Zealand Inc. and, in part, by the Medical Research Council of New Zealand.

References and Notes

(1) B. F. Cain, G. J. Atwell, and W. A. Denny, *J. Med. Chem.*, **18**, 1110 (1975).
(2) B. F. Cain, R. N. Seelye, and G. J. Atwell, *J. Med. Chem.*, **17**, 922 (1974).
(3) B. F. Cain, G. J. Atwell, and R. N. Seelye, *J. Med. Chem.*, **14**, 311 (1971).
(4) G. J. Atwell, B. F. Cain, and R. N. Seelye, *J. Med. Chem.*, **15**, 611 (1972).
(5) B. F. Cain, G. J. Atwell, and W. A. Denny, *J. Med. Chem.*, **19**, 772 (1976).
(6) B. F. Cain and G. J. Atwell, *J. Med. Chem.*, **19**, 1409 (1976).
(7) B. F. Cain and G. J. Atwell, *J. Med. Chem.*, **19**, 1124 (1976).
(8) M. J. Waring, *Eur. J. Cancer*, **12**, 995 (1976).
(9) J. C. Wang, *J. Mol. Biol.*, **89**, 783 (1974).
(10) B. F. Cain, G. J. Atwell, and R. N. Seelye, *J. Med. Chem.*, **12**, 199 (1969).
(11) J. W. Griffin and D. H. Hey, *J. Chem. Soc.*, 3334 (1952).
(12) J. H. Burckhalter, F. H. Tendick, E. M. Jones, P. A. Jones, W. F. Halcomb, and A. L. Rawlins, *J. Am. Chem. Soc.*, **70**, 1363 (1948).
(13) C. K. Banks, *J. Am. Chem. Soc.*, **66**, 1127, 1131 (1944).
(14) J. R. Keneford, J. S. Morley, J. C. E. Simpson, and P. H. Wright, *J. Chem. Soc.*, 1104 (1950).
(15) A. Maggiolo and A. P. Phillips, *J. Org. Chem.*, **16**, 376 (1951).
(16) O. G. Backeberg and J. L. C. Marais, *J. Chem. Soc.*, 381 (1942).
(17) A. Albert and N. Gledhill, *J. Soc. Chem. Ind., London*, **64**, 169 (1945).
(18) A. Albert, R. Goldacre, and E. Heymann, *J. Chem. Soc.*, 651 (1943).
(19) A. Albert, Ed., "The Acridines", 2nd ed, E. Arnold, London, 1966, p 489.
(20) B. C. Baguley, this laboratory, unpublished results.
(21) J. Clark and D. D. Perrin, *Q. Rev., Chem. Soc.*, **43**, 313 (1964).
(22) B. F. Cain and W. A. Denny, *J. Med. Chem.*, **20**, 515 (1977).
(23) A. Albert and E. P. Sergeant, Ed., "Ionization Constants of Acids and Bases", Methuen and Co., London, 1962.
(24) T. O. Soine, *J. Am. Pharm. Assoc.*, **33**, 141 (1944).
(25) R. Gaudy, *Can. J. Chem.*, **31**, 1060 (1953).
(26) W. S. Fones, R. Stander, and J. White, *J. Org. Chem.*, **16**, 708 (1951).
(27) R. H. Mizzoni, M. A. Hennesy, and C. R. Schloz, *J. Am. Chem. Soc.*, **76**, 2414 (1954).
(28) A. Müller and P. Krauss, *Monatsh. Chem.*, **61**, 219 (1932).
(29) R. C. Elderfield, W. J. Gensler, T. H. Bembry, F. Brody, L. Wiedenhold, and B. Newman, *J. Am. Chem. Soc.*, **68**, 1568 (1946).
(30) H. Schmid and P. Karrer, *Helv. Chim. Acta*, **31**, 1497 (1948).
(31) R. Winterbottom, J. W. Clapp, W. H. Miller, J. P. English, and R. D. Roblin, *J. Am. Chem. Soc.*, **69**, 1393 (1947).
(32) M. Frankel and P. Moses, *Tetrahedron*, **9**, 289 (1960).
(33) G. J. Atwell and B. F. Cain, *J. Med. Chem.*, **11**, 295 (1968).

Synthesis and Biological Action of Two Glucocorticoid Alkylating Agents[1]

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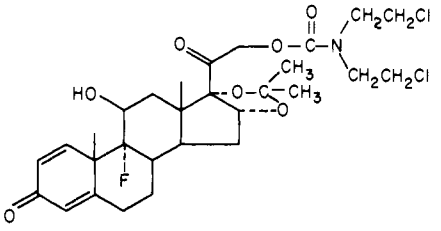
Two alkylating glucocorticoids have been synthesized in order to test the possibility of alkylating glucocorticoid receptors. The title compounds are 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 21-[bis(2-chloroethyl)carbamate] 16,17-acetonide (I) and 11β,17α,21-trihydroxypregn-4-ene-3,20-dione 21-[bis(2-chloroethyl)carbamate] (II), prepared from triamcinolone acetonide and cortisol, respectively, through the reaction of the C-21 hydroxyl group with phosgene and di-2-chloroethylamine in the presence of triethylamine. Both compounds are biologically active as inhibitors of the growth of cultured mouse fibroblasts and are able to compete for the specific binding of radiolabeled triamcinolone acetonide to the L929 cell receptor. The bis(2-chloroethyl)carbamate moiety is capable of reacting with nucleophilic groups as evidenced by the colorimetric reaction with 4-(*p*-nitrobenzyl)pyridine. Both the interaction with the receptor and inhibition of cell growth by these two glucocorticoids are reversible.

