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INDICATIONS FOR THE EXISTENCE OF ALTERNATIVE PATHWAYS OF STEROID SYNTHESIS VIA 21-HYDROXYPREGNENOLONE IN THE RAT ADRENAL CORTEX

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SUMMARY

After incubation of rat adrenals with [^{3}H]-acetate, specific radioactivity of cholesterol, pregnenolone and progesterone was lower than that of 11-desoxycorticosterone and corticosterone. ACTH decreased radioactivity incorporation into and specific radioactivity of cholesterol, while it increased incorporation into 11-desoxycorticosterone and corticosterone; specific radioactivity of 11-desoxycorticosterone increased, that of corticosterone remained constant. Specific radioactivity of 21-hydroxypregnenolone also decreased under the influence of ACTH, but it was higher than that of 11-desoxycorticosterone and corticosterone under all conditions. Thus, [^{3}H]-21-hydroxypregnenolone qualified as precursor of [^{3}H]-11-desoxycorticosterone and [^{3}H]-corticosterone.

Specific radioactivity of cholesterol from mitochondria was in the same order of magnitude as that of cholesterol from the remaining tissue fraction.

When the step "desmosterol—cholesterol" was inhibited by Triparanol®, radioactivity incorporation into cholesterol was blocked almost completely. However, specific radioactivity of 21-hydroxypregnenolone remained unchanged, and that of 11-desoxycorticosterone and corticosterone decreased only slightly.

Even if only a small, but highly labelled cholesterol pool were assumed to serve as steroid precursor, it appears most unlikely from these data that [^{3}H]-21-hydroxypregnenolone, -11-desoxycorticosterone and -corticosterone derived from [^{3}H]-cholesterol, -pregnenolone and -progesterone. From present results and data in the literature we suggested that the synthesis sequence "acetate—21-hydroxydesmosterol—21-hydroxypregnenolone—11-desoxycorticosterone—corticosterone" might explain discrepant specific radioactivity results, but would contribute only little to the mass of corticosteroids. From the decrease in 21-hydroxypregnenolone specific radioactivity under the influence of ACTH we suggested that the sequence "cholesterol—pregnenolone—21-hydroxypregnenolone—11-desoxycorticosterone—corticosterone", which would be of quantitative importance, might become operative during synthesis stimulation.

INTRODUCTION

A number of authors observed that the specific radioactivity of cholesterol was lower than that of the corticosteroids after perfusion [1, 2] or incubation [3–6] of adrenal tissue with radioactively labelled acetate. Two hypotheses are being discussed to explain these findings, which do not seem to fit into the generally accepted concept of cholesterol to be the central intermediate in steroid biosynthesis:

1. The "pool hypothesis": Only a very small, but highly labelled fraction of adrenal cortex cholesterol is metabolically active, i.e. synthesized from acetate and metabolized to steroids.

2. The "alternative pathway hypothesis": Corticosteroids are synthesized from acetate via (an) alternative pathway(s), in which cholesterol is not an intermediate.

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In previous reports [7, 8], we showed that ACTH caused a larger decrease in the specific radioactivity of corticosterone than in that of cholesterol and/or pregnenolone and/or progesterone, when rat adrenal quarters were incubated with either [4-^{14}C]-cholesterol, -pregnenolone or -progesterone. We concluded from these data that more endogenous precursor was incorporated into corticosterone during synthesis stimulation than was metabolized via the sequence "cholesterol—pregnenolone—progesterone". In the present communication, we wish to report on results from incubations of rat adrenals with [^{3}H]-acetate in the presence and absence of ACTH. 21-Hydroxypregnenolone, the only alternative among C21-steroids to progesterone as direct precursor of 11-desoxycorticosterone, was isolated in order to compare its specific radioactivity with that of cholesterol, pregnenolone, progesterone, 11-desoxycorticosterone and corticosterone. Cholesterol was isolated separately from mitochondria and from the remaining tissue fraction to reveal possible differences in specific radioactivity, which should be expected, if the pool hypoth-

esis were valid. Inhibiting the step "desmosterol—cholesterol" by Triparanol®, we examined the question of radioactivity incorporation into 21-hydroxypregnenolone, 11-desoxycorticosterone and corticosterone under conditions excluding cholesterol as intermediate.

EXPERIMENTAL

Instruments

"Dubnoff Metabolic Shaking Incubator": Will Scientific Inc. Homogenizer "Ultra-Turrax": Janke & Kunkel KG. Gaschromatograph "Aerograph 2100" with flame ionization detector: Varian GmbH. Radiochromatogram scanner Mod. 7201 and "Tri Carb" liquid scintillation spectrometer Mod. 3380/AAA 544: Packard Instrument Co.

Chemicals

Cholesterol (cholest-5-en-3β-ol): Merck AG. Pregnenolone (3β-hydroxy-5-pregnen-20-one): Calbiochem. Pregnenolone acetate (pregn-5-en-3β-acetoxy-20-one): Ikapharm. Progesterone (4-pregnen-3,20-dione): Calbiochem. 11-Desoxycorticosterone, DOC (21-hydroxy-4-pregnen-3, 20-dione): Mann Research. DOC acetate (4-pregnen-21-acetoxy-3, 20-dione): Merck AG. 21-Hydroxypregnenolone, 21-OH-pregnenolone (3β, 21-dihydroxy-5-pregnen-20-one): Ikapharm. 21-OH-Pregnenolone acetate (3β-21-dihydroxy-5-pregnen-20-one diacetate): Prepared by acetylation of 21-OH-pregnenolone and recrystallization from methanol. Corticosterone (11β, 21-dihydroxy-4-pregnene-3, 20-dione): Merck AG. Corticosterone acetate (11β, 21-dihydroxy-4-pregnene-3, 20-dione-21-acetate): Merck AG. Triparanol® (1-(*p*-diethylaminoethoxyphenyl)-1-(*p*-tolyl)-2-(*p*-chlorophenyl)-ethanol): Merrel Pharma. Adrenocorticotrophic hormone, ACTH, Cortrophine®: Organon GmbH. Other chemicals and solvents: Merck AG; all solvents were redistilled over 100 cm Raschig columns before use. Silica-gel t.l.c plates, Polygram SIL G/UV$_{254}$®: Macherey–Nagel & Co. Gas chromatography column filling, 3% OV 1 on 60–80 mesh Chromosorb W®: Varian GmbH.

