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Steroidal 21-Diazo Ketones: Photogenerated Corticosteroid Receptor Labels†

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ABSTRACT: The 21-diazo derivatives of 9α-fluoro- and 9α-bromo-21-deoxycorticosterone, 21-deoxycorticosterone, and progesterone were synthesized for use as photoaffinity labels for corticosteroid receptors. In the isolated toad bladder system, 9α-bromo-21-diazo-21-deoxycorticosterone was as active as *d*-aldosterone and more active than 9α-fluorocortisol in augmenting active Na^+ transport. The activities of 21-diazoprogesterone and progesterone were equal; both were much less potent than *d*-aldosterone, however. These results indicate that the 21-diazo derivatives had significant functional activity in the toad bladder system. The rat kidney slice system was used to estimate the relative affinities of the diazo steroids for aldosterone receptor sites by competition experiments. At 100-fold excess of competitor to [^{3}H]aldosterone, the order of affinities was 9α-fluoro-21-diazo-21-deoxycorticosterone > 9α-bromo-21-diazo-21-deoxycorticosterone > 21-diazoprogesterone. Moreover, 9α-bromo-21-diazo-21-deoxycorticosterone reduced binding of [^{3}H]aldosterone to cytoplasmic and nuclear forms of the receptor proportionately. On the basis of competition for [^{3}H]corticosterone binding, presumably to corticosteroid-binding globulin (CBG), the order of affinities was 21-diazo-21-deoxycorticosterone > 21-diazoprogesterone > 9α-bromo-21-diazo-21-deoxycorticosterone. These findings indicate that 21-diazo steroids may be suitable as photogenerated affinity labels for mineralocorticoid receptors. The tritiated derivative, [1,2-^{3}H]-9α-bromo-21-diazo-21-deoxycorticosterone (specific activity 25 Ci/mol) was synthesized and used in model experiments on photogenerated covalent binding to rat plasma proteins. Irradiation with uv light resulted in binding of [1,2-^{3}H]-9α-bromo-21-diazo-21-deoxycorticosterone to plasma proteins, that was resistant to extraction with methylene dichloride and did not exchange with unlabeled corticosterone. The diazocorticosteroids, therefore, may have the requisite functional and selectivity properties for photoaffinity labeling of corticosteroid-binding proteins. Further studies are needed, however, to assure that photogenerated labeling with these steroids was site specific.

A growing body of evidence indicates that induction of protein synthesis mediates the action of steroid hormones on growth, differentiation, and metabolism in target tissues (Feldman et al., 1972). The initial events involve binding to a steroid-specific receptor protein and attachment of the resulting complex to the genome.

Cytoplasmic receptors, characterized by specificity in binding steroid hormones with high affinity, have been demonstrated for all of the physiological steroids. Partial success has been achieved in the isolation of stereospecific aldosterone binding proteins from rat kidney (Herman et al., 1968). Use of affinity chromatography to purify these receptors, however, has been frustrated by release of the steroid ligand from the agarose matrix on addition of the cytosol fractions (Ludens et al., 1972). Recently, significant progress has been reported in the purification of uterine estrogen receptors by affinity chromatography (Sica et al., 1973). The lability of the corticosteroid receptor complexes and in particular the susceptibility of the aldosterone receptors to irreversible loss of binding activity, however, may compromise the application of this method to purification of corticosteroid receptors (Herman and Edelman, 1968).

Purification of corticosteroid receptors should be facilitated by covalent linkage of a labeled analog to the receptor. Affinity-labeled receptors could be purified by rigorous

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methods and the labeled site characterized by peptide analysis. Attempts to accomplish these goals have been reported previously, including the use of 4-mercuri-17β-estradiol, ω-substituted 3-alkyl ethers of 17β-estradiol, ω-diazo ketones of esters of 17α-hydroxyprogesterone, ω-substituted 16α-alkyl progesterone derivatives, and azide derivatives of estrogens (Chin and Warren, 1968; Katzenellenbogen et al., 1973; Liarkos and May, 1969; Solo and Gardner, 1968, 1971). Only 4-chloromercuri-17β-estradiol has been radiolabeled but the lability of the mercaptide bond impaired the effort to purify estradiol receptors with this reagent (Muldoon and Warren, 1969).

The steroid receptors constitute only a minute fraction of the soluble cellular proteins, probably less than 1/100,000 on a molar ratio. For example, rat kidney cytosol contains 2×10^{-14} mol of mineralocorticoid receptor sites/mg of cytosol protein, which, on a mole for mole basis, is a ratio of $\sim 1:5 \times 10^5$, assuming that cytosol proteins have an average molecular weight of $\sim 10^5$ (Funder et al., 1973a). Thus, if the steroid derivative is highly reactive, binding to nonspecific sites may exceed binding to receptor sites, by a prohibitive margin. Photoaffinity labeling offers a reasonable possibility of circumventing this difficulty since the binding reaction can be initiated by photoactivation after removal of the free steroid derivative (Knowles, 1970). A number of diazoestrogen derivatives have been prepared with reasonable affinities for the 17β-estradiol uterine receptors indicating that these derivatives may indeed be suitable as photoaffinity labels (Katzenellenbogen et al., 1973).

Our first goal was to prepare corticosteroid derivatives that might serve the stated purpose. We directed our efforts to the preparation of 21-diazo ketones since structure–function studies indicated that the C-21 site is probably inserted well within the binding site of the receptor (Edelman, 1968). Photolysis of a diazo ketone leads first to the corresponding carbene (Horner et al, 1951) which is capable of undergoing insertion reactions with C–C, C–H, O–H, C–O, and C–N bonds, and addition reactions to double bonds and aromatic rings (Hine, 1964). In principle, covalent bonding to any amino acid could occur by this route, and in fact bonding to tyrosine, histidine (Shafer et al., 1966), and alanine (Vaughan and Westheimer, 1969) has been demonstrated. Alternatively, the carbene can undergo the Wolff rearrangement (Wolff, 1902) to a ketene capable of acylating nucleophilic hydroxyl, amino, or thiol functions in amino acid residues (Figure 1). Thus, this method makes it possible to label hydrophilic or hydrophobic residues. The actual site of attachment will be influenced by the spatial disposition of the steroid ligand in relation to the protein surface.

In preliminary studies, we demonstrated photochemical carbene production from the steroidal diazo ketone systems by photolysis of 21-diazoprogesterone in methanol solution. The only product isolated was the ester derived from acylation of methanol by the ketene formed by the Wolff rearrangement of the steroidal carbene (Wolff, 1902). This is an expected product in methanol solution (Chaimovich et al., 1968) where the concentration of nucleophilic sites is high. In the receptor complex, however, where the steroid is expected to be surrounded by hydrophobic residues, the carbene insertion reactions may assume greater importance.

In this communication, we also describe the preparation of steroidal diazo ketones with relatively high affinities for renal mineralocorticoid receptor sites. The isolated toad bladder system was used to determine mineralocorticoid potency of some of these derivatives (Edelman, 1968). The covalent binding of a radiolabeled diazo ketone to plasma corticosteroid-binding proteins after photolysis was also demonstrated.

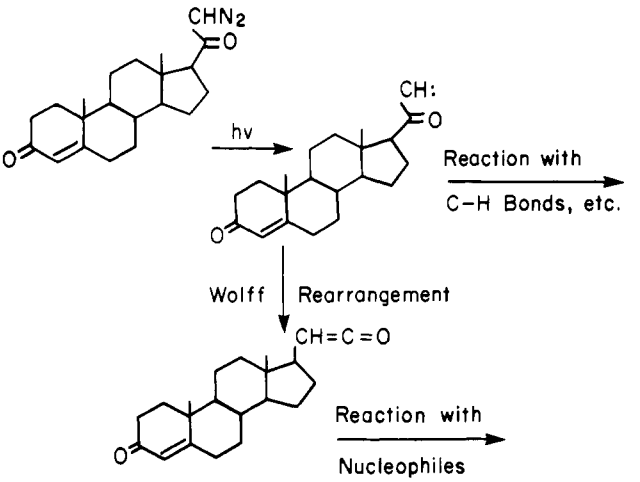

FIGURE 1: Photoactivation of 21-diazo ketones.