Several steroid derivatives with attached alkylating moieties have been found to be active against selected animal tumor systems. These include the cholesterol derivative, phenesterin[2] (NSC-104469), and two estradiol derivatives, estradiol mustard[3] (NSC-112259) and estracyt[4] (NSC-89199). A glucocorticoid alkylating agent has also been synthesized and demonstrated to be active against the L1210 murine leukemia model.[5] This compound, Leo 1031 (NSC-134087), is a chlorambucil ester of prednisolone at the 21 position that has recently been administered to a few patients with chronic lymphocytic leukemia[6] and lymphocytic lymphoma.[7] The rationale behind the development of these compounds was based upon two objectives. First, it was hoped that the passage of the alkylating agent across cell membranes might be facilitated by linking it to a more lipophilic moiety, like a steroid. Second, in the case of the sex hormone and glucocorticoid derivatives, it was hoped that two cytotoxic actions might be achieved—one resulting from hormone–receptor interaction and the second being due to a nitrogen mustard effect. The two actions could be produced by the intact compounds, or hydrolysis of the linkage between the steroid and alkylating moieties as a result of cellular esterase activity could yield two species with different cytotoxic mechanisms.

We have prepared two compounds with which to test the possibility of specifically alkylating the receptor molecule for glucocorticoids. The rationale for the compounds synthesized was as follows. In order to obtain specific alkylation it was considered optimal to utilize a steroid of very high affinity which would bind tightly to the receptor and permit us to rapidly eliminate the unbound compound without significant dissociation of the steroid–receptor complex. Accordingly, we have synthesized an alkylating derivative of the very potent glucocorticoid triamcinolone acetonide.[8] It was also thought preferable to utilize a relatively slow-reacting alkylating moiety. This might permit us to eliminate the unbound

compound before it could alkylate a significant number of proteins other than the receptor. The alkylating function is of the bis(β-chloroethylamine) type attached through a carbamate linkage to the C-21 oxygen atom of the steroid. The weak electron-withdrawing effect exerted on the nitrogen atom by the carbamate carbonyl is included in order to slow the rate of immonium ion formation. It would also seem optimal to have the alkylating function placed close to the steroid nucleus in order to enhance the probability of reaction with the receptor. The alkylating moiety is attached to the 21-hydroxyl group, permitting the rest of the steroid molecule to bind specifically to the receptor. We also synthesized the analogous derivative of cortisol, a glucocorticoid that dissociates much more rapidly from the receptor than triamcinolone acetonide.[9] This derivative (II) was prepared in order to permit us to examine the kinetics of the binding reaction. This study describes the synthesis of these two compounds, confirmation of structures, their activity as inhibitors of cell growth, and their chemical reactivity as alkylating agents.



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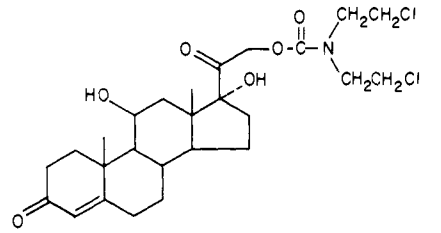

II

Experimental Section

Elemental analysis was performed by Midwest Microlab, Ltd., Indianapolis, Ind. Infrared spectra were recorded on a Perkin-Elmer 337 spectrophotometer. Mass spectra were recorded by solid probe at 25 eV on a Finnigan 3200 operating in the electron impact mode. Melting points were determined on a Thomas-Hoover apparatus and are corrected.

[1,2,3-^{3}H]Triamcinolone acetonide [21.6 Ci/mmol; radiochemical purity greater than 97% by thin-layer chromatography on silica gel GF in methylene chloride–methanol (24:1 v:v)] was purchased from New England Nuclear, Boston, Mass., and diluted to a specific activity of 10.6 or 5.3 Ci/mmol prior to use. Triamcinolone and its two isomeric acetophenone derivatives (structures presented in Figure 1) were provided by Drs. Patrick A. Diassi and Arnold Welch of The Squibb Institute for Medical Research, Princeton, N.J.

