The Pathways of Corticosteroid Biosynthesis by Homogenates of Adrenal Tissue from Rabbits Stimulated with Adrenocorticotropin*

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ABSTRACT: The mechanism by which adrenocorticotropic hormone stimulates rabbit adrenal tissue to produce cortisol was investigated by following the time course of the metabolism of pregnenolone-4-^{14}C by homogenates of adrenal tissue from adrenocorticotropin-stimulated rabbits. Both on the basis of ^{14}C content and total mass of products formed the sequence of intermediates between pregnenolone and cortisol appeared to be 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, and 11-deoxycortisol, although the data did not permit the definite exclusion of 17α,21-dihydroxypregnenolone as a possible intermediate between 17α-hydroxypregnenolone and 11-deoxycortisol. The rate of progesterone and corticosterone formation exceeded that of 17α-hydroxypregnenolone and cortisol, respectively. Results of incubations in which 1.0 μmole of unlabeled 17α-hydroxypregnenolone or 17α-hydroxyprogesterone was included as traps were consistent with the metabolic pathway from pregnenolone to cortisol indicated by the time-course studies.

The effect of daily injections of rabbits with adrenocorticotropin[1] for 21–28-day periods was originally reported by Kass *et al.* (1954). These investigators described a decrease in the amount of corticosterone[2] and an increase in the amount of cortisol secreted in the adrenal vein blood of the stimulated rabbits. Yudaev and Anfinogenova (1960) and Krum and Glenn (1965) essentially confirmed these results but were unable to obtain cortisol/corticosterone ratios greater than one as originally reported by Kass *et al.* (1954). Yudaev and Morozova (1965) studied *in vitro* corticosteroid biosynthesis by adrenal slices from long-term ACTH-stimulated rabbits and reported that the increase in cortisol biosynthesis observed was due to a partial inhibition of the 21-hydroxylase enzyme activity in the stimulated tissue. According to the authors the effects of this partial inhibition was to make more progesterone available for 17α hydroxylation and subsequent cortisol formation. Recently, I have published results of *in vitro* studies, similar to those reported by Yudaev and Morozova, in which evidence was presented for the preferential use of pregnenolone over progesterone in cortisol biosynthesis (Fevold, 1967). The data suggested that cortisol formation was promoted by the ACTH treatment through an increase in a 17α-hydroxylase activity which had a relative specificity for pregnenolone over progesterone. The present communication adds direct evidence for the existence of a cortisol biosynthetic pathway which bypasses progesterone in ACTH-stimulated rabbit adrenal tissue.

Experimental Section

Tissue Preparation. Male New Zealand white rabbits weighing 2.3–4.2 kg were injected daily for 28 days, or twice daily for 2 days, with 25–40 U of purified porcine ACTH in 0.2 ml of 5% beeswax in peanut oil containing 0.5% phenol. Control animals were injected with beeswax–peanut oil vehicle. Animals under light pentobarbital anesthesia were exsanguinated and the adrenals were removed, trimmed, and weighed. Adrenal glands from three to eight similarly treated rabbits were pooled and homogenized at 0° in sufficient Krebs–Ringer phosphate buffer (pH 7.35) to give a tissue concentration of 50 mg/ml. The buffer also contained 100 mg each of β-D-glucose and glucose 6-phosphate per 100 ml, 40 mM nicotinamide, 0.1 mM sodium fumarate, and 0.4 mM each of NADP, NAD, and ATP.

Incubation Procedure. Pregnenolone-4-^{14}C (Nuclear-Chicago) was chromatographically purified prior to use. The specific activity was adjusted to 1.82 μCi/μmole by the addition of nonradioactive pregnenolone (Sigma), and 0.165 μmole of this material was added to 25-ml incubation flasks in 0.2 ml of ethanol–propylene glycol (1:1, v/v). The ethanol

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[2] The following trivial names are used: corticosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; corticosterone 21-acetate, 11β,21-dihydroxy-4-pregnene-3,20-dione 21-monoacetate; cortisol, 11β,17α,21-trihydroxy-4-pregnene-3,20-dione; cortisol 21-acetate, 11β,17α-21-trihydroxy-4-pregnene-3,20-dione 21-monoacetate; 11-deoxycorticosterone, 21-hydroxy-4-pregnene-3,20-dione; 11-deoxycorticosterone acetate, 3,20-dioxo-4-pregnen-21-ylacetate; 11-deoxycortisol, 17α,21-dihydroxy-4-pregnene-3,20-dione; 11-deoxycortisol 21-acetate, 17α,21-dihydroxy-4-pregnene-3,20-dione 21-monoacetate; 17α,21-dihydroxypregnenolone, 3β,17α,21-trihydroxy-5-pregnen-20-one; 17α-hydroxypregnenolone, 3β,-17α-dihydroxy-5-pregnen-20-one; 17α-hydroxypregnenolone acetate, 3β,17α-dihydroxy-5-pregnen-20-one 3β-monoacetate; 17α-hydroxyprogesterone, 17α-hydroxy-4-pregnene-3,20-dione; 21-hydroxypregenolone, 3β,21-dihydroxy-5-pregnen-20-one; pregnenolone, 3β-hydroxy-5-pregnen-20-one; pregnenolone acetate, 20-oxo-5-pregnen-3β-yl acetate; progesterone, 4-pregnene-3,20-dione.