[4-^{14}C]-Cholesterol (58 mCi/mmol), [4-^{14}C]-pregnenolone (52, 8 mCi/mmol), [4-^{14}C]-progesterone (52, 8 mCi/mmol), [4-^{14}C]-DOC (54, 3 mCi/mmol), [4-^{14}C]-corticosterone (59, 3 mCi/mmol): New England Nuclear Corp.; all [^{14}C]-labelled compounds were examined for radiochemical homogeneity by chromatography in the systems used for isolation and by dilution with authentic material and recrystallization before they were used as internal standards. [^{3}H]-Acetic acid sodium salt (5, 2 Ci/mmol): Amersham Buchler & Co. PPO (2, 5-diphenyloxazole) and POPOP (1,2-di-(2-(5-phenyl)-oxazolyl)-benzene): Merck AG; scintillation cocktail: 4 g PPO and 50 mg POPOP in 1000 ml toluene.

Animals

Male Sprague–Dawley rats weighing 180–200 g. In one experiment male rats of 400–500 g were used.

Methods

1. *Tissue preparation and incubation.* Adrenals were extirpated under ether anesthesia and kept in ice-cold saline. After removal of fat and connective tissue, the glands were cut to quarters, or—after enucleation—to halves, and distributed statistically into preweighed 50-ml beakers containing 10 ml ice-cold saline. After weighing the beakers to determine "tissue weight", saline was replaced by 10 ml Krebs–Ringer bicarbonate glucose buffer pH 7.4 (KRBG) [9], and samples were preincubated for 60 min at 37° by gentle shaking under 95% O_2 and 5% CO_2. Preincubation media were discarded. Tissue was washed twice with 5 ml saline and transferred into fresh beakers containing 5 or 10 ml KRBG with 25 mCi [^{3}H]-acetate and the additions stated (ACTH, Triparanol). Triparanol had to be dissolved in diluted aqueous HCl. When this solution was added to KRBG, pH dropped to 7.25. Control samples without and with ACTH were adjusted to this pH. Main incubation was performed for 120 min under the conditions used for preincubation.

2. *Extraction and isolation of steroids.* After main incubation, samples were transferred into 100 ml centrifuge tubes containing 5 ml chloroform and [^{14}C]-labelled internal standards of the compounds to be isolated (with exception of [^{14}C]-21-OH-pregnenolone, which was not available). Beakers were carefully rinsed with chloroform, rinsings added to the samples. Amounts of internal standards were chosen not to exceed 1% of the samples steroid content, and for [^{14}C]-radioactivity to be within 5–30% of [^{3}H]-radioactivity after final purification. Samples were homogenized using a Ultra-Turrax® homogenizer and extracted with 3 times 10 ml chloroform by stirring. A water blank was carried throughout the procedure to guarantee the absence of radioactive contaminations and artifacts (gas chromatography) in final measurements. Combined chloroform fractions were dried over Na_2SO_4, filtered and evaporated. A liquid–liquid distribution followed between *n*-heptane and 90% methanol.

In two experiments internal standards [^{14}C]-pregnenolone, -progesterone, -DOC and -corticosterone were added to the samples after main incubation. Tissue was separated from the medium and rinsed 4 times with 4 ml 0.25 M sucrose; rinsings were added to the medium. The medium was extracted and distributed as described. Tissue was homogenized at 0°C in 6 ml 0.25 M sucrose for 2 min using a Potter–Elvehjem homogenizer and centrifuged for 25 min at 0°C and 800 ***g***. The supernatant was centrifuged for 20 min at 0°C and 20,000 ***g***, the pellet resuspended twice with 5 ml 0.25 M sucrose and recentrifuged. The final 20,000 ***g*** pellet contained the mitochondrial fraction. It was extracted after addition of internal

Table 1. Thin layer chromatographic systems and relative migration rates (R_F = relative to the solvent front; R_P = relative to progesterone)

	t.l.c.-I toluene: methanol = 92:8	t.l.c.-II *n*-heptane: benzene: ethyl acetate = 1:1:4	t.l.c.-III toluene: ethyl acetate: methanol = 15:3:2	t.l.c.-IV ethyl acetate: benzene: *n*-heptane = 1:1:1
R_F-progesterone	0.54	0.63	0.60	0.38
R_P-cholesterol	0.90	1.20	—	—
R_P-pregnenolone	0.65	0.95	—	—
R_P-DOC	0.65	0.70	—	—
R_P-21-OH-pregnenolone	0.42	—	—	—
R_P-18-OH-DOC	0.24	—	0.54	—
R_P-corticosterone	0.28	—	0.64	—
R_P-pregnenolone acetate	—	—	—	2.20
R_P-DOC acetate	—	1.00	—	—
R_P-21-OH-pregnenolone diacetate	—	—	—	2.00
R_P-corticosterone acetate	—	0.45	—	—

standard [^{14}C]-cholesterol, and the extract was subjected to liquid–liquid distribution. The 800 *g* pellet was combined with all 20,000 *g* supernatants to form the "tissue fraction". It was extracted after addition of internal standard [^{14}C]-cholesterol, and the extract distributed. *n*-Heptane phases of mitochondrial and tissue fractions were analyzed separately for cholesterol. The combined 90% methanol phases from medium, mitochondria and tissue fraction were analyzed for corticosteroids.

Cholesterol and corticosteroids were isolated in a succession of thin layer chromatographies on silica gel. Compositions of the t.l.c. systems and relative migration rates of the compounds are summarized in Table 1. Figure 1 shows the isolation scheme.