Triamcinolone Acetonide Alkylating Agent (I). Triamcinolone acetonide was prepared from triamcinolone by the method of Fried et al.[10] The recrystallized triamcinolone acetonide (400 mg, 1 mmol) was dissolved in a mixture of dioxane (10 mL) and tetrahydrofuran (THF) (4 mL), and 3 mL (3.78 mmol) of a 12.5% solution of phosgene in benzene (MC/B Manuf. Chemists) was added under anhydrous conditions. After stirring at room temperature for 24 h, excess phosgene and THF were removed by vacuum distillation at room temperature, producing 15 mL of a pale yellow liquid. A solution[11] of di-2-chloroethylamine (0.22 g, 1.5 mmol) in benzene (30 mL), previously mixed with triethylamine (0.15 g, 1.5 mmol), was added to the straw-yellow liquid dropwise over a period of 30 min while stirring at −10 °C, producing a cloudy mixture. The temperature was allowed to rise to room temperature over the next hour and stirring was continued for a total of 17 h. This mixture was diluted with 100 mL of ether and the precipitated triethylamine hydrochloride (160 mg, mp 254–257 °C) was removed by filtration. The filtrate was washed with 2% HCl (40 mL), 5% $NaHCO_3$ (40 mL), and distilled water (4 × 20 mL) and dried with Na_2SO_4, and the residual solvents were removed by vacuum distillation at room temperature. The resultant pale yellow solid (620 mg) was chromatographed on an alumina column (1.5 × 20 cm), using chloroform as eluent. The first 20 mL of eluate contained a yellow amorphous material and the following 60 mL contained the major component. The solvent was removed from this 60-mL portion in vacuo and the residue was recrystallized three times from chloroform (4 mL) and hexane (17 mL), with the production of 150 mg of rosettes of very electrostatic needles (yield 30%): mp 222–225 °C dec; $[\alpha]^{20}_D$ +104.7° (c 2.0, MeOH); $\lambda_{max}^{CH_3OH}$ 230 nm (ε 23 333); IR ν_{max}^{KBr} 3400, 1725–1705, 1665, 1620, 1480, 1300, 1130, 1088, 1065, 900, 770, 740, 660, and 555 cm^{-1}; *m/e* 601 (P − 1). Anal. ($C_{29}H_{38}Cl_2FNO_7$) C, H, N.

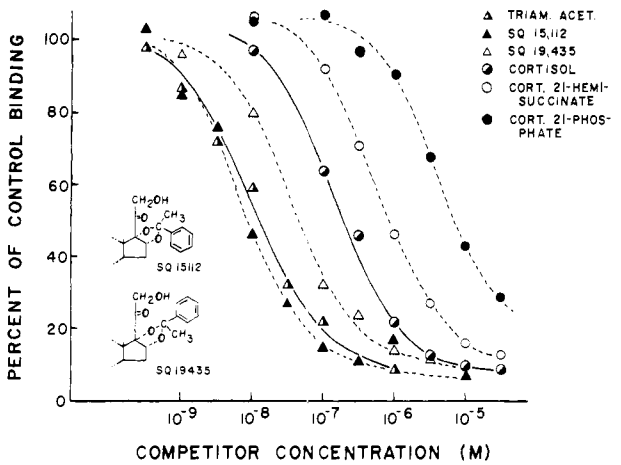

Figure 1. Ability of some substituted steroids to compete for the binding of [^{3}H]triamcinolone acetonide. Replicate 0.5-mL samples containing 0.4 mL of L cell 100 000g supernatant were incubated for 20 h at 0 °C with 1 × 10^{-8} M [^{3}H]triamcinolone acetonide and either vehicle or various concentrations of competing unlabeled steroid. The amount of bound radioactivity was determined on each sample at the end of the incubation. The data are expressed as the percent of the binding achieved in control samples containing vehicle vs. the concentration of the competing steroid. The structures of the two acetophenone derivatives of triamcinolone are also presented. Competitors are ▲, triamcinolone acetonide; ▲, SQ 15112; △, SQ 19435; ◑, cortisol; ○, cortisol 21-hemisuccinate; ●, cortisol 21-phosphate.

Cortisol Alkylating Agent (II). Cortisol (Schwarz/Mann, Orangeburg, N.Y.; mp 217–222 °C) was dissolved (360 mg, 1 mmol) in 10 mL of THF and then added at 0 °C, while stirring, to a mixture of THF (5 mL), benzene (5 mL), and 1 mL (1.26 mmol) of a solution of 12.5% phosgene in benzene. This solution was stirred for 30 min at 0 °C and then at room temperature for 1.5 h. After cooling to 0 °C, a solution of triethylamine (0.1 g, 1 mmol) in benzene (10 mL) was added over a period of 10 min with stirring. This mixture was allowed to reach room temperature and stirred for 30 min. The precipitated triethylamine hydrochloride (130 mg, mp 255–257 °C) was removed by filtration and the filtrate was concentrated in vacuo to a volume of approximately 10 mL. A solution[11] of di-2-chloroethylamine (0.22 g, 1.5 mmol) in benzene (30 mL), previously mixed with triethylamine (0.15 g, 1.5 mmol), was added dropwise to 10 mL of cortisol chloroformate solution over a period of 30 min, while stirring at 0 °C. The mixture became cloudy. THF (50 mL) was added and stirring was continued for 3 h at room temperature. Triethylamine hydrochloride (100 mg, mp 254–257 °C) was removed by filtration and the filtrate was washed with 2% HCl (30 mL), 5% $NaHCO_3$ (40 mL), and distilled water (4 × 20 mL) and dried with Na_2SO_4. After solvents were removed in vacuo at 45 °C, 460 mg of fluffy, white, amorphous residue remained. Thin-layer chromatography [silica gel, $CHCl_3$–CH_3OH (100:1.5)] of this material produced four spots with R_f values of 0.69, 0.60, 0.50, and 0.23. The major