was evaporated at 40° under nitrogen. Approximately 2 units of glucose 6-phosphate dehydrogenase (Sigma Type VI) was included in each flask with 2 ml of adrenal homogenate. The flasks were gassed with 95% O_2–5% CO_2, stoppered, and placed in a constant-temperature, shaking water bath at 37.5°. All incubations were performed in duplicate and terminated by the addition of 5 ml of dichloromethane–ethyl acetate (1:1, v/v). The flasks' contents were mixed thoroughly and kept at −20° until analyzed.

Extraction and Product Isolation Procedures. Approximately 0.05 μCi each of chromatographically purified ^{3}H-labeled cortisol (82.7 μCi/μg), corticosterone (158 μCi/μg), 11-deoxycorticosterone (110 μCi/μg), 11-deoxycortisol (30 μCi/μg), 17α-hydroxypregnenolone (40 μCi/μg), and 17α-hydroxyprogesterone (30 μCi/μg) were added to each flask to permit the estimation of per cent recoveries of these compounds. The incubation media were extracted four times with double volumes of dichloromethane–ethyl acetate (1:1, v/v). The combined extracts were evaporated and the residue was initially separated into four major fractions by paper chromatography in the heptane–formamide system (Zaffaroni, 1953), developed for 1 hr after the mobile phase had reached the end of the strip, followed by redevelopment in the benzene–formamide system. Each of the four fractions from these initial chromatograms was recovered by elution with 15.0 ml of ethanol. All subsequent chromatographic separations were performed on 2-cm strips of Whatman No. 1 filter paper or on Gelman ITLC-SAF thin-layer sheets. When performed, acetylations were carried out in 0.2 ml of pyridine–acetic anhydride (4:1, v/v).

Each of the four fractions recovered from the initial chromatogram, in order of decreasing polarity, was further purified and separated into its components as follows. Fraction I (cortisol, corticosterone, and 11-deoxycortisol) was chromatographed in the $CHCl_3$–formamide system and two subfractions, IA and IB, corresponding to the cortisol and corticosterone plus 11-deoxycortisol areas, were recovered by elution. Both fractions were acetylated. Fraction IA was again chromatographed in the $CHCl_3$–formamide system; the area corresponding to cortisol 21-acetate was eluted, and the recovered material was used for mass and radioactivity determinations. The acetylated IB fraction was chromatographed in heptane–benzene (1:1, v/v)–formamide for a total of 16 hr after the mobile phase had reached the end of the strip. The areas corresponding to corticosterone 21-acetate and 11-deoxycortisol 21-acetate were eluted and the recovered material was used for mass and radioactivity determinations.

Fraction II (17α-hydroxypregnenolone and 21-hydroxypregnenolone) was acetylated and rechromatographed in cyclohexane–ethyl acetate (3:1, v/v) on Gelman ITLC-SAF. The area corresponding to 17α-hydroxypregnenolone acetate was eluted and the recovered fraction was used for mass and radioactivity determinations and for further identification by recrystallization.

Fraction III (17α-hydroxyprogesterone and 11-deoxycorticosterone) was acetylated and rechromatographed on paper in heptane–benzene (1:1, v/v)–formamide. The areas corresponding to authentic 17α-hydroxyprogesterone and 11-deoxycorticosterone acetate were eluted. The 11-deoxycorticosterone acetate fraction was quantified without further purification. The 17α-hydroxyprogesterone fraction was rechromatographed in benzene–formamide. The ultraviolet absorbance of the eluate from this chromatogram was read against a chromatographic blank. Aliquots of the eluate were subsequently used for gas–liquid partition chromatographic analysis, radioactivity determinations, and recrystallization.

Fraction IV (pregnenolone and progesterone) was rechromatographed on paper in heptane–formamide. The area corresponding to pregnenolone and progesterone was eluted and the residue after evaporation of the solvent was acetylated and rechromatographed in the heptane–formamide system. The areas corresponding to progesterone and pregnenolone acetate were eluted and aliquots were taken for determination of ^{14}C content.

Quantitative Methods. All final paper chromatographic separations were performed on 2-cm strips of Whatman No. 1 filter paper that had been previously washed for 5 days with deionized water and for 5 days with freshly redistilled absolute methanol. Chromatographic fractions were recovered by elution with 15 ml of redistilled absolute ethanol, and one-tenth of each eluted sample was taken in duplicate for the determinations of ^{14}C and ^{3}H content by dual-channel liquid scintillation spectrometry. The mass of each isolated metabolite was determined spectrophotometrically on a second set of duplicate aliquots by the reaction of Porter and Silber (1950) for cortisol 21-acetate and 11-deoxycortisol 21-acetate, the blue tetrazolium reaction (Péron, 1962) for corticosterone 21-acetate and 11-deoxycorticosterone acetate, and the reaction of Oertel and Eik-Nes (1959) for 17α-hydroxypregnenolone.