3. *Quantitative analysis.* Cholesterol, pregnenolone (as pregnenolone acetate), progesterone, 21-OH-pregnenolone (as 21-OH-pregnenolone diacetate), DOC (as DOC acetate) and corticosterone (as corticosterone acetate) were analyzed quantitatively by gas chromatography with flame ionization detector. 3% OV 1 on 60–80 mesh Chromosorb W was used in U-shaped glass columns of 40–180 cm length and 3 mm width. Carrier gas was N_2. Flame gas for the FID was H_2 and compressed air. Column temperatures were 240–260° for cholesterol, 21-OH-pregnenolone diacetate and corticosterone acetate, 200–220° for pregnenolone acetate, progesterone and DOC acetate. Uniform and symmetrical peaks resulted for all compounds. Calibration curves of at least 10 measuring points were linear (r: 0.985–0.999) within the analytical ranges. The limit of sensitivity was about

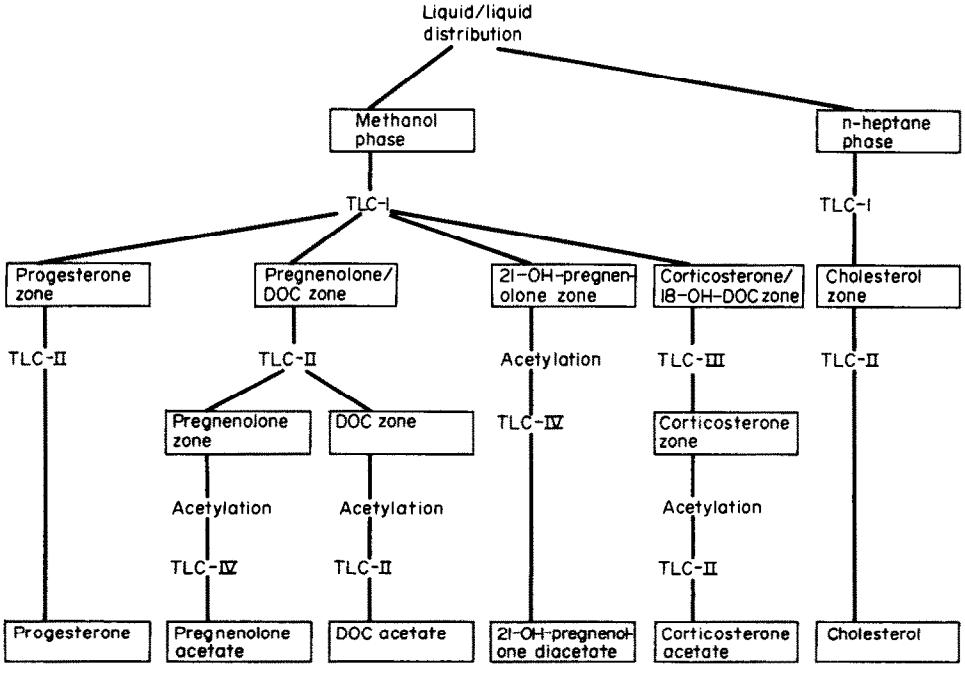

Fig. 1. Scheme for the isolation of cholesterol and corticosteroids from extracts of incubation samples.

10 pmol. Multiple injections of 2–4 μl were made of each compound dissolved in ethyl acetate resulting in precisions of 1–5% (SEM). [^{14}C]-Radioactivity was measured in at least 5 aliquots of each gas chromatography sample to estimate recoveries, and results were corrected for procedural losses.

4. *Radioactivity analysis.* In order to guarantee radiochemical homogeneity of [^{3}H]-radioactivity incorporated into the compounds isolated they were recrystallized from methanol–H_2O after addition of 20–50 mg of the corresponding authentic compound until constant ^{3}H: ^{14}C ratios or specific radioactivities (21-OH-pregnenolone) were obtained. Cholesterol was treated like the steroids in the ACTH dose response experiment. In all other experiments, cholesterol was brominated and debrominated prior to recrystallization by the method of Schwenk and Werthessen[10] to remove small amounts of highly labelled sterols ("high counting companions").* With exception of those of 21-OH-pregnenolone resulting [^{3}H]-radioactivities were corrected by the corresponding [^{14}C]-radioactivities recovered.

RESULTS

1. *Dose dependent effects of ACTH*

Adrenal quarters of 80 rats were incubated in 4 samples containing 0, 0.05, 0.5 and 2.5 I.U. ACTH/ml medium, respectively.

* Data resulting from each bromination/debromination and/or recrystallization step have been presented to the editor and referees.

Results of quantitative measurements and radioactivity analyses are summarized in Table 2. While the mass of cholesterol remained constant, radioactivity incorporation into cholesterol decreased with increasing ACTH concentrations. For [^{3}H]-pregnenolone and [^{3}H]-progesterone radiochemical homogeneity was not obtained due to sensitivity limits in radioactivity measurement. The mass of pregnenolone increased slightly under the influence of ACTH, that of progesterone increased continuously up to 2.7 fold at 2.5 I.U. ACTH/ml. Radioactivity incorporation from [^{3}H]-acetate into 21-OH-pregnenolone was proven by recrystallization to constant specific radioactivity, and corresponding masses could be measured quantitatively. [^{3}H]-Radioactivity incorporation into 11-desoxycorticosterone (DOC) was stimulated up to 11.1 fold, while the mass of DOC increased only up to 3.5 fold. In corticosterone relative stimulations of [^{3}H]-radioactivity and mass were in the same order of magnitude (6.2 and 5.7 fold, respectively, at 2.5 I.U. ACTH/ml).