Methods and Results

Preparation of the Diazo Ketones.[1] Steroid starting materials were purchased from Searle, Upjohn, or Steraloids and were the best bulk grade available. Other reagents and solvents (reagent grade) were purchased from Eastman Kodak, Aldrich, Mallinckrodt, or Baker and Adams.

Thin-layer chromatography (TLC) was carried out using 5 × 20 cm precoated silica gel plates (layer thickness 0.25 mm) (E. Merck) employing a benzene–ethyl acetate (1:1) solvent system. Spots were visualized using a spray solution of 10% ceric ammonium sulfate in 6.5 *N* H_2SO_4. Preparative thin layer chromatography was accomplished on 20 × 20 cm silica gel F-254 plates (layer thickness 2 mm) (E. Merck) and the bands were visualized under 254 nm uv illumination by fluorescence quenching. Column chromatography was performed using 100 g of 30–70 mesh silica gel (E. Merck)/g of steroid, eluting with benzene–ethyl acetate, 7:3, and collecting 5-ml fractions with an LKB fraction collector. Fractions containing the desired product(s) were identified by TLC and combined.

Melting points were determined with a Thomas-Hoover apparatus equipped with a corrected thermometer. Microanalyses were performed by the Micro-analytical Department, University of California, Berkeley, Calif. Nuclear magnetic resonance (NMR) spectra were obtained at a field strength of 60 MHz on samples in $CDCl_3$ on a Varian A-60A instrument, using tetramethylsilane as internal standard and given as ppm downfield (δ scale). IR spectra were made from samples in KBr discs using a Perkin-Elmer 457 spectrophotometer. Mass spectra were obtained on an AEI MS-902 instrument (70 eV) by Dr. Robert Weinkam. The catalytic tritiation was performed with the generous help of Dr. Chin-tzu Peng using a vacuum line system for tritium

[1] Abbreviated names used for compounds prepared in this study are: 21-diazoprogesterone, 21-diazopregn-4-ene-3,20-dione (**5**); 9α-bromo-21-diazo-21-deoxycorticosterone, 9α-bromo-21-diazo-11β-hydroxypregn-4-ene-3,20-dione (**15**); 9α-fluoro-21-diazo-21-deoxycorticosterone, 9α-fluoro-21-diazo-11β-hydroxyandrost-4-ene-3,20-dione (**20**); 21-diazo-21-deoxycorticosterone, 21-diazo-11β-hydroxypregn-4-ene-3,20-dione (**12**).

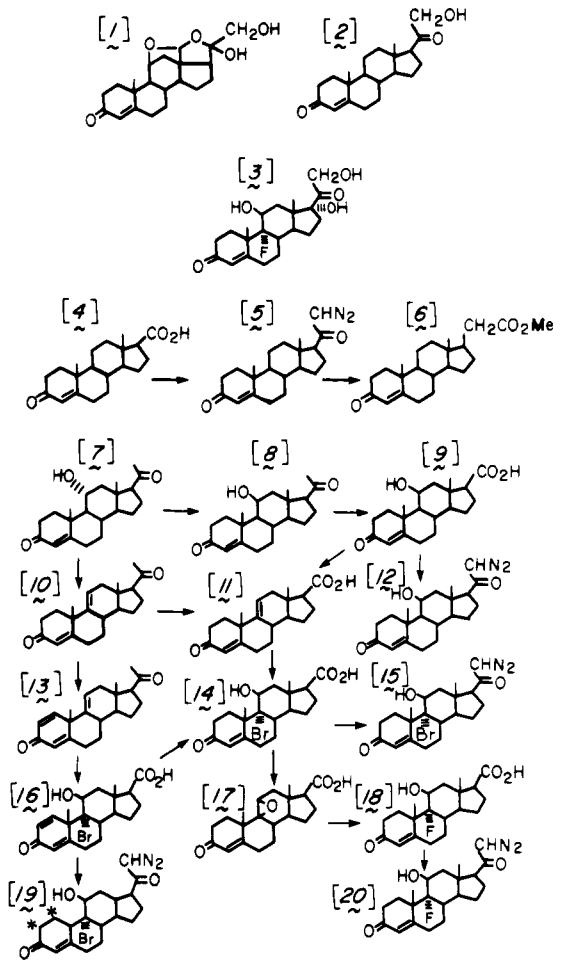

FIGURE 2: Pathways in the synthesis of 21-diazo steroids.

(Feinendegen, 1967). Figure 2 presents the reaction sequences in the preparation of the diazo steroids.

Ethyl 3-Oxopregn-4-en-21-oate (**6**) *from* **5** *by Photochemical Wolff Rearrangement* (*see Figure 1*). 21-Diazopregn-4-ene-3,20-dione (**5**) was prepared from 3-oxoandrost-4-ene-17β-carboxylic acid (**4**) in 83% yield using the method of Wilds and Shunk (1948) to give a product mp 176–178° after recrystallization from acetone (lit. mp 176–178°). This product (0.06 g in 60 ml of absolute ethanol) was irradiated with a 200-W Hanovia high-pressure mercury lamp. The solvent was evaporated and the spectrum of the residue showed only a small band at 2100 cm^{-1} (diazo ketone). Recrystallization of the crude material from ether–acetone gave colorless crystals: mp 182–185°, λ_{max}^{KBr} 1710 (CO_2Et), 1650 (C=O), 1605 (C=C) cm^{-1}; NMR δ 0.67, 1.20 (C-18 and C-19), 2.3 (doublet) (C-20 H), 4.10 (quartet), 1.43 (triplet) (ethyl ester) and 5.74 (C-4H) ppm; mass spectrum molecular weight (M^+) = 358. Anal. Calcd for $C_{23}H_{34}O_3$: C, 77.05; H, 9.56. Found: C, 76.87; H, 9.40.

11β-Hydroxypregn-4-ene-3,20-dione (**8**) *from 11α-Hydroxypregn-4-ene-3,20-dione* (**7**). A solution of 20 g of 11α-hydroxypregn-4-ene-3,20-dione in 300 ml of acetone was titrated with 8 *N* chromic acid. The solution was decanted from the green sludge which formed, concentrated to 100 ml by evaporation under reduced pressure, diluted with 200 ml of ether, washed with 10% NaOH (7 × 20 ml), and concentrated to 50 ml. Dilution with 400 ml of cold water and recrystallization of the precipitated product from acetone gave 16.8 g (85%) of 11-ketone. A mixture of 580 ml of benzene, 2.8 g of pyridine, and 3.0 ml of concentrated HCl was distilled until the vapor temperature reached 79°. The solution was cooled and 200 ml of redistilled ethylene glycol and 37.5 g of 11-ketone were added. Another 50 ml of benzene was distilled, a Dean–Stark water separator packed with 2.5 g of $MgSO_4$ was attached to the flask, and the mixture was refluxed for 16 hr; 4 g of NaOH in 4 ml of water was added to the cooled mixture and the benzene was evaporated under reduced pressure. The residue was added to 600 ml of ice–water and the product was collected, giving 45.5 g (98%) of bisketal, mp 162–167°, homogeneous on thin-layer chromatography. A stirred solution of 45.5 g of the bisketal in 700 ml of tetrahydrofuran was treated with excess lithium aluminum hydride for 1.5 hr and carefully decomposed by dropwise addition of 10% NaOH. The precipitated salts were removed by filtration. The filtrate was concentrated to 70 ml and diluted with 700 ml of acetone, and 5 ml of 6 *N* HCl was added dropwise. The mixture was kept for 18 hr at 25°, concentrated to 100 ml under reduced pressure, and diluted with 500 ml of water. The precipitated product was collected and weighed 26.7 g (71%) after drying, mp 183–185° (lit. 185–188°). The overall yield from 11α-hydroxypregn-4-ene-3,20-dione was 59%.