17α-Hydroxyprogesterone was quantified by determination of the ultraviolet absorbancy at 240 nm and by gas chromatographic analyses of the methoximetrimethylsilyl ether derivatives modified after the method of Van den Heuvel *et al.* (1967). Half of each 17α-hydroxyprogesterone sample was placed in 2-ml glass-stoppered tubes. Cholestane was added to each tube as an internal standard to permit a more accurate estimation of the portion of the sample injected. The dried samples were dissolved in 10 or 25 μl of 0.36 M methoxyamine hydrochloride (K & K Laboratories, Inc.) in pyridine, stoppered, and placed in a heating block at 65° for 30 min. After cooling at room temperature for 1 hr, 10 μl of bis(trimethylsilyl)amine and 5 μl of trimethylchlorosilane (Pierce Chemical Co.) were added to each sample. The samples were again heated at 65° for 30 min and left at room temperature overnight. Gas chromatographic analyses were performed using 1–4 μl of the reaction mixture in an F & M Model 402 gas chromatograph equipped with a flame ionization detector and a 4 ft × 4.0 mm i.d. glass column packed with 0.5% OV-1 on Gas-Chrom P (Applied Science Laboratories, Inc.). Oven, flash heater, and detector temperatures were maintained at 210, 250, and 230°, respectively. Helium was used as carrier gas at a pressure of 40 psi and a flow rate of 45 ml/min. Under these conditions the retention time of the methoximetrimethylsilyl ether derivative of 17α-hydroxyprogesterone relative to cholestane was 1.70.

Calculations. The values of both the per cent conversion of the substrate and the mass of each isolated metabolite were corrected for the per cent recovery as determined by the amount of the appropriate ^{3}H-labeled steroid recovered from the final chromatogram, unless otherwise indicated in the results. Values were also corrected for zero-time controls in which the organic solvent had been added to the flask immediately after homogenate addition. Samples were counted a sufficient time to achieve less than 2% error at a 95% confidence level. Cal-

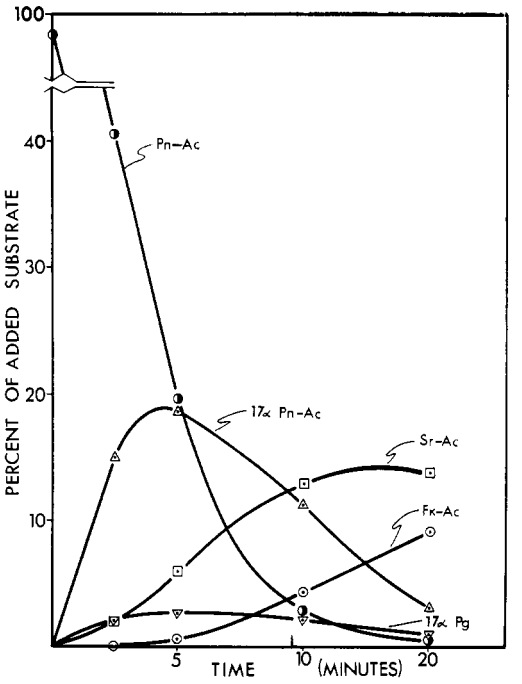

FIGURE 1: Time course of the metabolism of pregnenolone-4-^{14}C to 17α-hydroxy steroids by homogenates of adrenal tissue from rabbits stimulated twice daily for 2 days with 40 U of porcine ACTH. All products with the exception of 17α-hydroxyprogesterone were isolated and quantified as their acetate derivatives. Incubation conditions as described in text. (◐) Pregnenolone acetate (Pn-Ac), (△) 17α-hydroxypregnenolone acetate (17α-Pn-Ac), (⊡) 11-deoxycortisol 21-acetate (S_R-Ac), (⊙) cortisol 21-acetate (F_K-Ac), and (▽) 17α-hydroxyprogesterone (17α-Pg).

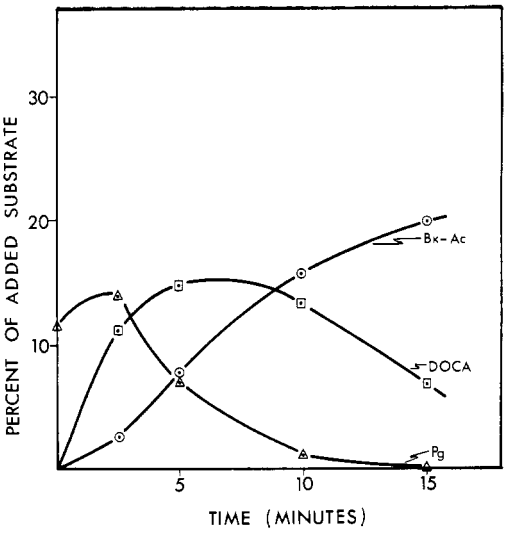

FIGURE 2: Time course of the formation of 17-deoxy steroids from pregnenolone-4-^{14}C by homogenates of adrenal tissue from rabbits stimulated twice daily for 2 days with 40 U of porcine ACTH. All products with the exception of progesterone were isolated and quantified as their acetate derivatives. Incubation conditions as described in text. (△) Progesterone (Pg), (⊡) 11-deoxycorticosterone acetate (DOCA), and (⊙) corticosterone 21-acetate (B_K-Ac).