Resulting specific radioactivities are shown in Fig. 2. In the control sample without ACTH specific radioactivity of DOC and corticosterone was about 12 times higher than that of cholesterol. While specific radioactivity of DOC increased with increasing ACTH concentrations (3 fold at 0.5 I.U. ACTH/ml), and that of corticosterone remained relatively constant, specific radioactivity of cholesterol decreased markedly (0.13 fold at 2.5 I.U. ACTH/ml). At the highest ACTH concentration, specific radioactivity of DOC was 302 times, that of corticosterone 103 times higher than that of cholesterol. Specific radioactivities

Table 2. Incubation of rat adrenal quarters with [^{3}H]-acetate (25 mCi/sample) in the absence of ACTH and in the presence of 0.05, 0.5 and 2.5 I.U. ACTH/ml medium

	ACTH 0	ACTH 0.05 I.U./ml	ACTH 0.5 I.U./ml	ACTH 2.5 I.U./ml
Cholesterol				
d.p.m. ^{3}H/100 mg	70,769	50,292	15,614	9737
nmol/100 mg	1187	1262	1332	1247
Pregnenolone (pregnenolone acetate)				
d.p.m. ^{3}H/100 mg	ϕ	ϕ	ϕ	ϕ
nmol/100 mg	0.221	0.326	0.331	0.319
Progesterone				
d.p.m. ^{3}H/100 mg	ϕ	ϕ	ϕ	ϕ
nmol/100 mg	0.569	0.686	1.071	1.539
21-OH-Pregnenolone (21-OH-pregnenolone diacetate)				
d.p.m. ^{3}H/100 mg	1201†	1737†	614†	455†
nmol/100 mg	0.0329†	0.0637†	0.1470†	0.0787†
11-Desoxycorticosterone (DOC acetate)				
d.p.m. ^{3}H/100 mg	1268	3643	9229	14,098
nmol/100 mg	1.706	3.437	3.870	5.971
Corticosterone (corticosterone acetate)				
d.p.m. ^{3}H/100 mg	11,563	30,485	56,365	71,940
nmol/100 mg	15.62	35.24	64.33	89.65

† Without correction of procedural losses.

Tissue weight/sample: Control 669 mg; 0.05 I.U. ACTH/ml: 626 mg; 0.5 I.U. ACTH/ml: 604 mg; 2,5 I.U. ACTH/ml: 602 mg. Radioactivity incorporation: d.p.m. ^{3}H/100 mg (tissue). Quantitative results: nmol/100 mg (tissue).

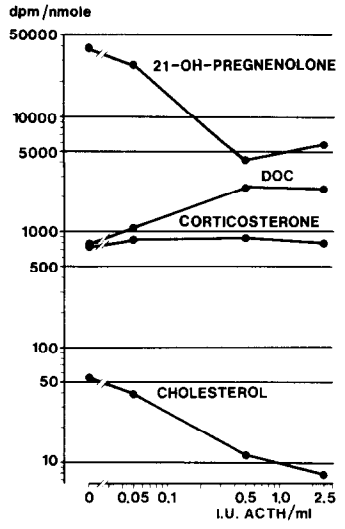

Fig. 2. Specific radioactivities after incubation of rat adrenal quarters with [^{3}H]-acetate and increasing concentrations of ACTH.

of pregnenolone and progesterone, as assessed from masses and sensitivity limits of radioactivity measurement, were also much lower than those of DOC and corticosterone. In contrast, specific radioactivity of 21-OH-pregnenolone was 49 times higher than that of DOC and corticosterone in the control sample. Under the influence of ACTH it decreased similarly to that of cholesterol (0.16 fold at 0.5 I.U. ACTH/ml), but remained higher than that of DOC under all conditions.

2. *Examination of the pool hypothesis*

Enzymes catalyzing cholesterol side chain cleavage are localized in the mitochondrion [11]. Therefore, the small pool of highly labelled cholesterol postulated in the pool hypothesis should be found within or closely connected to mitochondria. In 2 experiments, in which adrenals of 80 and 60 rats were incubated in a control sample each and a sample containing ACTH (6.4 and 7.1 I.U. ACTH/ml), cholesterol was isolated separately from mitochondria and from the remaining tissue fraction. Halves of adrenal capsules were used to eliminate interference by medullary cholesterol.

Results of quantitative and radioactivity analyses are summarized in Tables 3 (experiment 1) and 4 (experiment 2). In experiment 1 mitochondria contained less cholesterol than the tissue fraction. ACTH did not change the mass of mitochondrial cholesterol, but decreased that of cholesterol from the tissue fraction. In experiment 2 cholesterol masses were in the same order of magnitude in both fractions with no changes by ACTH. [^{3}H]-Radioactivity incorporation into cholesterol was unusually high in experiment 2. It exceeded that in experiment 1 by factor 8.3 in the tissue fraction and by factor 29.5 in mitochondria. Under the influence of ACTH tissue fraction [^{3}H]-cholesterol decreased by factor 0.15 in both experiments. Mitochondrial [^{3}H]-cholesterol decreased 0.18 fold in experiment 1 and 0.35 fold in experiment 2. These differences between both experiments may be due to the fact that in experiment 1 rats of 180–200 g, but in experiment 2 rats of 400–500 g body weight

Table 3. Incubation of adrenal capsule halves from rats weighing 180–200 g with [^{3}H]-acetate (25 mCi/sample) under control conditions (677 mg tissue) and in the presence of ACTH (6.4 I.U./ml medium) (539 mg tissue)

	Cholesterol			
	Control		ACTH	
	tissue fraction	mitochondria	tissue fraction	mitochondria
d.p.m. ^{3}H/100 mg	74,740	11,690	11,020	2151
nmol/100 g	2465	529.7	1323	560.2
	Pregnenolone (pregnenolone acetate)		Progesterone	
	control	ACTH	control	ACTH
d.p.m. ^{3}H/100 mg	ϕ	ϕ	ϕ	ϕ
nmol/100 mg	0.125	0.572	0.418	1.148
	DOC (DOC acetate)		Corticosterone (corticosterone acetate)	
	control	ACTH	control	ACTH
d.p.m. ^{3}H/100 mg	2870	113,300	57,148	411,169
nmol/100 mg	1.347	22.04	18.75	113.3
	21-OH-Pregnenolone (21-OH-pregnenolone diacetate)			
	control	ACTH		
d.p.m. ^{3}H/100 mg	2397†	6663†		
nmol/100 mg	0.0270†	0.327†		

† Without correction of procedural losses.