spot was at R_f 0.60. The R_f of cortisol is 0.23. The mixture (50 mg/plate) was separated on thick-layer chromatography plates (silica gel, 20 × 20 cm, 2 mm thick), using $CHCl_3$–CH_3OH (100:8) for processing. Each plate was chromatographed with the solvent system two times. The area of the major band (R_f 0.63 after the second solvent run) was collected and extracted with chloroform–acetone (1:1). The solvents were removed in vacuo, leaving 190 mg (yield 36%) of white, amorphous, electrostatic powder: mp 150–170 °C; $[\alpha]^{20}_D$ +123.5° (c 2.0, MeOH); $\lambda_{max}^{CH_3OH}$ 225 nm (ε 24 468), 320 (79 787). Attempts to crystallize this material from many solvent systems failed; however, it always yielded one spot on thin-layer chromatography. The infrared spectrum showed ν_{max}^{KBr} at 3425, 1725–1700, 1665, 1620, 1480, 1200, 1135, 1120, 1050, 950, 780, 740 and 650 cm^{-1}; *m/e* 530. Anal. ($C_{26}H_{37}O_6NCl_2$) C, H, N.

Cell Culture and Cell Fractionation. L929 mouse fibroblasts were grown in monolayer culture in Joklik medium as previously described.[12] The methods for the dose–response experiments for growth inhibition have also been presented in a previous publication.[13] In the work of this paper steroids were dissolved in dimethyl sulfoxide and added to the cultures in 0.1% of the volume of the growth medium. Controls contained the same concentration of vehicle.

Cells in log phase of growth were harvested from Roux bottles by scraping into cold Earle's saline. They were washed once by resuspension in several volumes of the saline solution and centrifugation at 600g at 0–4 °C. After washing, the cells were suspended in 1.6 vol of an iced hypotonic solution of 10 mM Tris buffer at pH 7.35 and 0.5 mM EDTA and homogenized with 15 strokes of a tight-fitting pestle in a Dounce-type glass homogenizer. The broken cell suspension was centrifuged at 27 000g at 0–4 °C for 10 min and the supernatant was centrifuged at 100 000g for 45 min at 2 °C. The 100 000g supernatant defines the soluble fraction of the cell as that term is used in this paper.

Incubation with Steroid and Binding Assay. The soluble preparation (0.4 mL), which contained 5–10 mg of 100 000g supernatant protein per milliliter, was incubated with steroid at 0 °C in a total incubation volume of 0.5 mL as previously described.[9] Radioactive triamcinolone acetonide was added from a stock solution dissolved in 10% ethanol. Nonradioactive steroids (including steroid alkylators) were dissolved in Me_2SO and added in 1% of the incubation volume. All control samples had equivalent concentrations of vehicle. Nonspecific binding was determined by incubating the mixture with 5 × 10^{-5} M dexamethasone as previously detailed.[9] The bound steroid was separated from the free compound on short Sephadex G-25 columns and the bound radioactivity assayed as described previously.[9]

Colorimetric Estimation of Alkylation Activity. The ability of the steroid derivatives to alkylate 4-(*p*-nitrobenzyl)pyridine was compared to that of mechlorethamine (HN_2). Solutions of HN_2 or steroid alkylator were prepared in 1.0% Me_2SO in water. Aliquots of solution, 3 mL, were incubated for 20 min in a boiling water bath with 4-(*p*-nitrobenzyl)pyridine in acetate buffer, pH 4.0, exactly as described by Sladek[14] in a modification of the method of Epstein et al.[15] In this assay an addition product is formed which gives rise to a purple color on the addition of base. The colored product is extracted into ethyl acetate and the absorbance measured at 540 nm exactly 6 min after the introduction of 5.0 N NaOH[14].

Results

Molecular Modifications that Permit Binding to the Receptor. In order to determine what portion of the glucocorticoid structure might be modified without eliminating binding to the specific receptor site, several compounds were evaluated for their ability to compete for the binding of radiolabeled triamcinolone acetonide. The competition potencies of compounds modified at the 21-carbon and on the acetonide group at positions 16 and 17 are compared with those of cortisol and triamcinolone acetonide in Figure 1. If the methyl group of the acetonide in the α position is replaced by a phenyl moiety (SQ 15112), the competitive potency of the steroid is unaltered. Phenyl substitution in the β configuration on the acetonide

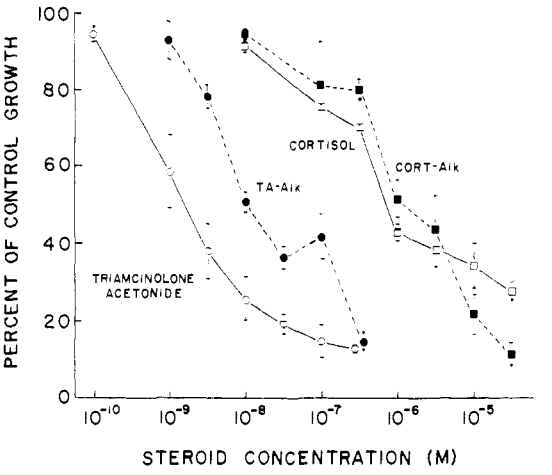

Figure 2. Ability of glucocorticoids and compounds I and II to inhibit the growth of fibroblasts in culture. Steroids were added to replicate cultures of L 929 cells in the early phase of growth and cells were harvested and counted 6 days later. Each value represents the mean and standard error of three replicate cultures expressed as a percent of the growth achieved in controls receiving vehicle alone. ○, triamcinolone acetonide; ●, I (TA-Alk); □, cortisol; ■, II (Cort-Alk).