culation of ^{3}H and ^{14}C content was done by programing the standard equations for dual-labeled samples (Nuclear-Chicago, Liquid Scintillation Manual) for an IBM 1620 data processing system.[3]

Identification Methods. In addition to identification of the isolated metabolites by chromatographic mobilities, pooled samples of 17α-hydroxypregnenolone acetate and 17α-hydroxyprogesterone from identical homogenate incubations were repeatedly crystallized to obtain constant specific activity after the addition of 25 mg of authentic compound. Crystals were weighed on a Cahn electrobalance in aluminum foil cups, and radioactivity was determined by liquid scintillation spectrometry.

After isolation by paper chromatography combined cortisol 21-acetate samples were chromatographed on microsilica gel columns (Sweat, 1954). Infrared analysis of the purified material was performed in micro-KBr pellets on a Beckman IR-4 spectrophotometer.[4]

All solvents used in the analytic procedures were either Spectrograde or were redistilled prior to use.

Results

The formation of ^{14}C-labeled 17α-hydroxylated products and the disappearance of pregnenolone-4-^{14}C substrate in incubations with 100 mg of pooled adrenal homogenate from rabbits injected twice daily for 2 days with 40 U of porcine ACTH are illustrated in Figure 1. Each point in Figures 1–4 represents the average of duplicate incubations. The pregnenolone values are not corrected for percent recovery. It appears that 17α-hydroxypregnenolone is the initial 17α-hydroxylated product formed under these conditions. 17α-Hydroxyprogesterone and 11-deoxycortisol were formed at about the same initial rate subsequent to the formation of 17α-hydroxypregnenolone, but 17α-hydroxyprogesterone did not accumulate to an appreciable extent. This indicates that the 3β-ol-dehydrogenase Δ^5-isomerase step is probably rate limiting in this metabolic sequence between pregnenolone and cortisol, perhaps due to saturation of the enzyme with the exogenous pregnenolone substrate. The isolated ^{14}C-labeled 17α-hydroxypregnenolone acetate and 17α-hydroxyprogesterone were repeatedly crystallized to obtain constant specific activity (Ta-

TABLE 1: Recrystallization of 17α-Hydroxypregnenolone Acetate.

Recrystzn No.	dpm/mg × 10^{-3} Crystals ^{14}C	Crystals ^{3}H	Supernatant ^{14}C	Supernatant ^{3}H	Solvent System
0	3.11	9.98			
1	3.43	11.20	2.59	8.46	EtOAc–heptane
2	3.59	11.70	3.30	10.86	EtOAc–EtOH
3	3.56	11.32	3.48	11.58	EtOH–H_2O
4	3.60	11.32	3.43	11.04	MeOH–H_2O

[3] University of Montana Computer Center.

[4] The infrared spectra were run by Mr. Charles Sloan under the direction of Dr. Graeme Baker, Montana State University.

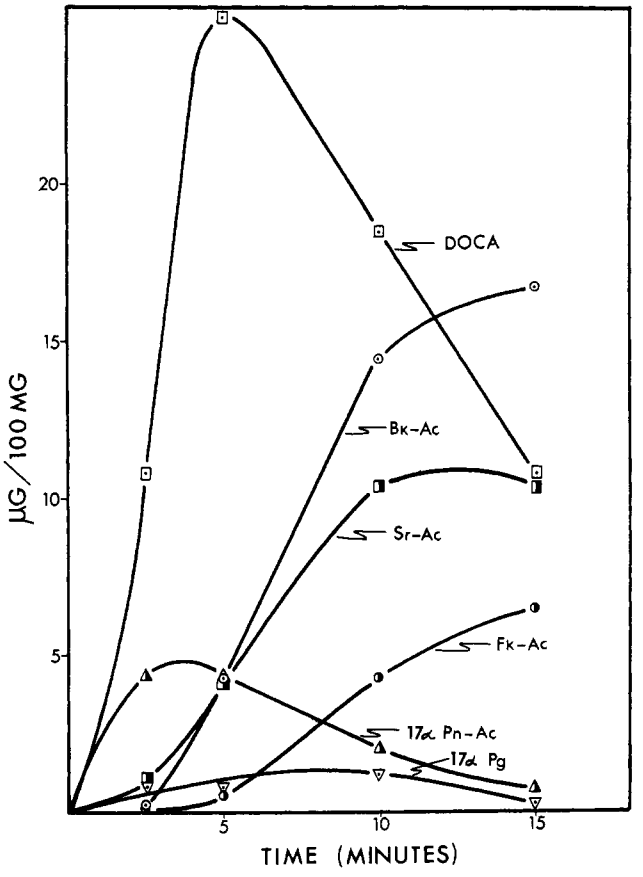

FIGURE 3: Time course of the formation of metabolic products by homogenates of adrenal tissue from rabbits stimulated twice daily for 2 days with 40 U of porcine ACTH. All products with the exception of 17α-hydroxyprogesterone were isolated and quantified as their acetate derivatives. Incubation conditions as described in text. (⊡) 11-Deoxycorticosterone acetate (DOCA), (⊙) corticosterone 21-acetate (B_K-Ac), (Δ) 17α-hydroxypregnenolone acetate (17α-Pn-Ac), (▽) 17α-hydroxyprogesterone (17α-Pg), (◨) 11-deoxycortisol 21-acetate (S_R-Ac), and (◑) cortisol 21-acetate (F_K-Ac).