Cholesterol was isolated separately from mitochondria and from the remaining tissue fraction. Radioactivity incorporation: d.p.m. ^{3}H/100 mg (tissue). Quantitative results: nmol/100 mg (tissue).

Table 4. Incubation of adrenal capsule halves from rats weighing 400–500 g with [^{3}H]-acetate (25 mCi/sample) under control conditions (381 mg tissue) and in the presence of ACTH (7.1 I.U./ml medium) (350 mg tissue)

	Cholesterol			
	Control		ACTH	
	tissue fraction	mitochondria	tissue fraction	mitochondria
d.p.m. ^{3}H/100 mg	617,697	345,340	90,230	121,910
nmol/100 mg	1743	1234	1584	1498
	Progesterone		21-OH-Pregnenolone (21-OH-pregnenolone diacetate)	
	control	ACTH	control	ACTH
d.p.m. ^{3}H/100 mg	ϕ	ϕ	3369†	5440†
nmol/100 mg	0.286	0.555	—	0.248†
	DOC (DOC acetate)		Corticosterone (corticosterone acetate)	
	control	ACTH	control	ACTH
d.p.m. ^{3}H/100 mg	6456	33,880	109,500	262,410
nmol/100 mg	1.828	6.177	27.67	82.53

† Without correction of procedural losses.

Cholesterol was isolated separately from mitochondria and from the remaining tissue fraction. Radioactivity incorporation: d.p.m. ^{3}H/100 mg (tissue). Quantitative results: nmol/100 mg (tissue).

were used. The data suggest that in older animals adrenocortical cholesterol synthesis from acetate is increased with increased accumulation of mitochondrial cholesterol. In spite of the differences in cholesterol, differences between both experiments were relatively small in the absolute values of steroids. ACTH was less effective in experiment 2. For pregnenolone (isolated in experiment 1) and progesterone (isolated in both experiments) again constant ^{3}H:^{14}C ratios could not be achieved. ACTH increased the mass of pregnenolone 4.6 fold, that of progesterone 2.7 and 1.9 fold. [^{3}H]-Radioactivity incorporation into DOC increased by factors 39 and 5.2, the mass of DOC by factors 16 and 3.4 under the influence of ACTH. Corticosterone showed comparable stimulations in [^{3}H]-radioactivity (7.2 and 2.4 fold) and mass (6.0 and 3.0 fold). Radioactivity incorporation from [^{3}H]-acetate into 21-OH-pregnenolone could be proven in both samples of each experiment, but we failed to measure the mass of this compound in the control sample of experiment 2.

Resulting specific radioactivities are shown in Fig. 3. The specific radioactivity of a fraction named "sterols" was calculated from mass and [^{3}H]-radioactivity of cholesterol before elimination of the "high counting companions" [10] by bromination, debromination and recrystallization. In experiment 1 the specific radioactivity of sterols from the tissue fraction was 5.3 (control) and 6.6 (ACTH) times higher than that of mitochondrial sterols. Specific radioactivity of tissue fraction cholesterol (30 d.p.m./nmol) was slightly higher than that of mitochondrial cholesterol (22 d.p.m./nmol). ACTH decreased the specific radioactivity of cholesterol from both fractions by factors 0.27 (tissue fraction) and 0.17 (mitochondria). Probably due to the excessive purification of cholesterol differences in specific radioactivity between cholesterol and the corticosteroids were even larger than those found in the ACTH dose response experiment. Specific radioactivity of cholesterol was exceeded by that of DOC 97 fold and by that of corticosterone 138 fold in the control sample, when cholesterol from mitochondria is compared. Under the influence of ACTH corticosteroids were 1 340 (DOC) and 945 (corticosterone) times higher in specific radioactivity

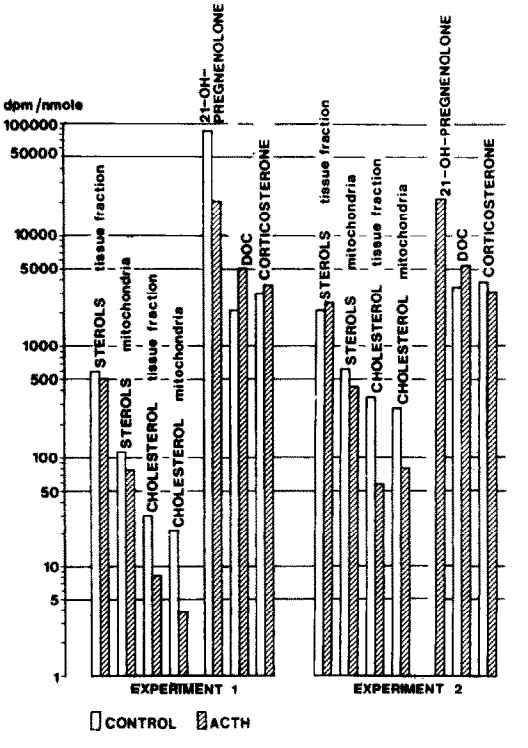

Fig. 3. Specific radioactivities after incubation of rat adrenal capsule halves with [^{3}H]-acetate under control conditions and in the presence of ACTH. Experiment 1: Rats of 180–200 g; experiment 2: Rats of 400–500 g.