11β-Hydroxy-3-oxo-androst-4-ene-17β-carboxylic Acid (**9**). A solution of 2.60 g (7.9 mmol) of 11β-hydroxypregn-4-ene-3,20-dione (**8**) in a mixture of 80 ml of dioxane (pure) and 15 ml of water was added dropwise, over a 2.5-hr interval, to a stirred solution of 1.7 ml (32.5 mmol) of bromine and 3.9 g of NaOH in a mixture of 34 ml of water and 30 ml of dioxane (pure) at 0°. After the mixture was stirred an additional 2.5 hr, 0.5 g of sodium bisulfite was added and the mixture was stirred for 15 min, and then concentrated under reduced pressure until solid material precipitated. After addition of 200 ml of water, 0.15 g of the precipitate was removed by filtration. The filtrate was acidified to pH 3 with dilute HCl at 0°, and the product was collected. Recrystallization from acetone gave 1.57 g (60%) of material, mp 257–260° (lit. mp 253–258° when obtained from adrenal tissue) (Mason et al., 1937).

3-Oxoandrosta-4,9 (11)-diene-17β-carboxylic Acid (**11**) *from* **10** *and* **9**. A solution of sodium hypobromite was prepared by adding 39 g (13.3 ml) of bromine to a solution of 28 g of NaOH in 180 ml of water at −5°, and diluting with 40 ml of *tert*-butyl alcohol. To the stirred solution there was added dropwise, during 45 min, a solution of 16.7 g of pregna-4,9(11)-diene-3,20-dione (**10**) in 80 ml of *tert*-butyl alcohol. After the addition was completed another 40 ml of *tert*-butyl alcohol was added and the mixture was stirred for a further 90 min. Sodium sulfite (2 g) was added to destroy excess oxidant and the solution was concentrated at 35° under reduced pressure. The residual aqueous suspension was extracted with ether to remove unreacted starting material, and the aqueous layer was diluted with 600 ml of water and acidified to pH 3 with 5 *N* HCl. The precipitated product was collected, washed with water, dissolved in chloroform, and dried over Na_2SO_4. Treatment with activated charcoal, isolation of the product, and recrystallization from chloroform gave 9.93 g (59%) of material, mp 253–256°.

A solution of 6.0 g of 11β-hydroxy-3-oxoandrost-4-ene-17β-carboxylic acid (**9**) in a mixture of 70 ml of acetic acid and 20 ml of HCl was boiled under reflux for 40 min and poured into water. The resulting precipitate was collected and recrystallized from acetone–hexane which yielded 2.94

g of product, mp 246–249° (lit. mp 240–250°) (Casanova et al., 1953). Anal. Calcd for $C_{20}H_{26}O_3$: C, 76.40; H, 8.34. Found: C, 76.67; H, 8.12.

21-Diazo-11β-hydroxypregn-4-ene-3,20-dione (**12**). Three milliliters of freshly distilled oxalyl chloride was added to a stirred suspension of 0.81 g of 11β-hydroxy-3-oxoandrost-4-ene-17β-carboxylic acid (**9**) in 40 ml of dry benzene at 0°. After the mixture was stirred for 3 hr at 0°, it was evaporated under reduced pressure at 25°. If the removal of oxalyl chloride was incomplete, lower yields result. A tenfold molar excess of ethereal diazomethane, previously dried over KOH pellets for 4 hr, was added to the residue and the suspension was stirred for 2.5 hr at 0°. The mixture was filtered and gave 0.25 g (28%) of product, mp 178° dec. The analytical sample, mp 177° dec, was obtained on recrystallization from chloroform. Anal. Calcd for $C_{21}H_{28}N_2O_3$: C, 70.76; H, 7.92; N, 7.86. Found: C, 70.48; H, 7.84; N, 7.84.

Pregna-1,4,9(11)-triene-3,20-dione (**13**). A stirred solution of 8.60 g of pregna-4,9(11)-diene-3,20-dione (**10**) in 400 ml of dry benzene was distilled until 30 ml of distillate was removed and 7.4 g of 2,3-dichloro-5,6-dicyanobenzoquinone (DDQ)[2] was added to the remainder. Another 30 ml of distillate was removed and the wine-red remaining solution was heated under reflux in a nitrogen atmosphere for 19 hr. At this time, thin-layer chromatography showed a trace of starting material. An additional 1 g of DDQ was added, the mixture was refluxed again for 4.5 hr, and then filtered and concentrated to 100 ml under reduced pressure. Ether (150 ml) was added to the concentrate and the resulting solution was washed with 10% NaOH (6 × 20 ml) and then dried ($MgSO_4$). A total of 8.1 g (95%) of product (mp 127–135°) was obtained by evaporation of the dried solution, recrystallization of the residue from ethyl acetate–hexane, and chromatography of the mother liquor on silica gel using chloroform. Anal. Calcd for $C_{21}H_{26}O_2$: C, 81.25; H, 8.44. Found: C, 80.95; H, 8.24.

9α-Bromo-11β-hydroxy-3-oxoandrost-4-ene-17β-carboxylic Acid (**14**) *from* **11**. A stirred suspension of 0.985 g of 3-oxoandrosta-4,9(11)-diene-17β-carboxylic acid (**11**) in 12 ml of peroxide-free dioxane containing 1.85 ml of 0.5 *N* perchloric acid was treated with 0.570 g of *N*-bromoacetamide in divided amounts over a 1-hr interval at 25° in the dark. After stirring the mixture for an additional hour at 25°, it was decomposed by addition of ~12 ml of 10% sodium sulfite solution until a negative reaction was given with starch iodide paper. Ice and chloroform (~30 ml each) were added, the layers were separated and the chloroform solution was washed once with ice–water. After drying (Na_2SO_4) the chloroform was evaporated under reduced pressure and the residue was treated with ether to effect crystallization. Recrystallization from acetone–ether gave 0.66 g of crystals, mp 155–159°, raised to 159–161° by an additional recrystallization. Mass spectrum: M^+ 411. Anal. Calcd for $C_{20}H_{27}BrO_4$: C, 58.40; H, 6.62. Found: C, 58.19; H, 6.76.

9α-Bromo-11β-hydroxy-3-oxoandrost-4-ene-17β-carboxylic Acid (**14**) *by Hydrogenation of 9α-Bromo-11β-hydroxyandrosta-1,4-dien-3-one-17β-carboxylic Acid* (**16**). A solution of 0.41 g of 9α-bromo-11β-hydroxyandrosta-1,4-dien-3-one (**16**) in 60 ml of redistilled tetrahydrofuran was hydrogenated at atmospheric pressure over 45 mg of 10% palladium on charcoal. After 1 equiv of H_2 was absorbed, the mixture was filtered and the residue from evaporation of the filtrate was recrystallized from acetone to give 0.23 g (56%) of colorless crystals, mp 158–160°, undepressed upon admixture with a sample of **14** obtained from precursor **11** (see previous procedure).

9α-Bromo-21-diazo-11β-hydroxypregn-4-ene-3,20-dione (**15**). To a stirred solution of 0.73 g of 9α-bromo-11β-hydroxy-3-oxoandrost-4-ene-17β-carboxylic acid (**14**) in 15 ml of dry benzene there was added 2.4 ml of freshly distilled oxalyl chloride in an ice bath. The cooled mixture was stirred for 30 min at 0° and then for 40 min at 25°. The resulting cloudy suspension was evaporated under reduced pressure, and the residue was dissolved in 15 ml of dry ether–benzene and filtered through a dried sintered glass filter. The filtrate was added slowly to a stirred ethereal solution of diazomethane at −5°, kept at −8° for 1 hr, and allowed to evaporate in the hood. The residue was dissolved in acetone, filtered through Celite, concentrated, and cooled to afford 0.11 g of product, mp 157–158°, which gave one spot on thin-layer chromatography. Recrystallization from acetone gave the analytical sample, mp 158–158.5°. Anal. Calcd for $C_{21}H_{27}BrN_2O_3$: C, 57.93; H, 6.25; N, 6.43; Br, 18.35. Found: C, 57.74; H, 6.23; N, 6.37; Br, 18.32.