bles I and II) after pooling corresponding fractions from the 2.5-, 5-, 10-, and 15-min incubations (Figures 1 and 3) and addition of authentic nonlabeled material as the final criterion of the identity of the two metabolites. The cortisol-^{14}C 21-acetate isolated from similar incubations has been previously

TABLE II: Recrystallization of 17α-Hydroxyprogesterone.

Recrystzn No.	dpm/mg × 10^{-2} Crystals ^{14}C	Crystals ^{3}H	Supernatant ^{14}C	Supernatant ^{3}H	Solvent System
0	2.80	93.7			
1	2.14	93.9	4.14	93.7	Acetone–EtOH
2	2.11	92.9	2.74	85.1	Acetone–H_2O
3	2.05	94.1	2.54	92.4	MeOH–H_2O
4	1.92	93.0	1.98	90.2	EtOH H_2O
5	2.20	92.6	2.18	103.1	MeOH–H_2O

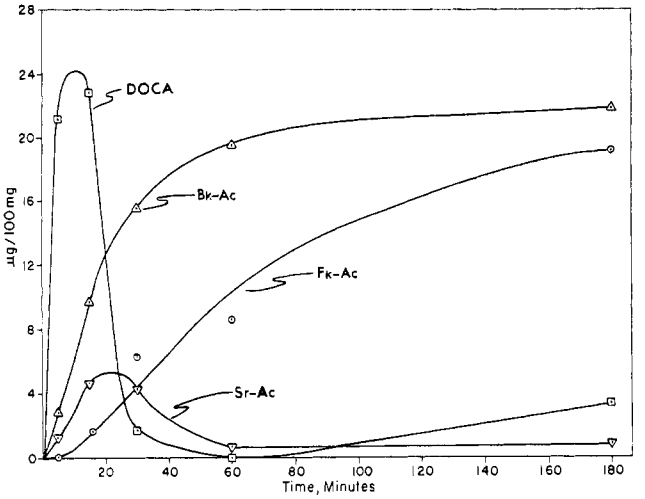

FIGURE 4: Time course of the formation of metabolic products by homogenates of adrenal tissue from rabbits stimulated for 28 days with 25–40 U of porcine ACTH. All products were isolated and quantified as their acetate derivatives. Incubation conditions as described in text. (⊡) 11-Deoxycorticosterone acetate (DOCA), (◬) corticosterone 21-acetate (B_K-Ac), (▽) 11-deoxycortisol 21-acetate (S_R-Ac), and (⊙) cortisol 21-acetate (F_K-Ac).

characterized by repeated crystallization (Fevold, 1968). The course of the metabolism of pregnenolone-4-^{14}C to corticosterone in these same incubations, for purposes of comparison, is illustrated in Figure 2. The progesterone values are not corrected for per cent recoveries or zero-time control values. Subtraction of the latter would have resulted in negative values over much of the curve. However, this was the only metabolic product found in quantities greater than 1% of the added substrate in the zero-time control incubations. These data demonstrate that the pathway of pregnenolone metabolism to corticosterone in this tissue was *via* progesterone and 11-deoxycorticosterone, and that the initial rate of progesterone formation exceeded that of 17α-hydroxypregnenolone formation (Figure 1).

To determine whether the measurement of radioactivity was indicative of the actual quantity of steroids being metabolized in these incubations, spectrophotometric measurements were made of the mass of 11-deoxycorticosterone, corticosterone, 17α-hydroxypregnenolone, 11-deoxycortisol, and cortisol as their acetate derivatives. Gas–liquid partition chromatographic analyses of the methoximetrimethylsilyl ether derivative of 17α-hydroxyprogesterone gave values somewhat smaller than those obtained from the ultraviolet absorption readings, presumably because of the greater specificity of the gas–liquid partition analyses. The gas–liquid partition analytical values were used in plotting the curve for 17α-hydroxyprogesterone in Figure 3. These data agree with the previous data (Figures 1 and 2) obtained from analysis of ^{14}C content of the metabolic products isolated from these incubations and confirm the sequence of metabolic reactions. They also suggest that the contribution of endogenous precursors to the formation of the products measured is minimal.