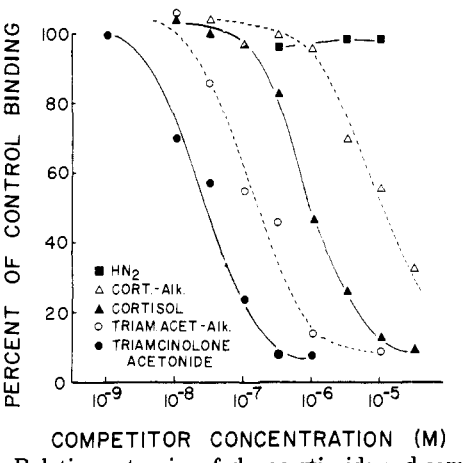

Figure 3. Relative potencies of glucocorticoids and compounds I and II in competing for the binding of radioactive triamcinolone acetonide to the soluble fraction of L cells. Methods are as described in the legend for Figure 1 except that [^{3}H]triamcinolone acetonide was present at a concentration of 2 × 10^{-8} M. ●, triamcinolone acetonide; ○, I (Triam Acet-Alk); ▲, cortisol; △, II (Cort-Alk). The solid squares show the binding achieved in the presence of mechlorethamine (HN_2).

(SQ 19435) produces a small reduction in the binding affinity. The presence of a 21-hemisuccinyloxy group produced only a moderate loss in competitive ability (K_D = 1.9 × 10^{-7} M) compared to that of cortisol (K_D = 4.3 × 10^{-8} M). However, the presence of phosphate at this position produced a marked reduction in binding affinity. From these observations it was concluded that substitutions could be made at either the 21 position or on the acetonide function without a total loss in ability to bind to the receptor. Principally for reasons of ease of chemical synthesis, the alkylating derivatives were prepared at position 21. As presented in the Experimental Section, elemental analysis, infrared data, and mass spectra for compounds I and II are appropriate for the structures presented.

Biological Activity of Steroid Alkylating Agents. Replicate cultures of fibroblasts were grown in the presence of various concentrations of triamcinolone acetonide, cortisol, or their alkylating derivatives. As presented in Figure 2, both compounds I and II are effective inhibitors

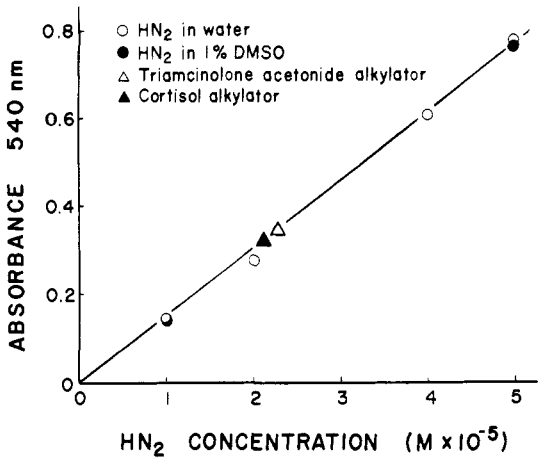

Figure 4. Comparison of the alkylating activity of I and II with that of mechlorethamine. Various concentrations of HN_2 dissolved in water (○) or in 1% Me_2SO (●) were incubated with nitrobenzylpyridine and assayed for the production of the alkylated derivative as described in the Experimental Section. The same assay was performed with 10^{-4} M I (△) or II (▲) dissolved in 1% Me_2SO. The absorbance recorded with HN_2 has been plotted vs. its concentration. The absorbance measured for 10^{-4} M I and II has been plotted on a line using the HN_2 data as a standard curve.

of cell growth, but they are somewhat less potent than the unmodified steroids.

The steroid alkylating agents compete against the specific binding of radioactive triamcinolone acetonide in a cell-free preparation (Figure 3). They are each about an order of magnitude less potent in this respect than their parent compounds. The concentration of radioactive triamcinolone acetonide in this experiment is greater than that required for occupation of all of the specific binding sites.[9] An apparent dissociation constant for I or II can be calculated from the competition curves by multiplying the measured dissociation constant of cortisol by the ratio of the concentration of I or II producing 50% inhibition of [^{3}H]triamcinolone acetonide binding to the concentration of cortisol that yields the same amount of competition. The dissociation constant for cortisol obtained by direct measurement of rate constants in the soluble fraction of L cells is 4.3×10^{-8} M.[9] Apparent dissociation constants for I and II calculated from the competition curves of Figure 3 are 7.5×10^{-9} and 4.3×10^{-7} M, respectively.

As shown by the solid squares in Figure 3, mechlorethamine, a highly reactive nitrogen mustard, does not affect the specific binding of triamcinolone acetonide in the concentration range examined. It is appropriate to ask whether the alkylating function on the steroid can react with nucleophilic groups and form covalent bonds. In order to answer this question we utilized a colorimetric assay for alkylation and compared the reactivity of I and II (at 10^{-4} M) to that of HN_2. As can be seen in Figure 4, both I and II reacted in a manner equivalent to a concentration of about 2×10^{-5} M HN_2. The parent steroids without the alkylating side chain produced no effect in the assay. The incubation of HN_2, I, or II with 4-(*p*-nitrobenzyl)pyridine was carried out far beyond the time required for maximum extent of covalent bond formation. Thus, I and II under the conditions of this assay are capable of about 20% as much reaction with the pyridine compound as HN_2.