Table 5. Incubation of rat adrenal quarters with [^{3}H]-acetate (25 mCi/sample) under control conditions (624 mg tissue), in the presence of ACTH (8,9 I.U./ml medium) (606 mg tissue), in the presence of Triparanol® (0,5 mM) (585 mg tissue), and in the presence of both ACTH and Triparanol (637 mg tissue)

	Control	ACTH	Triparanol	Triparanol/ACTH
Cholesterol				
d.p.m. ^{3}H/100 mg	55,280	19,500	3788	2075
nmol/100 mg	1173	1024	992.7	897.7
Pregnenolone (pregnenolone acetate)				
d.p.m. ^{3}H/100 mg	ϕ	ϕ	ϕ	ϕ
nmol/100 mg	1.286	0.616	0.583	0.389
21-OH-pregnenolone (21-OH-pregnenolone diacetate)				
d.p.m. ^{3}H/100 mg	4305†	3397†	3441†	2238†
nmol/100 mg	0.118†	0.150†	0.0749†	0.0911†
11-Desoxycorticosterone (DOC acetate)				
d.p.m. ^{3}H/100 mg	3119	11,930	2510	3551
nmol/100 mg	3.009	4.822	1.919	2.496
Corticosterone (corticosterone acetate)				
d.p.m. ^{3}H/100 mg	30,838	99,691	22,045	37,589
nmol/100 mg	10.29	31.42	9.769	23.55

† Without correction of procedural losses.
Radioactivity incorporation: dpm ^{3}H/100 mg (tissue). Quantitative results: nmole/100 mg (tissue).

than mitochrondrial cholesterol. Calculations from masses and sensitivity limits of radioactivity measurements proved again that not only the specific radioactivity of cholesterol, but also that of pregnenolone and progesterone was much lower than that of DOC and corticosterone. According with results from the ACTH dose response experiment specific radioactivity of 21-OH-pregnenolone was higher than that of DOC (control: 42 times; ACTH: 4.0 times) and corticosterone (control: 29 times; ACTH: 5.6 times). Aside from the fact that specific radioactivities of sterols and cholesterol were markedly higher and ACTH was less effective in experiment 2, results of experiment 1 were confirmed by those of experiment 2.

3. *Inhibition of the step "desmosterol—cholesterol"*

Triparanol has been shown to inhibit the reduction of the 24-en double bond of sterols [12, 13] and, thus, the transformation of desmosterol to cholesterol [14, 15]. In order to examine radioactivity incorporation from [^{3}H]-acetate into corticosteroids adrenals of 60 rats were incubated in 4 samples, one of which served as control, one contained ACTH (8.9 I.U./ml), one Triparanol (0.2 mg/ml), and one ACTH and Triparanol.

Quantitative results and results of radioactivity analyses are summarized in Table 5. The mass of cholesterol remained relatively constant under all conditions. ACTH caused a 0.35 fold decrease in [^{3}H]-cholesterol. Under the influence of Triparanol [^{3}H]-radioactivity incorporation into cholesterol was reduced by factor 0.07, and when ACTH was added together with Triparanol, incorporation further decreased by factor 0.5. Again, for pregnenolone constant ^{3}H:^{14}C ratios were not obtained. [^{3}H]-Radioactivity incorporation into 21-OH-pregnenolone was proven under all conditions, and masses of the compound could be measured. As in preceeding experiments, ACTH caused larger increase in radioactivity (3.8 fold) than in mass (1.6 fold) of DOC. [^{3}H]-Radioactivity (0.8 fold) and mass (0.6 fold) of DOC were reduced only slightly by Triparanol. Additional ACTH stimulated [^{3}H]-radioactivity incorporation into DOC by factor 1.4 and the mass of DOC by

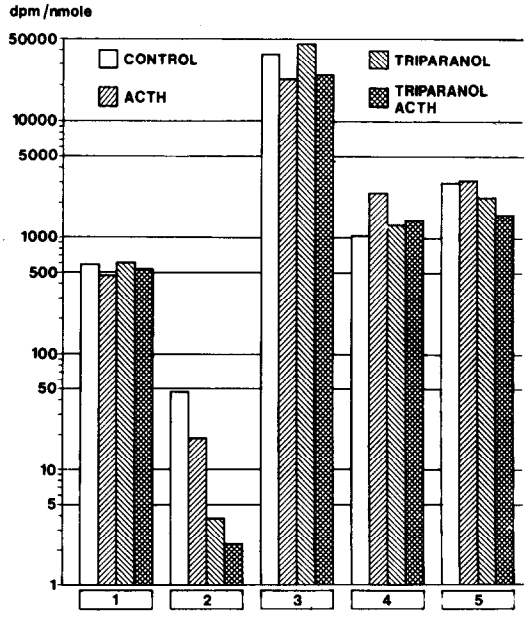

Fig. 4. Specific radioactivities after incubation of rat adrenal quarters with [^{3}H]-acetate under control conditions, in the presence of ACTH, in the presence of Triparanol®, and in the presence of both Triparanol and ACTH. 1: Sterols; 2: Cholesterol; 3: 21-OH-Pregnenolone; 4: DOC; 5: Corticosterone.

factor 1.3. In Corticosterone ACTH stimulated [^{3}H]-radioactivity (3.2 fold) and mass (3.1 fold) to the same extent as observed in previous experiments. Triparanol caused a slight reduction of [^{3}H]-radioactivity by factor 0.7 and left the mass of corticosterone unchanged. In the presence of Triparanol ACTH stimulated [^{3}H]-radioactivity 1.7 fold and mass 2.4 fold.

Resulting specific radioactivities are shown in Fig. 4. That of sterols remained in the same order of magnitude under all conditions. The pattern of specific radioactivities resulting from the control and the ACTH sample confirms results of the preceeding experiments. Again, specific radioactivity of cholesterol and pregnenolone (assessed) was far below, and that of 21-OH-pregnenolone was markedly above that of DOC and corticosterone. Under the influence of Triparanol specific radioactivity of cholesterol decreased by factor 0.08 with further decrease by factor 0.6 under concomittant action of ACTH. In contrast, specific radioactivity of 21-OH-pregnenolone was not changed by Triparanol. In DOC and corticosterone Triparanol alone had also very little effect on specific radioactivities, but it caused slight reductions by factors 0.6 (DOC) and 0.5 (corticosterone) in the sample stimulated by ACTH.