9α-Bromo-11β-hydroxyandrosta-1,4-dien-3-one-17β-carboxylic Acid (**16**). To a stirred ice-cold solution of 3.9 g of sodium hydroxide in 34 ml of water there was first added 5.2 g (1.66 ml) of bromine and then 28 ml of dioxane (pure). The resulting solution was added, during 1 hr, to a stirred, ice-cold solution of 2.85 g of pregna-1,4,9(11)-triene-3,20-dione (**13**) in a mixture of 80 ml of dioxane and 15 ml of water. After 30 hr at 0°, the mixture still contained unreacted starting material as shown by thin-layer chromatography. More oxidant (from 0.7 g of sodium hydroxide, 1.0 g of bromine, 6 ml of water, and 5 ml of dioxane) was added, the mixture was kept at 5° for 18 hr, and then decomposed with 0.2 g of sodium bisulfite. The mixture was extracted with ether and the aqueous layer was concentrated to half volume and acidified at 0° with 6 *N* HCl. The product was collected and recrystallized from acetone to give 2.31 g of material, mp 245–252°. To a stirred solution of 2.92 g of the foregoing product in 270 ml of dioxane kept at 5°, there was added 2.70 g of *N*-bromoacetamide in one portion. After the addition of 4.5 ml of 0.2 *N* perchloric acid the mixture was kept at 5° for 18 hr, when thin-layer chromatography showed no $\Delta^{9,11}$ material remaining. The mixture was concentrated in vacuo until solid material began to appear and diluted with 250 ml of water, and the precipitated product was collected. By recrystallization from acetone 2.80 g (73%) of material, mp 168–171°, was obtained. Anal. Calcd for $C_{20}H_{25}BrO_4$: C, 58.69; H, 6.16; Br, 19.52. Found: C, 58.78; H, 6.59; Br, 19.27.

9β,11β-Oxido-3-oxoandrost-4-ene-17β-carboxylic Acid (**17**). A suspension of 5.0 g of crude 9α-bromo-11β-hydroxy-3-oxoandrost-4-ene-17β-carboxylic acid (**14**) in a mixture of 50 ml of water and 50 ml of acetone was titrated to pH 8 with 2 equiv of 0.5 *N* NaOH at 25°. The acetone was removed under reduced pressure and the remaining aqueous solution was filtered to remove a small amount of solid material. The filtrate was carefully acidified to pH 3 with 3 *N* HCl and the precipitated product was collected, dissolved in chloroform, and washed with brine (saturated solution of NaCl). Evaporation of the dried extract gave 4.0 g (99%) of product, mp 221–225°. A sample obtained in another experiment was recrystallized from acetone giving

[2] Abbreviations used are: DDQ, 2,3-dichloro-5,6-dicyanobenzoquinone; scc, short-circuit current; CBG, corticosteroid-binding globulin.

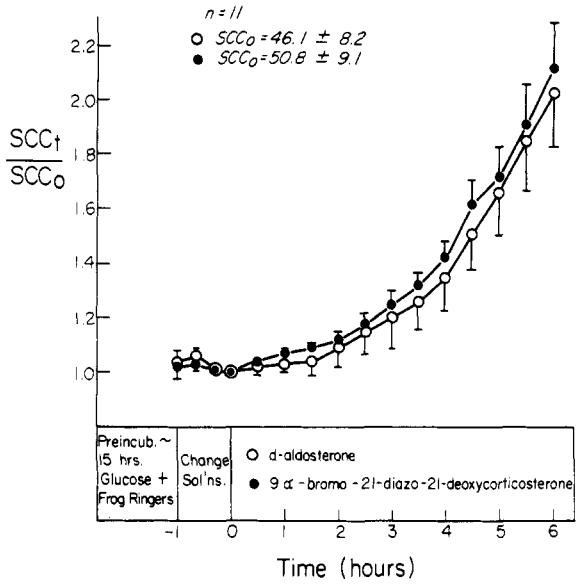

FIGURE 3: The effects of *d*-aldosterone and 9α-bromo-21-diazo-21-deoxycorticosterone on active Na^+ transport across the toad bladder. scc_t/scc_0 denotes the short-circuit current at time "*t*" divided by that at time-zero in each hemibladder. The vertical bars represent ±1 SE and the circles the mean values. *N* denotes the number of pairs of hemibladders and scc_0, the absolute short-circuit current at time-zero (mean ±SE) expressed in $\mu A/2.54$ cm^2. Pairs of hemibladders were preincubated in steroid-free glucose-frog Ringers solution for 15 hr. All media were then replaced with fresh media and the steroids were added to both serosal and mucosal media at time-zero (final concentration = 5×10^{-8} *M*).

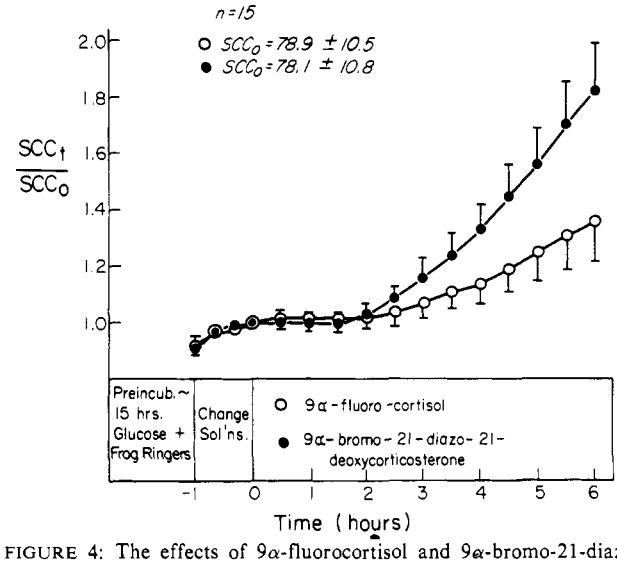

FIGURE 4: The effects of 9α-fluorocortisol and 9α-bromo-21-diazo-21-deoxycorticosterone on active Na^+ transport across the toad bladder. The conventions used in this figure are defined in the legend of Figure 3.

crystals, mp 231–233°. Anal. Calcd for $C_{20}H_{26}O_4$: C, 72.70; H, 7.93. Found: C, 72.47; H, 8.10.

9α-Fluoro-11β-hydroxy-3-oxoandrost-4-ene-17β-carboxylic Acid (**18**). To a magnetically stirred solution prepared from 11 ml of anhydrous liquid HF previously added to 22.5 ml of redistilled dry tetrahydrofuran in a polyethylene erlenmeyer flask, there was slowly added, at −70°, 4.75 g of 9β,11β-oxido-3-oxoandrost-4-ene-17β-carboxylic acid (**17**) dissolved in 38 ml of dry, ethanol-free chloroform. The flask was stoppered and the mixture was stirred at −60° for 2.5 hr and then allowed to warm to 0° during an additional 1.5 hr. The deep red mixture was poured onto ice and extracted with chloroform. The chloroform layer was washed with water and brine, dried, treated with activated charcoal, and evaporated under reduced pressure. The product was recrystallized from methanol–chloroform to give 2.61 g of crystals, mp 282–283°, mass spectrum M^+ 350. Anal. Calcd for $C_{20}H_{27}FO_4 \cdot 0.5H_2O$: C, 66.83; H, 7.85; F, 5.29. Found: C, 67.35; H, 7.62; F, 5.26.