Longer term kinetic data (Figure 4) obtained using tissue from animals injected with 40 U of ACTH daily for 28 days extends the data in Figure 3 and shows typical precursor–product relationships between 11-deoxycortisol and cortisol

and between 11-deoxycorticosterone and corticosterone, indicating that 21 hydroxylation and the 3β-ol-dehydrogenase Δ^5-isomerase reactions precede 11β hydroxylation in this tissue. The data also illustrate that the final cortisol concentration can approach that of corticosterone under these conditions, although the rate of cortisol formation is considerably less. Measurement of ^{14}C incorporation into the metabolic products showed essentially the same relationships as illustrated in Figure 4. The cortisol 21-acetate fractions isolated from these incubations were pooled, purified by column chromatography on silica gel, and the infrared spectrum was determined. The spectra of the isolated material and authentic cortisol 21-acetate are shown in Figure 5 and substantiate the identity of the two materials.

Table III illustrates results obtained in incubations in which 1.0-μmole traps of either nonlabeled 17α-hydroxyprogesterone or 17α-hydroxypregnenolone were included with the 82.5 μM (0.165 μmole in 2.0 ml) pregnenolone-4-^{14}C substrate. Homogenate equivalent to 100 mg of adrenal tissue from rabbits stimulated twice daily for 2 days with 40 U of ACTH was used in these experiments. All values are averages of duplicate incubations. The data corroborate the kinetic data in that 1.0 μmole of either 17α-hydroxyprogesterone or 17α-hydroxypregnenolone did not prevent the metabolism of pregnenolone-^{14}C to ^{14}C-labeled 17α-hydroxypregnenolone in 5-min incubation periods, but the 17α-hydroxypregnenolone trap did inhibit further transformation of the 4-^{14}C-labeled 17α-hydroxypregnenolone to 17α-hydroxyprogesterone and 11-deoxycortisol in 15-min incubations. The 17α-hydroxyprogesterone trap caused larger amounts of both ^{14}C-labeled 17α-hydroxypregnenolone and 17α-hydroxyprogesterone to remain after 15-min incubations when compared with control incubations without the nonlabeled traps present, while impairing the further transformation of the labeled intermediates to 11-deoxycortisol. These data are consistent with the pathway of pregnenolone metabolism to 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, and 11-deoxycortisol in that order.

Discussion

Previous data from our laboratory which compared the efficacy of pregnenolone-4-^{14}C and progesterone-4-^{14}C as precursors for cortisol synthesis by ACTH-stimulated rabbit adrenal tissue (Fevold, 1967) suggested that the 17α-hydroxylase utilized pregnenolone preferentially. Several groups have reported evidence for the existence of a pathway of cortisol biosynthesis involving 17α hydroxylation of pregnenolone in adrenal tissue of human (Lipsett and Hökfelt, 1961; Mulrow and Cohn, 1961; Mulrow *et al.*, 1962; Weliky and Engel, 1962, 1963; Klein and Giroud, 1967; Cameron *et al.*, 1968; Cameron and Griffiths, 1968; Whitehouse and Vinson, 1968), monkey (Lantos *et al.*, 1968), pig (Yudaev and Pankov, 1964), and beef (Ewald *et al.*, 1965) origin. Most of these conclusions have been based on either the relative extent of the conversion of exogenous pregnenolone and progesterone into 17α-hydroxylated products, or on the ability of the tissue to form and/or utilize 17α-hydroxypregnenolone in the formation of cortisol. If only the amount of the exogenous labeled precursor converted into a specific product is followed in such experiments, the problem of pool size and intracellular compartmentation is ignored; and if both mass and radioactivity of the products

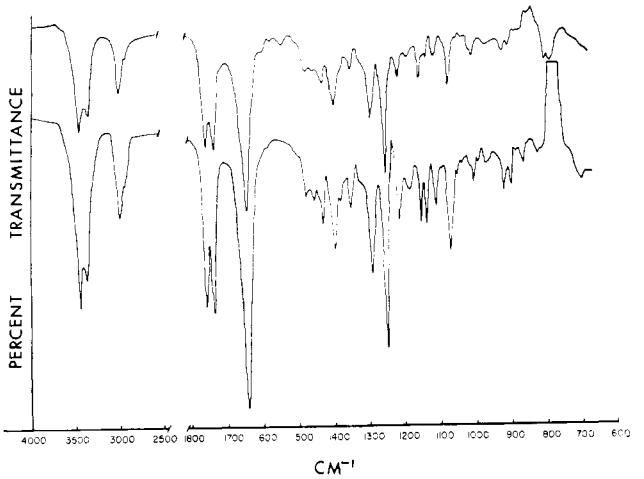

FIGURE 5: Infrared spectra of authentic cortisol 21-acetate (lower curve) and the acetate derivative of material isolated from incubations of homogenates of adrenal tissue from ACTH-stimulated rabbits (upper curve).