If the steroid alkylating agents form covalent products with the specific glucocorticoid binding component, they are not formed in sufficient amount to be observed kinetically. This is shown in the experiment presented in

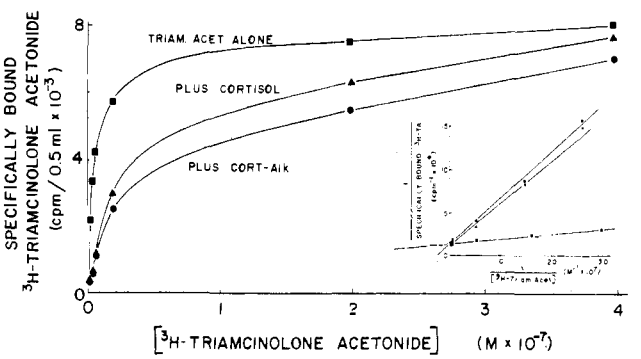

Figure 5. Kinetics of inhibition of binding by the cortisol alkylating agent. Replicate aliquots of 100 000g supernatant were incubated in the presence of vehicle, 10^{-7} M cortisol, 10^{-6} M II, or 3×10^{-5} M dexamethasone for 4 h in ice. Various concentrations of [^{3}H]triamcinolone acetonide were then added, and the incubations were continued for 18 h. The bound radioactivity was determined as described in the Experimental Section and the nonspecific binding (that found in the presence of 3×10^{-5} M competing dexamethasone) was substracted from each value. The specific binding in each sample, expressed as thousands of cpm bound per 0.5-mL incubation, is plotted vs. the molar concentration of free radioactive triamcinolone acetonide. The inset presents a double reciprocal plot of the same data. ■, specific binding of triamcinolone acetonide after a 4-h preincubation in the presence of vehicle alone; ▲, after preincubation and subsequent incubation in the presence of 10^{-7} M cortisol; ●, in the presence of 10^{-6} M II (Cort-Alk).

Figure 5. Replicate aliquots of supernatant were incubated for 4 h with 10^{-7} M cortisol or 10^{-6} M II. The specific receptor sites are 85% occupied when they are in equilibrium with this concentration of cortisol at 0 °C.[9] At the end of 4 h of preincubation, radiolabeled triamcinolone was added and exchange binding was allowed to proceed for an additional 18 h. As can be seen from Figure 5, at high concentrations triamcinolone acetonide binds nearly maximally in the presence of either cortisol or II and the kinetics of the binding are of the competitive type (inset, Figure 5). This experiment was done with cortisol and its alkylating analogue because the dissociation rate of cortisol ($t_{1/2}$ = 60 min) is rapid enough to permit nearly complete exchange during a reasonable time interval.[9]

The effect of steroid alkylating agent on cell growth is also reversible. This is shown in the experiment presented in Figure 6. In this case cells at low density were exposed to triamcinolone acetonide and I for 48 h at 37 °C and then the drugs were removed. The effect of the steroid and the alkylator analogue reversed in approximately the same manner after drug removal.

Discussion

Substituting glucocorticoids at the 21-carbon position often results in a loss of biological activity,[16] including loss of antileukemic potency.[17] Several compounds are exceptions, however, and it is clear from the experiments of Figures 1 and 3 that large functional groups can be attached at this position with only moderate diminution in ability to compete for specific binding. Since these experiments were conducted at 0 °C, it is highly unlikely that this competition is due only to products resulting from enzymatic hydrolysis of the 21-carbamate linkage. Furthermore, equivalent competition can be demonstrated in binding experiments of only a few minutes duration (data not presented). In order to test the possibility that significant hydrolysis occurs under the experimental conditions employed, we would require compound I and II synthesized with radioactive labels in the steroid and alkylating moieties.

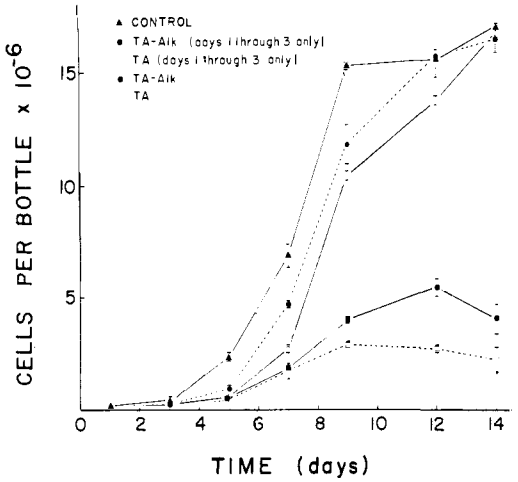

Figure 6. Reversibility of growth inhibition by triamcinolone acetonide and triamcinolone acetonide alkylating agent. Vehicle, triamcinolone acetonide, or I was added to replicate 15-mL monolayer cultures of L cells on day 1, 18 h after they were inoculated. The final concentration of the steroid or steroid alkylator was 3×10^{-7} M. On day 3 the medium was changed in all bottles and half of the steroid-containing cultures were switched to steroid-free medium. The medium in all cultures was renewed every other day thereafter and the number of cells per bottle was counted. ▲, cultures exposed to vehicle alone; ●, cultures grown in the presence of 3×10^{-7} M I (TA-Alk) from days 1 to 3 and then in the presence of vehicle; ○, cultures exposed to triamcinolone acetonide on days 1–3 only; ■, maintained in the continuous presence of I; □, triamcinolone acetonide continuously present. Each value represents the mean and standard error of counts determined on three replicate cultures.