[1,2-^{3}H]-9α-Bromo-21-diazo-11β-hydroxypregn-4-ene-3,20-dione (**19**). This reaction was conducted using a vacuum line apparatus designed for the transfer of tritium. A solution of 0.37 g of 9α-bromo-11β-hydroxy-3-oxoandrosta-1,4-diene-17β-carboxylic acid (**16**) in 20 ml of redistilled tetrahydrofuran containing 40 mg of 10% palladium on charcoal was introduced into a glass vessel equipped for magnetic stirring. Carrier-free tritium gas (50 Ci) was introduced from the vacuum line and the hydrogenation vessel was sealed. The contents of the vessel was stirred for 4 hr and the excess tritium was pumped off. The mixture was filtered and the filtrate was evaporated. The residue was slurried in 15 ml of acetone, and 0.82 g of 9α-bromo-11β-hydroxyandrost-4-en-3-one-17β-carboxylic acid (**14**) was added. The resulting slurry was kept overnight, the supernatant layer was then removed by aspiration, and the residue was washed twice with cold acetone; 80 ml of dry benzene and 3.5 ml of oxalyl chloride was added to the remaining solid material, and the solution was stirred for 3.5 hr. Then excess ethereal diazomethane was added and the mixture was kept at 25° for 18 hr. After evaporation of the mixture, the product was purified by preparative thin-layer chromatography using 2-mm silica gel layers and benzene–ethyl acetate, 8:3, solvent system. The product isolated from this procedure was recrystallized from acetone–ether, and had a specific activity of 25 Ci/mol. It decomposed rapidly in the crystalline state but was more stable when kept in benzene at 4°.

9α-Fluoro-21-diazo-11β-hydroxyandrost-4-ene-3,20-dione (**20**). This compound was prepared in the same way as 9α-bromo-21-diazo-11β-hydroxypregn-4-ene-3,20-dione (**15**), using 0.83 g of 9α-fluoro-11β-hydroxy-3-oxoandrost-4-ene-17β-carboxylic acid (**18**) as starting material. After recrystallization from methanol–chloroform, 0.15 g of pure product was obtained, mp 210–211°. Anal. Calcd for $C_{21}H_{27}FN_2O_3$: C, 67.36; H, 7.27; N, 7.48. Found: C, 67.61; H, 7.19; N, 7.26.

Physiological and Renal Receptor Experiments

Materials. The conventional reagents (analytical grade) were obtained from Baker and Adams or Mallinckrodt Co., [1,2-^{3}H]-*d*-aldosterone (50 Ci/mol) and [1,2-^{3}H]corticosterone (40 Ci/mol) were purchased from New England Nuclear, Inc. 9α-Fluorocortisol was a gift of the Upjohn Co. and the remaining unlabeled steroids were A grade from Calbiochem Co.

Physiological Methods. The toads (*Bufo marinus*, indigenous to Colombia) were purchased from Tarpon Zoo, Tarpon Springs, Fla., and kept without food, with access to water, for about 1 week. Active Na^+ transport across the epithelium of the toad bladder was measured in vitro by the short-circuit (scc) technique of Ussing and Zerahn (1951). Urinary hemibladders were excised after double pithing the toads and each hemibladder was mounted as a diaphragm in chambers filled with frog-Ringers solution (10 ml each on the mucosal and serosal sides) containing glucose (10 m*M*), penicillin G (100 U/ml), and streptomycin (0.1

Table I: Response of the Short-Circuit Current to Diazo Steroid Derivatives.[a]

No. of Pairs	Steroid	Concn (M)	scc_6/scc_0	p
11	9α-Bromo-21-diazo-21-deoxycorticosterone	5×10^{-8}	2.12 ± 0.17	
	d-Aldosterone	5×10^{-8}	2.03 ± 0.20	n.s.
12	9α-Bromo-21-diazo-21-deoxycorticosterone	{5×10^{-8}}		
	{+ *d*-aldosterone}	5×10^{-8}	1.70 ± 0.13	
	d-Aldosterone	5×10^{-8}	1.53 ± 0.09	n.s.
15	9α-Bromo-21-diazo-21-deoxycorticosterone	5×10^{-8}	1.82 ± 0.17	
	9α-Fluorocortisol	5×10^{-8}	1.36 ± 0.14	s.
10	9α-Bromo-21-diazo-21-deoxycorticosterone	5×10^{-7}	1.61 ± 0.12	
	9α-Fluorocortisol	5×10^{-7}	1.67 ± 0.16	n.s.
7	9α-Bromo-11β-OH-3-oxo-androst-4-ene-17β-carboxylic acid (9)	5×10^{-7}	0.77 ± 0.06	s.
	d-Aldosterone	7×10^{-8}	1.47 ± 0.17	
8	21-Diazoprogesterone	5×10^{-6}	0.95 ± 0.17	
	d-Aldosterone	7×10^{-8}	1.62 ± 0.19	s.
10	21-Diazoprogesterone	5×10^{-6}	1.71 ± 0.10	
	{+ *d*-aldosterone}	{7×10^{-8}}		n.s.
	d-Aldosterone	7×10^{-8}	1.66 ± 0.08	
12	21-Diazoprogesterone	5×10^{-5}	1.31 ± 0.39	
	d-Aldosterone	7×10^{-8}	2.31 ± 0.55	s.
10	21-Diazoprogesterone	5×10^{-5}	1.49 ± 0.18	
	Progesterone	5×10^{-5}	1.59 ± 0.21	n.s.
8	21-Diazoprogesterone	5×10^{-5}	1.50 ± 0.19	
	Saline		0.95 ± 0.14	s.

[a] Paired hemibladders were preincubated in steroid-free glucose–Ringers solution for 15 hr. At time-zero, the steroids were added to the serosal and mucosal bathing media at the indicated concentrations and the scc was measured at 30-min intervals. The ratio scc_6/scc_0 denotes the short-circuit current at 6 hr divided by that at time-zero for each hemibladder. Results are given as mean ±SE. The "p" values of the differences between each set of hemibladders was computed by the student T test. n.s. denotes not significant and s significant at the 5% level or less.

mg/ml) as described previously (Porter and Edelman, 1964). The hemibladders were incubated at 24° for 15 hr to remove endogenous steroids. All solutions were then replaced with fresh, frog-Ringers solution containing glucose (5 mM). "Time-zero" was designated as the time of addition of the test steroids to the media. The protocols are given in the legends of Figures 3 and 4, and Table I.

Physiological Results. Mineralocorticoid agonist or antagonist activity of the diazo derivatives was assessed by comparison with the maximum increase in scc elicited by *d*-aldosterone, 9α-fluorocortisol, or progesterone (concentrations given in legends of Table I and Figures 3 and 4). The derivatives that were assayed in this system were 9α-bromo-21-diazo-21-deoxycorticosterone and 21-diazoprogesterone. The time courses of the response to *d*-aldosterone and 9α-bromo-21-diazo-21-deoxycorticosterone are shown in Figure 3. The response to *d*-aldosterone is characterized by a concentration-independent latent period of 60–120 min and a monotonic increase in scc over the next 4–5 hr to a steady-state level that is from 1.5 to 3 times as great as the control level (Edelman, 1968; Porter and Edelman, 1964). The maximum effect of *d*-aldosterone on the scc is obtained at a concentration of 5×10^{-8} M (Porter, 1968). Surprisingly, 9α-bromo-21-diazo-21-deoxycorticosterone elicited a response of the same magnitude as *d*-aldosterone at this concentration. The high degree of mineralocorticoid activity of 9α-bromo-21-diazo-21-deoxycorticosterone was also indicated by a comparison with the activity of 9α-fluorocortisol, the most potent of the synthetic mineralocorticoids. At a concentration of 5×10^{-8} M, 9α-fluorocortisol elicited about half of the effect of this diazosteroid (Figure 4). These and the remaining results are summarized in Table I and presented as the ratio of the scc at 6 hr to that at time-zero (i.e., scc_6/scc_0). At a concentration of 5×10^{-7} M, 9α-fluorocortisol and 9α-bromo-21-diazo-21-deoxycorticosterone elicit the same increases in scc, presumably by saturating the steroid-sensitive receptor–effector system. That 9α-bromo-21-diazo-21-deoxycorticosterone augments Na^+ transport by the same pathway as *d*-aldosterone is implied by the finding that simultaneous addition of both compounds had only a marginally (n.s.) greater effect on the scc than *d*-aldosterone alone (Table I). The synthetic precursor, **9**, which has an hydroxyl group in place of the diazomethyl function did not stimulate the scc and may have depressed it slightly, indicating that contamination with the precursor **9** could not account for the activity of the diazo steroid.

