3.07:0.93.

All melting points reported were determined under a microscope and are not corrected. All elemental analyses were performed by Elec. Microanalytical Laboratories, Los Angeles, Calif. (U.S.A.).

Column chromatography of steroid nucleotides

The crude material was dissolved in a minimum amount of water and applied to a Dowex-2 ion exchange column (formate form), measuring 5 cm in height and 0.5 cm diameter. 200–300 ml of water, 300 ml of 0.001 *N*, 300 ml of 0.01 *N* and 300 ml of 0.1 *N* formic acid were used as eluents, the volume of the individual sub-fractions being 20 ml and the flow rate approximately 2 ml/min. Each individual fraction eluted from the column was assayed for adenine, employing u.v. absorption at 260 mμ. An aliquot of each fraction containing adenine was evaporated *in vacuo* and the residue submitted to the color reaction with ethanol–sulfuric acid (1:2, v/v) characteristic for Δ5-3β-hydroxy-steroids. As shown in Fig. 1, steroid nucleotides were eluted with 0.001 *N* formic acid. All fractions containing adenine and Δ5-3β-hydroxy-steroids were combined and evaporated to dryness *in vacuo*.

Paper chromatography of steroid nucleotides

Two solvent systems were used for paper chromatography of steroid nucleotides: *n*-butanol–1 *N* NH_4OH[8] and isobutyric acid–0.1 *M* Versene–*N* NH_4OH (100:1.6:60 v/v/v)[9]. After ascending paper chromatography in 50 % methanol[14] essentially as described in a previous publication[14] descending paper chromatography was performed. For paper chromatography in the solvent system *n*-butanol–1 *N* NH_4OH, 6 h equilibration was allowed prior to development of the paper chromatogram with the mobile phase. The chromatograms were run to the front, which required between 18 and 20 h for both systems. The spots containing steroid nucleotides were located

by u.v. absorption and either the ZIMMERMANN reaction indicative of 17-ketosteroids or the ethanol–sulfuric acid reaction. The steroid nucleotides finally were eluted using 50 % methanol and evaporated to dryness *in vacuo*.

Enzymic hydrolysis of 5-pregnen-3β-ol-20-one-ADP

34.5 μg 5-pregnen-3β-ol-20-one-ADP dissolved in 0.1 ml 0.25 *M* Na-succinate–HCl-buffer of pH 6.5 were pipetted into each of four graduated centrifuge tubes. To 2 of the tubes 0.01 ml of a crude phosphodiesterase preparation[13] (= A) containing approx. 5.5 units/ml were added and 0.01 ml of the purified phosphodiesterase preparation (= B) containing 6.3 units/ml to the remaining two tubes. All samples, including controls with buffer and enzyme were diluted to 0.2 ml and incubated at 37° for 1 h. Following the incubation, 10 ml of water was pipetted into each tube. All samples were acidified to pH 3 using 1 *N* sulfuric acid and extracted immediately 3 times with 10 ml ethyl acetate–butanol (1:1, v/v). In order to hydrolyze pregnen-3β-ol-20-one-ADP, not cleaved during incubation, 1 ml concentrated sulfuric acid was added to the aqueous phase of each sample. After 30 min in a boiling water bath all samples were extracted 3 times with 10 ml ethyl acetate. The combined ethyl acetate–butanol extracts (A_1 and B_1) containing 5-pregnen-3β-ol-20-one-phosphate were washed once with 10 ml water and the combined ethyl acetate extracts (A_2 and B_2) containing free 5-pregnen-3β-ol-20-one once with 10 ml 1 *N* sodium hydroxide and twice with 10 ml water. All extracts were evaporated, the residues dissolved in methanol and aliquots assayed for Δ5-3β-hydroxy-steroids, using the control extracts as blanks. Additional aliquots of A_1 and B_1 were analyzed for phosphorus. The remaining material of all extracts was chromatographed on paper: A_1 and B_1 with the appropriate controls in *n*-butanol–1 *N* NH_4OH, A_2 and B_2 with controls in the solvent system propylene glycol–methyl cyclohexane[15] which enabled the isolation of 5-pregnenolone.

Paper electrophoresis

Paper electrophoresis was performed using 0.05 *M* acetate buffer (pH 5.6). At a current of 5 mA for 16 h at 25°, the mobility of the steroid nucleotides to the anode ranged between 7.3 and 10.3 cm. The spots were located by u.v. absorption and the color reactions previously described; these spots were eluted and the eluates assayed for adenine and steroid.

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THE GLOBULIN OF CALF THYMUS NUCLEI AND THE *IN VITRO* INCORPORATION OF [^{14}C]ADENOSINETRIPHOSPHATE INTO GLOBULIN-RNA

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SUMMARY

Investigation of the globulin fraction of isolated thymus nuclei has shown that the globulin contains RNA and is a polydisperse ribonucleoprotein complex. The globulin fraction is able to incorporate [8-^{14}C]ATP into its RNA moiety. Partial fractionation of the globulin reveals that the fractions have different rates of [^{14}C]ATP incorporation.

INTRODUCTION

The ability of isolated cell nuclei to incorporate nucleic acid precursors *in vitro* has been demonstrated by a number of investigators in calf thymus, and in rabbit liver and appendix[1–4]. These precursors have been shown to be incorporated into the nucleic acids and are closely related to protein synthesis[1]. Recently a "pH 5 enzyme" has also been found in isolated nuclei from calf thymus and liver[5,6], and chicken kidney[6] which catalyzes pyrophosphate–ATP exchange in the presence of amino acids, thus being similar to the "pH 5 enzyme" prepared from cytoplasmic supernatant by ZAMECNIK and others[7,8]. These findings, therefore, substantiate the belief that RNA plays an important role in the metabolic processes of the cell nucleus. In the light of these studies, further examination of the relationship between nuclear RNA and nuclear protein fractions would appear to be of interest.

Abbreviations: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; ATP, adenosine triphosphate; AMP, adenosine monophosphate; CTP, cytosine triphosphate; Tris, tris(hydroxymethyl)aminomethane; RNase, ribonuclease.