DISCUSSION

Present results, which show that the specific radioactivity of cholesterol was lower than that of DOC and corticosterone after incubation of rat adrenal tissue with [^{3}H]-acetate, and that ACTH decreased radioactivity incorporation into cholesterol while it stimulated incorporation into DOC and corticosterone, confirm corresponding observations reported by a number of investigators [1–6, 16]. In addition, this study revealed that not only specific radioactivity of cholesterol but also that of pregnenolone and pregesterone was lower than that of DOC and corticosterone. These findings agree with results of previous experiments with rat adrenal quarters and precursors [4-^{14}C]-cholesterol, -pregnenolone or -progesterone [7, 8]. Therefore, if the pool hypothesis were accepted to explain discrepancies in specific radioactivities, the existence of a small, metabolically active and highly labelled pool must be assumed not only for cholesterol but also for pregnenolone and progesterone. The alternative pathway hypothesis would have to be extended, too. Pregnenolone and progesterone in addition to cholesterol must be considered to be bypassed in the synthesis of DOC and corticosterone from acetate.

We could prove that radioactivity from [^{3}H]-acetate was incorporated into 21-OH-pregnenolone, the only alternative among C21-steroids to progesterone as direct precursor of DOC. Its specific radioactivity was higher than that of DOC and corticosterone under all conditions. Therefore, [^{3}H]-21-OH-pregnenolone might well have been the precursor of [^{3}H]-DOC and [^{3}H]-corticosterone in our experiments. Transformation of 21-OH-pregnenolone to DOC by homogenized bovine adrenals has been shown by Berliner *et al.*[17]. Pasqualini *et al.*[18] demonstrated transformation of 21-OH-pregnenolone to DOC and corticosterone by slices of hyperplastic human adrenals and radioactivity incorporation from [^{3}H]-21-OH-pregnenolone into DOC and corticosterone by slices of rat and rabbit adrenals. From which precursor did the highly labelled [^{3}H]-21-OH-pregnenolone derive in our experiments? Pasqualini *et al.*[18] demonstrated that rat and rabbit adrenals are able to synthesize 21-OH-pregnenolone from pregnenolone *in vitro*, and the same has been shown for fetal and adult sheep adrenals by Vinson[19]. If, in the present study, [^{3}H]-21-OH-pregnenolone derived from cholesterol via pregnenolone, the specific radioactivity of assumed fractions of metabolically active cholesterol and pregnenolone must have been at least in the same order of magnitude as that of 21-OH-pregnenolone, i.e. up to 5000 times higher than that of total cholesterol. If such a highly labelled cholesterol pool existed, it should be found within or closely connected to mitochondria, since the mitochondrion is the morphological site of cholesterol side chain cleavage [11]. Therefore, after incubation of adrenocortical tissue with radioactively labelled acetate, markedly higher specific radioactivity should be expected for mitochondrial cholesterol than for cholesterol from the remaining tissue fraction. Our experiments did not prove this assumption. The specific radioactivity of mitochondrial cholesterol was in the same order of magnitude or even lower than that of cholesterol from the tissue fraction. The specific radioactivity of total sterols, i.e. of cholesterol before elimination of small amounts of highly labelled sterols [10], was definitely lower in mitochondria than in the tissue fraction. This is not surprising, since the enzymes transforming squalene to cholesterol are localized in the "microsomal" fraction of the cell [20, 21, 22]. Although these data cannot disprove the pool hypothesis, they certainly do not support it. Our results rather suggest that newly synthesized [^{3}H]-cholesterol was randomly distributed from cytoplasma into all cellular cholesterol fractions. In one experiment, in which older rats were used, radioactivity incorporation into and specific radioactivity of cholesterol was unusually high (Table 4, Fig. 3). In spite of obviously increased cholesterol synthesis from acetate and accumulation of cholesterol in mitochondria the specific radioactivities of 21-OH-pregnenolone, DOC and corticosterone were in the same order of magnitude as those obtained in a preceding experiment with normal cholesterol synthesis (Table 3, Fig. 3). This, again, does not favour the suggestion that [^{3}H]-cholesterol served as the major precursor of [^{3}H]-21-OH-pregnenolone, -DOC and -corticosterone. Discrepancies between our results and those of Ichii and Kobayashi[23], who found slightly higher specific radioactivity in mitochondrial "cholesterol"

than in "cholesterol" from nuclei and supernatant, may be due to procedural differences.

More conclusive answers to the question "pool or alternative pathway hypothesis?" may be found in results from the experiment with Triparanol (Table 5, Fig. 4). Triparanol reduced radioactivity incorporation into cholesterol to 6.9% and specific radioactivity of cholesterol to 8.0% of corresponding control values. The specific radioactivity of sterols remained unchanged indicating that sterol synthesis from acetate was not influenced by Triparanol. This is in good agreement with the suggested effect of this compound, specific inhibition of the step "desmosterol—cholesterol" [12–15]. Although radioactivity incorporation into cholesterol was inhibited almost completely, there were only slight reductions of incorporation into DOC and corticosterone, and specific radioactivities of 21-OH-pregnenolone, DOC and corticosterone remained in the order of magnitude of corresponding control values. These data confirm results reported by Hall[24], who found incorporation of [^{14}C]-acetate into testosterone by testis slices of rabbits pretreated with Triparanol, although the synthesis of [^{14}C]-cholesterol was blocked. Therefore, it appears most unlikely that, in our experiments, cholesterol was an important intermediate in the synthesis of [^{3}H]-21-OH-pregnenolone, -DOC and -corticosterone. Since we have found that Elipten® (2-(*p*-aminophenyl)-2-ethyl glutaric imide), an inhibitor of cholesterol side chain cleavage [25], completely inhibited the incorporation of [1-^{14}C]-acetate into corticosteroids by rat adrenal quarters [26], a sterol that can serve as substrate to the side chain cleaving enzymes must be considered to be the precursor of 21-OH-pregnenolone.

The cholesterol side chain cleavage system is known to be of limited substrate specificity as far as the structure of the side chain is concerned [27–32]. However, it seems to be relatively specific for the 3β-ol-5-en structure of the sterol nucleus [28, 38]. Therefore, of known sterols desmosterol would qualify as steroid precursor. Desmosterol has been shown to be a possible intermediate in corticosteroid biosynthesis [14, 15, 34]. Since a sequence "desmosterol—pregnenolone—21-OH-pregnenolone" must be excluded because of the low specific radioactivity of pregnenolone in our experiments, we tend to the view that 21-hydroxylation may occur prior to side chain cleavage, and, thus, 21-hydroxydesmosterol may be the precursor of 21-OH-pregnenolone. It has been shown recently that a 21-hydroxylated sterol can be substrate to the side chain cleavage system by Burstein *et al.*[35], who demonstrated the conversion of (20$\bar{S}$)-20,21-dihydroxycholesterol to 21-OH-pregnenolone by bovine adrenocortical mitochondrial preparations. The assumption of an alternative pathway with the sequence "21-OH-desmosterol—21-OH-pregnenolone—DOC—corticosterone"could easily explain the fact that the specific radioactivity of cholesterol, pregnenolone and progesterone was lower than that of DOC and corticosterone after incubation of rat adrenals with [^{3}H]-acetate.

Marked increases of radioactivity incorporation into DOC and corticosterone under the influence of ACTH (Tables 2–5) allow the conclusion that the synthesis of [^{3}H]-DOC and -corticosterone via the proposed alternative pathway was stimulated by ACTH. It appears logical to suggest that the effect of ACTH within the alternative pathway is comparable to that within the cholesterol pathway, i.e. stimulation of sterol side chain cleavage. Increased transformation of sterols to 21-OH-pregnenolone reduces the amount of sterol precursors to be transformed to cholesterol and, thus, explains the decrease in radioactivity incorporation into and in specific radioactivity of cholesterol under the influence of ACTH. An effect of ACTH prior to sterol side chain cleavage, which might favour the synthesis of sterols and steroids but decrease cholesterol formation, cannot be excluded. Morris and Gorski[36] proposed corresponding actions for LH in rabbit ovary cells, when they found decreased labelling from [^{14}C]-acetate of 7-dehydrocholesterol and cholesterol but increased labelling of other sterols.

The decrease in specific radioactivity of 21-OH-pregnenolone during synthesis stimulation by ACTH (Figs 2–4) suggests isotope dilution by increased incorporation of low specific radioactivity precursors into 21-OH-pregnenolone. The large pool of lowly labelled adrenocortical cholesterol may be accepted as source of lowly labelled 21-OH-pregnenolone. Increased transformation of cholesterol to pregnenolone under the influence of ACTH may subsequently increase the transformation of pregnenolone to 21-OH-pregnenolone [18, 19].

Figure 5 combines conclusions from present results and from literature data in a suggested succession of biosynthetic steps. Dotted arrows indicate a sequence through which most of the radioactivity might have been incorporated from [^{3}H]-acetate. Solid arrows represent the synthesis pathways for lowly labelled DOC and corticosterone. The scheme proposed provides a number of advantages: 1. It explains the discrepancies observed in specific radioactivities in a rather logical way. 2. It involves only enzyme systems that are known to be operative in adrenocortical steroid synthesis. 3. There is no need to postulate small, metabolically active pools of cholesterol, pregnenolone and progesterone, which are hard to define morphologically and functionally. 4. It does not contradict the fact that *in vivo* up to 90% of the corticosteroids derive from blood cholesterol in the rat [37].

In order to gain approximative values on the quantitative importance of the suggested pathways via 21-OH-pregnenolone as compared to the progesterone pathway, we assumed that specific radioactivity of progesterone was in the same order of magnitude as that of cholesterol. From specific radioactivities of cholesterol, 21-OH-pregnenolone and DOC we cal-

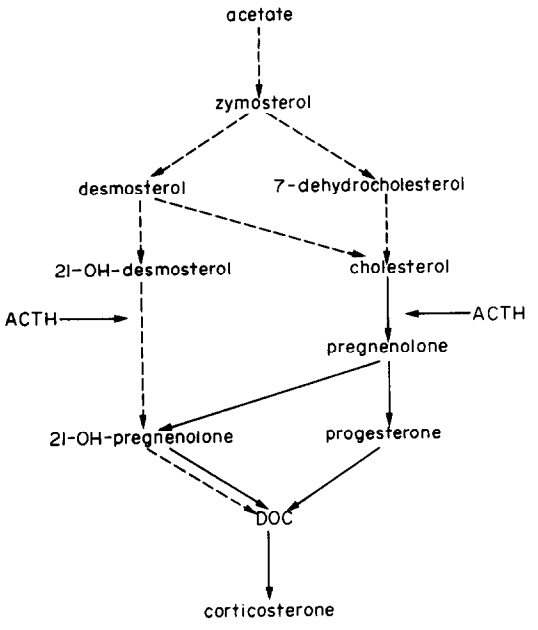

Fig. 5. Suggested pathways of corticosteroid synthesis in the rat adrenal cortex from acetate (----- radioactivity from [^{3}H]-acetate) and cholesterol (—— mass).

Table 6. Percentages of the mass of DOC that may have derived from 21-OH-pregnenolone; calculated from specific radioactivities of 21-OH-pregnenolone, cholesterol and DOC

Specific radioactivities from:	Control	ACTH
Fig. 2	1.9%	40.8%†
Fig. 3		
experiment 1	2.4%‡	25.2%‡
experiment 2	—	24.8%‡
Fig. 4	2.7%	10.9%

† ACTH concentration: 2.5 I.U./ml.
‡ Cholesterol from the tissue fractions.

culated the percentage of the mass of DOC that could have derived from 21-OH-pregnenolone. Results are summarized in Table 6. They show that the 5-en-3β-ol pathway seems to be of minor importance in the absence of ACTH. However, during synthesis stimulation by ACTH, an average of 25% of total DOC may have been formed from 21-OH-pregnenolone, most of it probably through the sequence "cholesterol—pregnenolone—21-OH-pregnenolone—DOC". Therefore, the pathway "acetate—zymosterol—desmosterol—21-OH-desmosterol—21-OH-pregnenolone—DOC—corticosterone" seems to be of mere academic interest to explain discrepant results of specific radioactivities from *in vitro* experiments. In contrast, the sequence "cholesterol—pregnenolone—21-OH-pregnenolone—DOC—corticosterone" must be considered to contribute quantitatively to the biosynthesis of corticosteroids.

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