Progesterone and 11-deoxycorticosterone are distinctly less potent than *d*-aldosterone or 9α-fluorocortisol in the toad bladder system (Edelman, 1968). As indicated in Table I, 21-diazoprogesterone (which may also be considered an analog of 11-deoxycorticosterone) had no agonist or antagonist activity at 5×10^{-6} M but elicited about 25% of the maximum response at 5×10^{-5} M, as compared with 7×10^{-8} M *d*-aldosterone. The addition of the 21-diazo moiety yielded a compound with about the same mineralocorticoid potency as progesterone, since the 21-diazo derivative was as active (at 5×10^{-5} M) as the parent compound. The saline control clearly establishes that the 21-diazo steroid is significantly active at this high concentration.

Renal Receptor Affinity

Binding Experiments. Male, Sprague-Dawley rats (100–200 g body wt) were obtained from Simonsen Labs., Inc., and maintained on standard Purina laboratory chow until use. The rats were surgically adrenalectomized and maintained for 3–5 days on standard Purina lab chow and 0.9% (9 g/l.) saline drinking water. Rats were killed by exsanguination via cardiac puncture under ether anesthesia. The kidneys were removed, decapsulated, halved, and rinsed in ice-cold incubating solution (Na^+ = 133, K^+ = 6,

Table II: Competition of Various Steroids and Diazo Analogs with [^{3}H]Aldosterone for Renal Cytoplasmic Receptors.[a]

Competing Steroid	% of Cytoplasmic Binding of [^{3}H]-Aldosterone	No. Expt
d-Aldosterone	6	12
9α-Fluorocortisol	10	12
11-Deoxycorticosterone	15	4
Corticosterone	17	8
Progesterone	22	6
9α-Fluoro-21-diazo-21-deoxycorticosterone	18	2
9α-Bromo-21-diazo-21-deoxycorticosterone	39	10
21-Diazoprogesterone	50	10

[a] Kidney slices from adrenalectomized rats were incubated with 5 × 10^{-9} *M* [^{3}H] aldosterone and either no additions or competing unlabeled steroid at a concentration of 5 × 10^{-7} *M*. The amount bound in the cytoplasmic fraction was determined by filtration through G-50 [fine] Sephadex. The results are expressed as percent of the binding in the control fractions (i.e., no "cold" steroid added).

Table III: Competition of Diazo Steroids with [^{3}H] Aldosterone for Renal Cytoplasmic Receptors.[a]

Concn of Competing Steroid	5 × 10^{-9} *M*	5 × 10^{-8} *M*	5 × 10^{-7} *M*
d-Aldosterone	48 (2)	12 (2)	6 (12)
9α-Bromo-21-diazo-21-deoxycorticosterone	100 (2)	79 (6)	39 (10)
21-Diazoprogesterone	100 (2)	83 (8)	50 (10)

[a] Kidney slices from adrenalectomized rats were incubated with 5 × 10^{-9} *M* [^{3}H] aldosterone and (1) either no additions, or (2) competing unlabeled steroid at the indicated concentrations for 30 min at 25°. Cytoplasmic binding of [^{3}H] aldosterone was determined by filtration through G-50 Sephadex. The results are expressed as percent of the binding, expressed in moles of [^{3}H] aldosterone/milligram of protein, in the control slices (i.e., no "cold" steroid added). The number of experiments, each in duplicate, is given in parentheses.

Mg^{2+} = 0.5, Ca^{2+} = 1, Cl^- = 134, Tris-HCl = 5, glucose = 5, and KH_2PO_4 = 6; all in mmoles/l.; pH 7.4 (at 0°C)). Kidney slices, 275 μ in thickness, were made with a McIlwain tissue chopper and the slices rinsed under suction. [^{3}H]Aldosterone (final concentration = 5 × 10^{-9} *M* ± various concentrations of competing steroid) was added to the flasks and the kidney slices were incubated for 30 min in a 25° water bath with continuous agitation. The slices were then rinsed under suction on nylon mesh and homogenized in 0.25 *M* sucrose–3 m*M* $CaCl_2$ with 8 strokes of a Potter-Elvejhem homogenizer. This and all subsequent procedures were performed at 0–4°. The homogenates were centrifuged at 30,000*g* for 30 min to obtain the high speed supernatant fraction; 1-ml aliquots of the supernatants were passed through 3.6 ml of G-50 fine Sephadex to separate free from protein-bound [^{3}H]aldosterone. Aliquots of the void volume, which contained the protein-bound steroid, were taken for radioassay (Fanestil and Edelman, 1966) and determination of protein concentration by the method of Warburg and Christian (1942).

In the experiments with [1,2-^{3}H]corticosterone and [1,2-^{3}H]-9α-bromo-21-diazo-21-deoxycorticosterone (**19**), binding to plasma proteins, primarily corticosteroid-binding globulin (CBG), was assessed by incubating aliquots of diluted plasma (1:60 with homogenizing medium) from adrenalectomized rats with the tritiated steroids for 15 min at 37° in a shaking water bath. Bound steroid was measured by Sephadex chromatography as described above. The relative affinities of three diazo steroids (9α-bromo-21-diazo-21-deoxycorticosterone, 21-diazo-21-deoxycorticosterone, and 21-diazoprogesterone) for the CBG binding site was estimated in competition experiments with [1,2-^{3}H]corticosterone (5.2 × 10^{-8} *M*) under the same incubation conditions as above. To evaluate the extent of covalent binding of a diazo steroid to plasma proteins, [1,2-^{3}H]9α-bromo-21-diazo-21-deoxycorticosterone was incubated with diluted plasma, from adrenalectomized rats, for 15 min at 37°. Aliquots were irradiated with uv light (200-W Hanovia high-pressure mercury lamp) for 5 min either before or after passage through G-50 Sephadex (fine) columns to remove free ^{3}H-labeled steroid. All samples were then extracted with methylene dichloride 5:1 (v:v) at room temperature for 30 min. The mixture was centrifuged at 2500*g* for 5 min at 4°. The organic phase was dried in a counting vial and assayed for ^{3}H content as described above.

The extent of covalent binding was also evaluated by resistance to exchange with unlabeled corticosterone. Diluted plasma from adrenalectomized rats was incubated with [1,2-^{3}H]-9α-bromo-21-diazo-21-deoxycorticosterone (final concentration = 2.6 × 10^{-4} *M*) for 15 min at 37°. Half of the incubation mixture was irradiated with uv light for 5 min. Various concentrations of corticosterone (up to 2.6 × 10^{-4} *M*) were then added to aliquots of both the irradiated and control mixtures and incubated for an additional 15 min at 37°. The residual bound ^{3}H-labeled steroid content was assessed by passage through G-50 Sephadex columns as described above.

Results of the Binding Experiments. The ability of the diazo analogs to compete for aldosterone binding sites was assessed in kidney slices prepared from adrenalectomized rats (Marver et al., 1972). In the first set of experiments, the analogs were added at 100-fold the concentration of [^{3}H]aldosterone (final concentration = 5 × 10^{-9} *M*). The results in Table II indicate that the 21-diazo function significantly reduced the affinity of the steroid for the aldosterone-binding sites (compare 9α-fluoro-21-diazo-21-deoxycorticosterone to 9α-fluorocortisol, 21-diazoprogesterone to progesterone or deoxycorticosterone). These results are in contrast to those obtained in the toad bladder system. Nevertheless, 9α-fluoro-21-diazo-21-deoxycorticosterone retained considerable affinity for these sites.

To obtain a quantitative index of the affinities of diazo derivatives for renal aldosterone-binding sites, competition studies were carried out at three concentration levels of the competitors. The results in Table III indicate that 9α-bromo-21-diazo-21-deoxycorticosterone has about 1/100th of the affinity of *d*-aldosterone for this site and that 21-diazoprogesterone has a significantly lower affinity than the 9α-bromo derivative.