are measured, it is not possible to be sure that the two exogenous substrates whose efficiency as precursors are being compared are equally accessible to the enzymes involved. In a few of the previous investigations with human adrenal tissue (Cameron and Griffiths, 1968; Whitehouse and Vinson, 1968), kinetic data have been presented to more definitively illustrate the sequence of biosynthetic reactions between tracer amounts of pregnenolone and cortisol. The authors assumed that the metabolism of the exogenous tracer reflected endogenous metabolism. Our data show that a similar pathway exists in ACTH-stimulated rabbit tissue (Figures 1 and 4) and that this is a quantitatively significant pathway in terms of the total amount of cortisol formed under these experimental conditions. In human adrenal tissue (Weliky and Engel, 1963; Klein and Giroud, 1967) there appears to be a relative lack of a 3β-ol-dehydrogenase Δ^5-isomerase enzyme complex to convert pregnenolone into progesterone, based on the observations that little exogenous pregnenolone was converted into progesterone and 17-deoxycorticosteroids, while progesterone

TABLE III: Effect of Nonlabeled Intermediate Traps on Pregnenolone-4-^{14}C Metabolism by ACTH-Stimulated Rabbit Adrenal Homogenates.

	% Substrate					
	5 min			15 min		
Trap[b]	17α-Pn[a]	17α-Pg	S_R	17α-Pn	17α-Pg	S_R
None	29.0	2.6	6.0	5.8	1.1	13.8
17α-Pn	29.7	1.2	1.7	21.5	0.5	3.2
17α-Pg	30.2	2.2	0.9	36.9	15.3	2.4

[a] 17α-Pn = 17α-hydroxypregnenolone; 17α-Pg = 17α-hydroxyprogesterone; S_R = 11-deoxycortisol. [b] Traps were 1.0 μmole added in 0.05 ml of propyleneglycol.

was efficiently converted into 17-dexoycorticosteroids and smaller amounts of 17-hydroxycorticosteroids. In ACTH-stimulated rabbit tissue, however, there appears to be no deficiency in the conversion of pregnenolone into progesterone, as the latter is the most rapidly formed initial product of pregnenolone-4-^{14}C metabolism (Figure 2). This difference could be due to the fact that the unstimulated rabbit tissue normally synthesizes predominantly 17-deoxycorticosteroids from pregnenolone (Fevold, 1967). The relative amounts of 17-deoxy- and 17-hydroxycorticosteroids formed in the ACTH-stimulated rabbit tissue, and in others with similar pathways of pregnenolone metabolism, would be principally determined under these conditions by the results of a competition for pregnenolone substrate between a 17α-hydroxylase and a 3β-ol-dehydrogenase enzyme system. The fact that the stimulated rabbit adrenals can secrete more cortisol than corticosterone (Kass *et al.*, 1954) and that the ratio of cortisol to corticosterone production increases from 0.26 to 0.75 as the pregnenolone substrate concentration is decreased from 660 to 82.5 μM in *in vitro* incubations (Fevold, 1967) suggests that the products of pregnenolone metabolism are determined by the kinetic properties of the enzymes competing for the pregnenolone substrate, rather than by the amounts of the enzymes present. Whether this is also true in human tissue cannot be determined until *in vitro* studies are performed using varied substrate concentrations.

It is not clear from the results of the present studies (Figures 3 and 4) or from studies with human tissue (Pasqualini, 1964; Whitehouse and Vinson, 1968) whether the 17α-hydroxypregnenolone is metabolized solely to 17α-hydroxyprogesterone, or if 21 hydroxylation precedes 3β-ol dehydrogenation and Δ^5 isomerization. In the present studies and in those of Whitehouse and Vinson (1968), the amount of 17α-hydroxyprogesterone isolated was too small to permit exclusion of 17α,21-dihydroxypregnenolone as a possible intermediate between 17α-hydroxypregnenolone and 11-deoxycortisol, while Pasqualini *et al.* (1964) demonstrated the conversion of 17α,21-dihydroxypregnenolone into cortisol. However, in our investigations no significant quantities of a metabolic product with chromatographic mobilities similar to 17α,21-dihydroxypregnenolone were observed, although it was not specifically analyzed for in the separation scheme. The data of Cameron and Griffiths (1968) are more definitive on this point, showing a rapid increase in 17α-hydroxyprogesterone formation immediately following 17α-hydroxypregnenolone in experiments with human tissue.

The results of the experiments in which nonlabeled 17α-hydroxyprogesterone or 17α-hydroxypregnenolone were included as traps (Table III) suggest that 17α-hydroxyprogesterone is the primary product formed from 17α-hydroxypregnenolone, since the amount of ^{14}C label in the 17α-hydroxypregnenolone remained higher for a longer period of time when nonlabeled 17α-hydroxyprogesterone was present than in control incubations without it. The relatively large amount of nonlabeled 17α-hydroxyprogesterone was apparently inhibiting by mass action the transformation of 17α-hydroxypregnenolone-^{14}C to 17α-hydroxyprogesterone. This evidence is, however, merely indicative, as it is also possible that 1.0 μmole of 17α-hydroxyprogesterone might competitively inhibit the possible 21 hydroxylation of 17α-hydroxypregnenolone. It is evident from the longer term kinetic studies that 11-deoxycortisol is an immediate precursor of cortisol (Figure 4), although judging from the relative amounts of each product, it is possible that not all the cortisol was formed from 11-deoxycortisol. In the ACTH-stimulated rabbit adrenal tissue, then, cortisol synthesis most likely follows a reaction sequence from pregnenolone to 17α-hydroxypregnenolone, 17α-hydroxyprogesterone, 11-deoxycortisol, and cortisol, with the possibility that 21 hydroxylation may precede the 3β-ol-dehydrogenase Δ^5-isomerase step.