The relative contribution of steroid (hormone) and alkylating effects to the cytotoxicity of steroidal alkylators has not been well defined. Estradiol mustard, for example, has hormonal action[3b] but the extent of the alkylating activity is not clear. After intraperitoneal injection of estradiol mustard into rats, there is a depletion of uterine cytoplasmic receptor and concomitant translocation to the nucleus.[18] This process occurs only after a delay of several hours, suggesting that estradiol mustard may be slowly converted to a metabolite (possibly free estradiol) which is able to bind to the receptor. Estradiol mustard has been shown to compete for the binding of radioactive estradiol in rat uterine cytosol.[19] However, the calculated dissociation constant (3×10^{-6} M) is approximately four orders of magnitude weaker than that for estradiol itself. As the competition observed in cell-free preparations could have been produced by contamination of the mustard preparation with as little as 0.02% free estradiol, there is no clear evidence that this drug can bind directly to the receptor in the unaltered form.

It is important to note that the simultaneous administration of estradiol and the alkylating function (given as phenester, NSC-116785) as separate entities is more effective against mammary carcinoma (R3230AC) in the rat than would be predicted from an addition of the effects of each agent alone.[20] The effect of coadministration of the two agents separately, however, is also greater than that of estradiol mustard. If the alkylating moiety is important in contributing to the cytotoxic action of estradiol mustard, the mechanism of that contribution is not at all clear.

The glucocorticoid alkylating agents synthesized in this work were designed to permit binding to the specific receptor site. From our observations we would also predict that the 21-chlorambucil ester of prednisolone (Leo 1031) can interact with the receptor and produce a hormone effect. There is no indication that the alkylating moiety contributes to the cytotoxicity of either Leo 1031 or the compounds presented in this paper. We would presume on the basis of the reversibility of both the binding and the growth inhibitory effect (presented in Figures 5 and 6, respectively) that there is no significant alkylation of the receptor and insufficient alkylation (at the concentrations examined) of other cellular constituents to provide a therapeutically useful advantage over the steroid alone.

While the desired specific alkylation of the receptor was not seen in this work, some synthetic possibilities for glucocorticoids are suggested. For example, the ability of the acetophenone derivatives to compete directly for specific binding suggests that the α-phenyl moiety would be an appropriate site for the attachment of a spin label useful for studying glucocorticoid–receptor interaction. The goal of achieving a receptor-site alkylating steroid is important enough to warrant continued studies. Such a steroid would certainly be very useful in expanding our understanding of steroid–receptor interactions. We are currently pursuing this possibility by synthesizing compounds with photoactive groups at the 21 position and on the 16,17-acetonide moiety. These steroids may have the potential of binding to the receptor site in a covalent manner after ultraviolet irradiation.

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References and Notes

(1) This work was presented at the 173rd National Meeting of the American Chemical Society, Division of Medicinal Chemistry, New Orleans, La., March 1977.
(2) E. P. Vollmer, D. J. Taylor, I. J. Masnyk, D. Cooney, B. Levine, C. Piczak, and L. Trench, *Cancer Chemother. Rep.*, 4, 103 (1973).
(3) (a) M. E. Wall, G. S. Abernethy, F. I. Carroll, and D. J. Taylor, *J. Med. Chem.*, 13, 810 (1969); (b) E. P. Vollmer, D. J. Taylor, I. J. Masnyk, D. Cooney, B. Levine, and C. Piczak, *Cancer Chemother. Rep.*, 4, 121 (1973).
(4) R. Y. Kirdani, J. Müntzing, M. J. Varkarakis, G. P. Murphy, and A. A. Sandberg, *Cancer Res.*, 34, 1031 (1974).
(5) A. H. Evenaar, E. H. R. Wins, and L. M. van Putten, *Eur. J. Cancer*, 9, 773 (1973).
(6) L. Brandt, I. Könyves, and T. R. Möller, *Acta Med. Scand.*, 197, 317 (1975).
(7) T. R. Möller, L. Brandt, I. Könyves, and L. G. Lindberg, *Acta Med. Scand.*, 197, 323 (1975).
(8) Abbreviations used: triamcinolone acetonide, 9α-fluoro-11β,16α,17α,21-tetrahydroxypregna-1,4-diene-3,20-dione 16,17-acetonide; triamcinolone acetonide alkylating agent, triamcinolone 21-[bis(2-chloroethyl)carbamate] 16,17-acetonide; dexamethasone, 9α-fluoro-16α-methyl-11β,-17α,21-trihydroxypregna-1,4-diene-3,20-dione; cortisol, 11β,17α,21-trihydroxypregn-4-ene-3,20-dione; cortisol alkylating agent, cortisol 21-[bis(2-chloroethyl)carbamate]; Me_2SO, dimethyl sulfoxide; HN_2, mechlorethamine.
(9) W. B. Pratt, J. L. Kaine, and D. V. Pratt, *J. Biol. Chem.*, 250, 4584 (1975).
(10) J. Fried, A. Borman, W. B. Kessler, P. Grabowich, and E. F. Sabo, *J. Am. Chem. Soc.*, 80, 2338 (1958).

(11) W. C. J. Ross and J. G. Wilson, *J. Chem. Soc.*, 3616 (1959).
(12) B. W. Kemper, W. B. Pratt, and L. Aronow, *Mol. Pharmacol.*, **5**, 507 (1969).
(13) W. B. Pratt and L. Aronow, *J. Biol. Chem.*, **241**, 5244 (1966).
(14) N. E. Sladek, *Cancer Res.*, **31**, 901 (1971).
(15) J. Epstein, R. W. Rosenthal, and R. J. Ess, *Anal. Chem.*, **27**, 1435 (1955).
(16) I. E. Bush, *Pharmacol. Rev.*, **14**, 317 (1962).
(17) J. J. Jaffe, G. A. Fisher, and A. D. Welch, *Biochem. Pharmacol.*, **12**, 1081 (1963).
(18) R. E. Shepherd, K. Huff, and W. L. McGuire, *J. Natl. Cancer Inst.*, **53**, 895 (1974).
(19) R. B. Everson, R. W. Turnell, J. L. Witliff, and T. C. Hall, *Cancer Chemother. Rep.*, **58**, 353 (1974).
(20) R. B. Everson, T. C. Hall, and J. L. Witliff, *Cancer Chemother. Rep.*, **57**, 353 (1973).

Quantitative Relationships between Steroid Structure and Binding to Putative Progesterone Receptors[1]

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Relationships between chemical structure of androst-4-en-3-one derivatives and their affinity for putative progesterone receptors are described. The binding affinity for 55 derivatives can be expressed by the equation log relative binding affinity (rabbit receptor) = 1.79 + 0.18 (±0.11) π_a + 1.45 (±0.21) π_b + 0.010 (±0.002) (surface area in hydrophobic pockets) − 0.012 (±0.003) (surface area out of hydrophobic pockets) − 0.99 (±0.21) MK − 0.33 (±0.08) (conformational changes). For this equation, r = 0.88. The equation successfully predicts the affinities of other compounds in the literature. The importance of the surface area terms is discussed.

A number of QSAR studies relating the structure of steroids to their pharmacological activity have appeared in recent years,[2-6] following our initial report in this area. Although there has been controversy regarding appropriate parameters to employ in such regressions, certainly the major difficulty in these studies has been their attempt to relate animal pharmacology to simple physiochemical vectors. Since animal results represent the sum of a number of part processes (receptor affinity, drug metabolism, drug distribution, and intrinsic activity), the coefficient for each variable in the regression will be the *net average* for the effect on *all* of these activities. The attempt to *average these very different contributions* is probably responsible, far more than the choice of a particular steric or hydrophobic parameter, for the scatter observed in the regressions.

There is a growing body of evidence which indicates that induction of protein synthesis mediates the action of steroid hormones on growth, differentiation, and metabolism in target tissues.[7] 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. Thus, in principle, it is possible to study a *single* part process, namely, drug–receptor affinity, in the case of the steroids.

The advantage of examining a single part process is seen in a brilliant study from Hansch's laboratory.[8-10] The enzyme affinities of over 1000 enzyme inhibitors were analyzed in terms of enzymic space around each substituent through the use of multiparameter regression techniques. Several important principles emerged from this monumental work. First, the usual qualitative relationships so familiar to the medicinal chemist could be expressed in quantitative fashion for hundreds of compounds. Second, there is difficulty in parameterizing the hydrophobic term by a single variable—enzymic space is better represented in terms of polar and nonpolar pockets. Third, the activity of a molecule can best be represented by setting up terms for *several positions* in the molecule.

Table I. Bondi Surface Area

Radical	Surface area, Å²		Surface area, Å²
–C–	0	–O–	10.0
–C–H	9.5	=CH–	17.9
$>CH_2$	22.4	$=CH_2$	30.9
$-CH_3$	35.2	–C≡	16.3
>C=CH–	23.1	≡CH	28.9
>C=O	27.6	Phenyl	88.5

As a result of our recent study on the thermodynamics of steroid–receptor interactions,[11] we have been able to approach these difficulties in parameterization from a somewhat different standpoint. We found[11] that binding forces for steroid–receptor interactions are primarily hydrophobic, except for specific polar interactions, and that it is possible to use surface area[12] as a parameter for hydrophobic bonding.[13] Second, the conformation of the A ring in glucocorticoids, which we have shown to be covariant with biological activity,[14] can be parameterized in the equation by the C-3 to C-17 distance. Third, a polar interaction term, analogous to the hydrophobic term, must be included.

In the present study, we describe the extension of these efforts to the progestational receptor system using the data of Kontula et al.[15]

Methods. Multiple regression analysis was accomplished with a stepwise regression program adapted for the PROPHET[16] time-sharing system. Octanol–water partition coefficients were taken from the compilations of Hansch and co-workers[17] and surface area of various substituents was taken from the work of Bondi[12] (Table I). The value used was the *net change* in surface area. Thus, for a 6α-methyl substituent, the net change is obtained by subtracting 22.4 Å², the value for the C-6 $-CH_2-$ group, and adding 9.4 Å² (C-6 –CH) plus 35.2 Å² (6α-CH_3), giving an