Steroid hormone action apparently involves the translocation of a cytoplasmic steroid–receptor complex to the nucleus and binding of this complex to the genome (Feldman et al., 1972; Marver et al., 1972; Raspé, 1970). Inhibition of the formation of the cytoplasmic [^{3}H]aldosterone–receptor complex by the diazo derivative, therefore, should reduce nuclear uptake of the complex proportionately. As shown in Table IV, at a concentration ratio of 100-fold, 9α-bromo-21-diazo-21-deoxycorticosterone reduced formation of the renal cytoplasmic [^{3}H]aldosterone–receptor complex to

Table IV: Competition of a Diazo Analog with [^{3}H]Aldosterone for Renal Cytoplasmic and Nuclear Receptors.[a]

	Cytoplasm		Nuclear Fractions: Tris-Soluble		Nuclear Fractions: KCl Extract	
	Amount[b] Bound	% Bound	Amount[b] Bound	% Bound	Amount[b] Bound	% Bound
Control	20.9	100	58.3	100	10.8	100
+9α-Bromo-21-diazo-21-deoxycorticosterone	3.4	16	6.5	11	1.3	12

[a] Kidney slices from adrenalectomized rats were incubated in 5.2×10^{-9} *M* [^{3}H] aldosterone ± 5.2×10^{-7} *M* 9α-bromo-21-diazo-21-deoxycorticosterone at 25° for 1 hr. Nonspecific binding was determined by addition of 9α-fluorocortisol (final concentration = 5.2×10^{-7} *M*) to parallel flasks. [b] Amounts bound are expressed as moles ($\times 10^{15}$)/milligram of protein. $n = 2$ experiments, in duplicate.

16% of the control value and nuclear uptake of the complex to 11% (Tris-soluble form) and 12% (chromatin-bound form) of their respective control values.

Covalent Binding to Plasma Protein. The potential utility of a diazo steroid depends on its ability to binding covalently to the receptor. To demonstrate covalent binding to a protein, however, requires labeling of the steroid derivative. As indicated above, we prepared [^{3}H]-9α-bromo-21-diazo-21-deoxycorticosterone [**19**] (25 Ci/mol). Unfortunately, the specific activity of this product is 100–1000-fold less than that required for studies on the crude renal extracts. As an alternative, we chose plasma protein as a model system to test covalent binding capability because CBG contains an abundance of corticosterone binding sites (Westphal, 1971). The feasibility of using plasma proteins as a model system was evaluated by studies on competition of diazo derivatives for [^{3}H]corticosterone binding sites, presumably in CBG.

Plasma from adrenalectomized rats was incubated with 5.2×10^{-8} *M* [^{3}H]corticosterone, with or without competing steroid, for 15 min at 25° ($n = 4$ experiments, each in duplicate). The amount bound was determined by filtration through G-50 (fine) Sephadex. At concentrations of 5×10^{-7} and 5×10^{-6} *M*, 21-diazo-21-deoxycorticosterone reduced binding of [^{3}H]corticosterone to 44 and 6% of the control values, respectively. At these concentrations, 21-diazoprogesterone reduced binding to 83 and 52%, respectively. At concentrations of 5×10^{-6} and 5×10^{-5} *M*, 9α-bromo-21-diazo-21-deoxycorticosterone reduced binding to 94 and 68%, respectively. Thus, 21-diazo-21-deoxycorticosterone had a relatively high affinity for [^{3}H]corticosterone binding site, 21-diazoprogesterone, an intermediate affinity, and 9α-bromo-21-diazo-21-deoxycorticosterone, a relatively low affinity. The relatively low affinity of the 9α-bromo derivative is in accord with earlier findings that 9α-halogenated steroids exhibit decreased binding to CBG (Westphal, 1971). Despite the relatively low affinity of the 9α-bromo derivative for CBG, the very high steroid binding capacity of these proteins made it possible to detect binding of the ^{3}H derivative.

Diluted rat plasma was incubated with [^{3}H]-9α-bromo-21-diazo-21-deoxycorticosterone (final concentration = 2.6×10^{-6} *M*) and aliquots were exposed to uv light. Covalent binding was assessed by CH_2Cl_2 extraction. In the control samples (not exposed to uv), 50% of the ^{3}H-labeled diazo derivative was CH_2Cl_2 extractable and in the uv-activated sample only 10% was extractable (Table V). In these preliminary trials, the free and bound steroids were not separated prior to irradiation, which allows unbound steroid to react with "nonspecific" binding sites after irradiation. To explore the possibility of minimizing covalent binding to nonspecific sites, aliquots of the incubation mixtures were filtered through G-50 Sephadex columns before exposure to uv light. The results in Table V indicate that activation reduced extractability from 60 to 16%, implying covalent binding. The marked reduction in total yield may indicate a higher percentage of binding to specific sites.

Table V: The Effect of uv Light on Methylene Dichloride Extractability of [^{3}H]-9α-Bromo-21-diazo-21-deoxycorticosterone from Rat Plasma.[a]

Filtration through G-50 Sephadex	Activation State	^{3}H-Labeled Steroid Concn (cpm/ml)	CH_2Cl_2 Extractable ^{3}H-Labeled Steroid: cpm/ml	CH_2Cl_2 Extractable ^{3}H-Labeled Steroid: % Extracted
No	No	25,000	12,283	50
	Yes	21,500	2,100	10
Yes	No	1,920	1,120	60
	Yes	1,920	314	16

[a] Plasma from adrenalectomized rats was incubated with [^{3}H]-9α-bromo-21-diazo-21-deoxycorticosterone (2.6×10^{-6} *M*) for 15 min at 37° and either immediately activated by exposure to uv light for 5 min or filtered through G-50 (fine) Sephadex to remove free ^{3}H-labeled steroid and then activated. Methylene dichloride was added to the plasma and vigorously mixed, and the sample was centrifuged. The organic layer was removed and an aliquot dried in a counting vial and the radioactivity measured. $n = 1$ experiment, in duplicate in each category.

The presence of photogenerated binding was explored further by susceptibility to displacement by unlabeled corticosterone. Diluted plasma was incubated with [^{3}H]-9α-bromo-21-diazo-21-deoxycorticosterone for 15 min at 37° and aliquots were irradiated with uv light. Various concentrations of corticosterone (2.6×10^{-6} to 2.6×10^{-4} *M*) were then added to each pair of samples and incubated for a further 15 min at 37°. All aliquots were cleared of free ^{3}H-labeled steroid by filtration through G-50 Sephadex. As shown in Figure 5, corticosterone displaced 70% of the ^{3}H-labeled diazo derivatives in the control samples and the extent of displacement was concentration dependent. In contrast, corticosterone, even in 100-fold excess, had no effect on the binding of the ^{3}H-labeled diazo derivative after irradiation. From the relative amounts displaceable by corticosterone in the controls and the total bound after irradiation, it appears that 20% of the tightly bound derivative may have been to corticosteroid specific sites, but further studies are needed to verify this inference.

Discussion

Aldosterone (**1**), 11-deoxycorticosterone (**2**), and 9α-fluorocortisol (**3**) are potent mineralocorticoids in anurans and

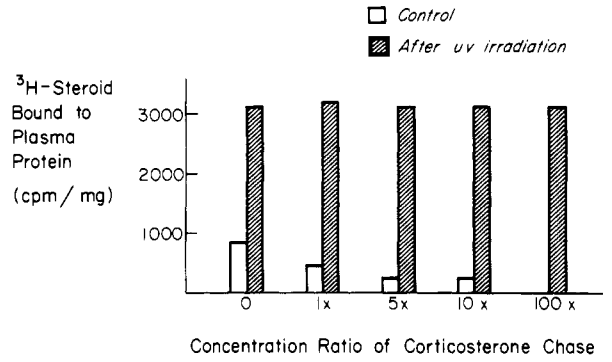

FIGURE 5: The effect of uv irradiation on exchangeability of [^{3}H]-9α-bromo-21-diazo-21-deoxycorticosterone (2.6×10^{-6} *M*) for 15 min at 37°. One of each pair of samples was irradiated with uv for 5 min. Unlabeled corticosterone or the diluent was then added to each pair at various concentrations (1X = 2.6×10^{-6} *M*) and all samples were reincubated for 15 min at 37°. The residual amount of ^{3}H-labeled steroid bound was determined by filtration through G-50 (fine) Sephadex. *N* = 1 experiment, in duplicate.

mammals and have high affinities for the mineralocorticoid receptors in the rat kidney (Edelman, 1968; Herman et al., 1968; Swaneck et al., 1970). In view of the anticipated difficulties in preparing 18-substituted steroids closely related to aldosterone, we elected to prepare the 21-diazo analog of 11-deoxycorticosterone (**2**) and 9α-halogenated, 11β-hydroxy 21-diazo ketones. These compounds, where the diazo group replaces the 21-hydroxy group in **2** and **3**, were expected to have the required structural features to retain affinity for the receptor.

After many trials with several types of starting material for the preparation of the diazo ketones, the following methods were the most convenient and gave the best yields. Commercial 11α-hydroxyprogesterone (**7**) was oxidized at C-11 with chromic acid in acetone and the resulting triketone was converted to the corresponding 3,20-bisketal with ethylene glycol in the presence of pyridinium chloride. Reduction of the bisketal and removal of the protecting groups gave 11β-hydroxyprogesterone (**8**). Treatment of **8** with sodium hypobromite gave the etio acid (**9**) identical with material obtained from adrenal tissue (Mason et al., 1937). The acid **9** was converted to the 11β-hydroxy diazo ketone (**12**) with oxalyl chloride and diazomethane. To prepare the 9α-halogenated derivative of **12**, the 9,11-olefin **11** was obtained from **9** by dehydration (HCl–HOAc). Compound **11** was also prepared by dehydration of **7** to give the olefin **10**, followed by hypobromite oxidation. Addition of HOBr, generated from *N*-bromoacetamide and perchloric acid, to **11** gave the bromohydrin **14**, which furnished the corresponding diazo ketone **15** upon treatment with oxalyl chloride and diazomethane. Finally, the 9α-fluoro diazo ketone **20** was synthesized by dehydrohalogenation of **14** to give the epoxide **17**, which was opened with HF to form the fluorohydrin **18** which was then converted to 9α-fluoro-21-diazo-11β-hydroxyandrost-4-ene-3,20-dione (**20**).

The method chosen for the synthesis of radiolabeled **19** was as follows. Dehydrogenation of **10** with 2,3-dichloro-5,6-dicyanobenzoquinone gave the 1,4,9(11)-triene **13**, which was converted to the bromohydrin **16** by side chain degradation followed by HOBr addition. Selective hydrogenation at atmospheric pressure of the 1,2-double bond in **16** with palladium on charcoal in tetrahydrofuran medium gave **14**, identical with the previous sample. Under the same conditions using tritium gas, the reduction of **16** gave labeled **14** which was converted to labeled **19**. The conditions employed should yield a product of high specific activity (Feinendegen, 1967), and the crude product indeed had high specific activity. Moreover, the isolated product had a specific activity of only 25 Ci/mol. However, it decomposed rapidly in the crystalline state although it was more stable in benzene solution.

Over the last decade attempts to purify steroid receptors have been impeded by the paucity of these proteins with respect to total cell protein, and in the case of mineralocorticoid receptors by the lability of the receptors during physical manipulations (Herman and Edelman, 1968). In this report, we have described the synthesis of 21-diazo analogs of 9α-bromocorticosterone, 9α-fluorocorticosterone, corticosterone, and progesterone. The mineralocorticoid activities of two of these derivatives were tested in the isolated toad bladder system. 9α-Bromo-21-diazo-21-deoxycorticosterone was equipotent with *d*-aldosterone at 5×10^{-8} *M* and significantly more effective than 9α-fluorocortisol (Table I). In addition, 21-diazoprogesterone proved to be as effective as progesterone in stimulating active Na^+ transport. These results indicate that functional competence may be retained after diazo substitution at C-21 and are in accord with the observation that the activities of diazoestrogens bear a relationship to that of the parent steroid (Katzenellenbogen et al., 1973). Although the isolated toad bladder system is convenient for an exploration of the functional properties of these analogs, total yields of cells are small; as a result, this tissue is impractical for receptor studies. Thus, for studies on affinities of the analogs for corticosteroid receptor sites, we chose the rat kidney system which has been well-characterized with respect to both mineralocorticoid and glucocorticoid receptors (Feldman et al., 1973; Funder et al., 1973a,b; Marver et al., 1972).

Rat kidney cytosol contains stereospecific receptors for *d*-aldosterone, previously designated type I sites and two distinct receptors for corticosterone, designated type II and type III sites (Feldman et al., 1973). The latter are distinguishable by their affinities for dexamethasone; type II sites have a high affinity for dexamethasone and type III sites very low affinity for this steroid. Similarly, *d*-aldosterone has a negligible affinity for type III sites. In the present study, [^{3}H]-*d*-aldosterone was used as the reference ligand. On the basis of previous experience with this system and at a concentration of [^{3}H]aldosterone of 5×10^{-9} *M*, about 75% of the amount bound should be to type I sites and the remainder to type II sites (Funder et al., 1973a). The results in Tables II and III indicate that the relative affinities for the aldosterone binding sites were 9α-fluoro-21-diazo-21-deoxycorticosterone > 9α-bromo-21-diazo-21-deoxycorticosterone > 21-diazoprogesterone. The most promising analogs for selective labeling of aldosterone receptor sites are the 21-diazo derivatives of 9α-fluoro- and 9α-bromo-21-deoxycorticosterone. Further studies are needed on the utility of these compounds, however, since the 9α-bromosteroid possessed only 1% of the affinity of *d*-aldosterone for the [^{3}H]aldosterone binding sites in rat kidney cytosol. Despite this relatively low affinity, with adequate controls designed to distinguish between specific and nonspecific binding, this compound might prove adequate as an affinity label, particularly if partial purification of the receptor proteins was achieved prior to initiation of covalent labeling. It is also possible that one or more of these derivatives, probably 21-diazo-21-deoxycorticosterone, would be suitable as an affinity label for glucocorticoid receptor sites.

The "active" component of the cytoplasm steroid–receptor complex translocates into the nucleus and binds to the genome (Feldman et al., 1972). The competition studies summarized in Table IV indicate that 9α-bromo-21-diazo-21-deoxycorticosterone reduces cytoplasmic binding and nuclear uptake of [^{3}H]aldosterone proportionately. This finding lends credence to the inference that this diazo derivative occupies aldosterone binding sites of the specific "receptor."

The advantage of photoaffinity labels as markers during purification of steroid receptors is based on the capability of forming the covalent bond after almost all of the free steroid has been removed from the medium. This procedure was used in the studies with the plasma protein model system (cf. Table V). Photoactivation in the presence of the free diazo derivative resulted in excess covalent binding, probably to reactive sites on any protein available to the photogenerated intermediate. The results in Figure 5 suggest that the photogenerated irreversible binding was to steroid-specific sites but further studies are required to establish that this actually was the case. Moreover, further progress depends on solving the problem of introducing an appropriate marker, such as tritium, without evoking decomposition of the derivative. The technique used to tritiate 9α-bromo-21-diazo-21-deoxycorticosterone was expected to yield a specific activity of 25 Ci/mmol but after purification the product had a specific activity of only 25 Ci/mol. This level of activity was sufficient for studies on covalent binding to plasma proteins but is at least 100-fold less than required for isolation of corticosteroid receptors. Thus, improved techniques for preparing high specific activity, stable derivatives need to be developed. Alternatively, the utility of the diazo steroids might be enhanced by devising nonradioactive markers or detecting the steroid by very sensitive techniques (e.g., mass spectrometry, Raman spectroscopy).

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