The mechanism by which ACTH effects the stimulation of the synthesis of cortisol is not completely clear. It is apparent, however, that the appearance or increase in the 17α-hydroxylase which utilizes pregnenolone as substrate can explain the previous observations (Kass *et al.*, 1954; Krum and Glenn, 1965; Yudaev and Anfinogenova, 1960; Fevold, 1967) that the dramatic increase in cortisol secretion or biosynthesis is accompanied by a decrease in corticosterone production. Since the present data (Figures 1 and 2) indicate that progesterone is not an important intermediate in cortisol biosynthesis, they do not support the hypothesis of Yudaev and Morozova (1965) that the increased cortisol production by ACTH-stimulated rabbit adrenal tissue was due to a partial inhibition of the 21-hydroxylase, making more progesterone available for 17α hydroxylation. The molecular mechanism by which the 17α-hydroxylase activity is stimulated by ACTH is of considerable interest, since it does not seem to be readily explained by the current hypotheses concerning the mechanism of ACTH action (Halkerston, 1968).

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The Effect of Guanidinium, Carbamoylguanidinium, and Guanylguanidinium Salts on the Solubility of Benzoyl-L-tyrosine Ethyl Ester and Acetyltetraglycine Ethyl Ester in Water*

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ABSTRACT: The effects of the chloride, bromide, iodide, and thiocyanate salts of guanidinium, carbamoylguanidinium, and guanylguanidinium cations on the water solubility of benzoyl-L-tyrosine ethyl ester, a model hydrophobic compound, and acetyltetraglycine ethyl ester, a model peptide and amide compound, have been examined. Regardless of the cation used, the solubility of both model compounds increases progressively through the series chloride < bromide < iodide < thiocyanate. This anion series parallels the effectiveness of these anions as denaturants of several proteins. When the anion is held constant, the cation effect on the solubility of benzoyl-L-tyrosine ethyl ester is guanidinium < guanylguanidinium < carbamoylguanidinium and the cation effect on the solubility of acetyltetraglycine ethyl ester is guanidinium < carbamoylguanidinium < guanylguanidinium.

It is concluded that the protein denaturing effectiveness of these guanidinium, carbamoylguanidinium, and guanylguanidinium salts is due to their ability to increase the solubility of protein hydrophobic and peptide amide groups.

The denaturing effectiveness of salts of guanidinium, carbamoylguanidinium, and guanylguanidinium cations toward rabbit muscle aldolase, ovalbumin, and bovine serum albumin increases through the series: $Cl^- < Br^- < I^- < CNS^-$ (Castellino and Barker, 1968a). This anion series parallels that obtained by Hofmeister (1888) for a large number of ionic phenomena including precipitation of proteins. When combined with neutral inorganic salts, essentially the same anion series exists for denaturation of collagen (Bello *et al.*, 1962; von Hippel and Wong, 1962), ribonuclease (von Hippel and Wong, 1964), DNA (Hamaguchi and Geiduschek, 1962), and fumarase (Massey, 1953). When the anion is held constant the cation effect on the denaturation of rabbit muscle aldolase, ovalbumin, and bovine serum albumin increases through the series guanidinium < carbamoylguanidinium < guanylguanidinium (Castellino and Barker, 1968a).

This report deals with the effect of the chloride, bromide, iodide, and thiocyanate salts of guanidine, carbamoylguanidine, and guanylguanidine on the solubility of models for the hydrophobic and amide groups which are exposed to the solvent in the denatured state of a protein. A model hydrophobic compound (BTEE, I)[1] and a model peptide (ATGEE, II) were examined and the effectiveness of the salts in solubilizing the compounds was compared with the effect of urea.

Materials and Methods

BTEE was purchased from Cyclo Chemical Co. and used without further purification.

Acetyltetraglycine Ethyl Ester-^{14}C. Tetraglycine ethyl ester was prepared according to the method of Fischer (1904) and twice recrystallized from ethanol. Acetylation was performed by dissolving 1.5 g of tetraglycine ethyl ester in 150 ml of 40% aqueous pyridine and slowly adding a total of 2.5 ml of acetic anhydride-1-^{14}C (New England Nuclear Corp.) at 4°. Crystals appeared upon concentration in a rotary evaporator under water-aspirator pressure. The product was recrystallized from water to constant specific activity, and a final yield of 60% was obtained. The product melted at 267° with decomposition, in good agreement with the mp of 264° reported for this compound by Robinson and Jencks (1965a). The specific activity of the ATGEE-^{14}C was 22,215 cpm/mg at approximately 70%

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[1] Abbreviations used are: BTEE, benzoyltyrosine ethyl ester; ATGEE, acetyltetraglycine ethyl ester; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene.