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Fluorescent Chemoaffinity Labeling. Potential Application of a New Affinity Labeling Technique to Glucocorticoid Receptors†

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ABSTRACT: A new, general methodology for affinity labeling is proposed. We call this method chemo-, or chemically activated, affinity labeling (CAL) since the addition of a specific chemical is required to initiate the affinity labeling process. Chemoaffinity labeling thus combines control over the timing of covalent bond formation, a distinguishing feature of photoaffinity labeling (PAL), with the advantages of the relatively stable and specific "reactive" functional groups found in conventional, or electrophilic, affinity labeling (EAL). Our chemical initiator for CAL is *o*-phthalaldehyde (OPTA), which rapidly reacts with primary amines and thiols to give 1-(alkylthio)-2-alkylisoindoles in high yield [Simons, S. S., Jr., & Johnson, D. F. (1978) *J. Org. Chem. 43*, 2886–2891]. Since these isoindoles are intensely fluorescent [Simons, S. S., Jr., & Johnson, D. F. (1978) *Anal. Biochem. 90*, 705–725], this CAL reaction will usually result in fluorescent chemoaffinity (FCA) labeling, or FCAL. In order to obtain a covalent receptor–steroid complex, we have prepared three hydrolytically stable glucocorticoid derivatives which should participate in this CAL reaction. All three steroids exhibit high affinity for the glucocorticoid receptors from rat liver hepatoma tissue culture (HTC) cells and induce the enzyme tyrosine aminotransferase (TAT). In model studies with OPTA and an added amine or thiol, each steroid gives a good yield of the desired isoindole, two of which are strongly fluorescent and display good stability and response to changes in solvent polarity. Thus, these synthetic steroids show excellent promise for being effective FCA labels for glucocorticoid receptors. OPTA is also a new cross-linking reagent, and cross-linked protein–isoindole derivatives readily display energy transfer from tryptophan to isoindole.

Affinity labeling has become a powerful method for studying the binding sites of ligand–macromolecule complexes (Baker et al., 1961; Singh et al., 1962; Jakoby & Wilchek, 1977). Presently, there are two general methods of affinity labeling (Wold, 1977). Conventional, or electrophilic, affinity labeling (EAL) (Baker et al., 1961) relies on the presence of a substituent (HNuc; usually a nucleophile) in the binding cavity of the macromolecule (B) which can attack a chemically reactive functional group (X; e.g., an α-halo ester) attached to the ligand (A). While these reactions are reasonably specific for a given X–HNuc combination, no control over the reaction is possible since covalent bond formation occurs upon approximation of the two groups. The frequency of approxi-

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mation of X and HNuc may be low but, since A–X is usually relatively stable, high levels of conversion to A–Nuc–B are possible even for slow reactions (Pons et al., 1976; Khan & Rosner, 1977; Bhatnager et al., 1978).

In the second general method, i.e., photo- or photoactivated affinity labeling (PAL) (Singh et al., 1962), photolysis converts a suitable group Z to a highly reactive Z* (such as a carbene) which will react with virtually any substituent of B to give the product A–Z′–B. Reaction is almost instantaneous but occurs only after irradiation, thus permitting control over the moment of affinity labeling. Unfortunately, the yields of A–Z′–B are usually low since A–Z is converted all at once to the very reactive A–Z*, which undergoes nonproductive side reactions (Singh et al., 1962; Chowdhry et al., 1976; Marver et al., 1976; Katzenellenbogen et al., 1977). Only the recently reported nitrophenyl ethers seem not to be restricted by this objectionable property (Jelenc et al., 1978).

In this paper we introduce a new approach to affinity labeling which appears to combine some of the advantages of the above two methods. We call this new method chemoaffinity labeling (CAL) because the addition of a chemical O is required to activate the formation of a covalent linkage between groups M on the ligand (A) and N on the macromolecule (B). This reaction would be most useful if it is specific for the functional groups M and N. CAL allows the same level of control of timing as seen with PAL since no reaction can occur before the addition of O. As with EAL, all the components should be stable, thus permitting high levels of conversion to product even in slow reactions. Finally, the high levels of nonspecific bond formation that are encountered in EAL can be reduced in PAL and CAL by removing the nonspecifically bound A–Z, or A–M, before irradiating, or adding O to, the solution.

We have developed a reaction that appears to fulfill all the above requirements for CAL (Simons & Johnson, 1978a). Furthermore, the product is usually intensely fluorescent (Simons & Johnson, 1978b). Hence, we call this approach fluorescent chemoaffinity (FCA) labeling, or FCAL. Herein we describe these results and our efforts to apply FCA labeling to the receptor for glucocorticoid hormones.

Experimental Procedures

Instrumentation. Melting points were determined on a Fisher-Johns hot-stage melting point apparatus and are uncorrected. Perkin-Elmer 237B grating infrared and Carey 14 spectrophotometers were used to record IR and UV spectra, respectively. NMR spectra were acquired at 100 MHz (JEOL FX-100 spectrometer). Low-resolution mass spectra were obtained on Hitachi Perkin-Elmer RMU-6E [electron impact (EI) mode] or Finnigan 1015D [chemical ionization (CI) mode] spectrometers. A JOEL JMS-015G-2 spectrometer with an Ionomet photoplate was used for the high-resolution mass spectra. Analyses were performed by the Microanalytical Section of the Laboratory of Chemistry, NIAMDD, Bethesda, MD.

Materials. Cystamine dihydrochloride (Aldrich) was used as received. Cortisol was purchased from Sigma Chemical Co. Dexamethasone was a gift from Dr. T. Y. Shen of Merck Sharp & Dohme while [^{3}H]dexamethasone (~25 Ci/mol) was obtained from Amersham. The preparation of 21-mercaptocortisol (**2**) and dexamethasone 21-mesylate will be outlined elsewhere (Simons et al., unpublished experiments). Buffers (Simons et al., 1979a) and other reagents (Simons & Johnson, 1978a,b) have been previously described.

21-[(Mercaptobutyl)thio]dexamethasone (**3**). To 10 equiv each of 1,4-butanedithiol (3.09 mmol, 362.6 μL; Aldrich) and triethylamine (430.7 μL; Aldrich) at 0 °C under N_2 in 0.74 mL of dry acetone were added, dropwise, over 20 min, aliquots of a cold solution of dexamethasone 21-mesylate (0.309 mmol, 145.4 mg) in 6.46 mL of dry acetone. After 1 h at 0 °C, the reaction solution was added to 150 mL of 0 °C H_2O, and the pH was lowered to ~3 by the addition of 3.09 mmol of HCl. The subsequent addition of petroleum ether caused the product to precipitate out at the water–petroleum ether interface. This solution was stirred at 0 °C for 45 min, at which point the petroleum ether was removed and fresh petroleum ether (30 mL) was added for an additional 45 min at 0 °C. Dissolution in methylene chloride of the filtered solid, after 3 × 35 mL washes of 0 °C H_2O, followed by drying over $MgSO_4$ and removal of solvent under reduced pressure, gave 152.2 mg (99.2% yield) of crude thiol. Preparative thin-layer chromatography (35:10 $CHCl_3$–acetone on two 2000-μm silica gel GF plates from Analtech) and extraction of the desired band with 3 × 50 mL of ethyl acetate gave an 85% overall yield of the thiol as an almost TLC-pure, colorless oil which soon crystallized (mp 169.7–172.0 °C). Recrystallization from $CHCl_3$–*i*-Pr_2O gave material (mp 172.5–173.3 °C) with a trace of suspected disulfide but which was analytically pure. Anal. Calcd for $C_{26}H_{37}FO_4S_2$: C, 62.87; H, 7.51. Found: C, 62.46, 62.40; H, 7.60, 7.62. Exact mass calcd for $C_{26}H_{37}FO_4S_2$: 496.2116. Found: 496.2108. Mass spectrum *m/e* (rel intensity) 497 (MH^+, 30) and 377 (100) in the CI mode with ammonia and *m/e* (rel intensity) 496 (M^+, 4), 478 (M – H_2O, 1), 333 (72), 315 (56), 223 (64), 147 (69), 135 (59), and 121 (100) in the EI mode; IR (Nujol) 3500, 1668, 1631, 1225, and 888 cm^{-1}; UV (EtOH) 238 nm (ϵ 1.59 × 10^4).

21-[(Aminopropyl)thio]dexamethasone (**4**). 3-Aminopropanethiol hydrochloride was a gift from Dr. Daniel Klayman (Walter Reed Army Institute of Research). To prepare the free amine, 2.51 mL of 4.2 M KOH in methanol was added to an equal volume of the amine hydrochloride (10.53 mmol, 1.345 g) in MeOH at room temperature in a 15-mL centrifuge tube. Two extractions of the solid mass with methylene chloride (~10 mL), each time accompanied by centrifugation (1500*g*), and removal of solvent under reduced pressure gave a quantitative yield (0.983 g) of the crude amine. Sublimation (65 °C at ~30 mmHg) afforded the pure amine (corrected mp in a sealed capillary tube = 110.5–111.3 °C; lit. (Dictionary of Organic Compounds, 1965) mp 112–113 °C) which was stored under N_2 at −20 °C.

To 253.9 mg of dexamethasone 21-mesylate (0.540 mmol) in 2.5 mL of dimethylformamide was added 1.5 equiv of a 0.251 M solution of the aminothiol in dimethylformamide (0.809 mmol, 3.22 mL). After 50 min at room temperature, the reaction mixture was added to 65 mL of 0 °C H_2O containing 1.05 equiv of NaOH to give a light yellow cloudy solution. The solid was removed by filtration, washed with 4 × 30 mL of 0 °C H_2O and dried under vacuum over $CaCl_2$ to give 225.0 mg (89.6% yield) of the crude amine (dec 164.0–165.5 °C). Recrystallization from 3% H_2O in acetonitrile caused the formation of large amounts of suspected cyclic imine but the recrystallized solid (dec 169.5–171.0 °C) was analytically pure. Anal. Calcd for $C_{25}H_{36}NO_4FS$: C, 64.48; H, 7.79; N, 3.01. Found: C, 64.70, 64.50; H, 7.96, 7.75; N, 3.45, 3.04. IR (Nujol) ~3350, 1689, 1666, 1657, 1606, and 887 cm^{-1}; mass spectrum *m/e* 466 (MH^+, 55), 448 (100), 428 (58), and 410 (15) in the CI mode with ammonia.

The hydrochloride salt (**4**·HCl) was prepared by dissolving 76.3 mg of **4** (0.164 mmol) in 2.3 mL of 0.8 M HCl in EtOH at 0 °C. Filtration and removal of solvent under reduced pressure gave 81.5 mg (99.0% yield) of the salt as a cream

colored powder (dec 233.5–238.0 °C). All attempts to recrystallize this material failed. Mass spectrum *m/e* (rel intensity) 466 (MH^+ for free amine, 12), 448 (100), and 428 (7) in the CI mode with ammonia. The parent ion was not observable in an exact mass determination. The highest observed peak corresponded to M – H_2O. Anal. Exact mass calcd for $C_{25}H_{34}NFSO_3$: 447.2244. Found: 447.2260. IR (Nujol) ~3490, 3360, ~3060, 2040, 1702, 1658, 1613, 1609, and 888 cm^{-1}; NMR (D_2O) 7.50 (d, $J \simeq 9.6$ Hz, 1 H), 6.40 (d, $J \simeq 9.6$ Hz, 1 H), 6.21 (s, 1 H), 4.38 (d, $J \simeq 10$ Hz, 1 H), ~3.89 (d, $J \simeq 17$ Hz, 1 H), ~3.58 (d, $J \simeq 17$ Hz, 1 H), 3.10 (t, $J = 7.1$ Hz, ~2 H), 2.67 (t, $J = 7.1$ Hz, ~2 H), 1.95 (t of t, $J = 7.4$ Hz, ~2 H), 1.53 (s, ~3 H), 1.09 (s, ~3), and 0.86 ppm (d, $J = 6.8$ Hz, ~3 H); UV (EtOH) 238 nm (ϵ 1.64 × 10^4).

Cells. The growing of HTC cells on monolayer or in spinner flasks, the whole cell assay for tyrosine aminotransferase induction, and the cell-free competition assay for binding to receptor have been described elsewhere (Simons et al., 1979a). The chromatographic procedures with Sephadex G-25 (Simons et al., 1976a) and DEAE-cellulose (Sakaue & Thompson, 1977) have been previously described.

Preparation of Isoindoles. The various additional materials and the procedure for the preparation of the *o*-phthalaldehyde (OPTA)–ethanethiol–*n*-propylamine adduct (i.e., ET adduct) have been reported previously (Simons & Johnson, 1978a,b).

For the preparation of the isoindole from **2**, the thiol (1.90 mg, 5.01 μmol) was dissolved in 250 μL of fresh tetrahydrofuran, cooled to 0 °C, and then treated with 5 μL of 1 M OPTA and then 5 μL of 1 M *n*-propylamine, both in 95% EtOH. This solution was then diluted in 95% EtOH for fluorescence measurements. Mass spectrum *m/e* (rel intensity) 536 (MH^+, 4), 379 (4), 347 (57), and 192 (100) in the CI mode with ammonia. With larger scale preparations (e.g., 18.9 mg of **2**), the reaction product could be concentrated and chromatographed on preparative TLC plates (alumina, 1000 μm; $CHCl_3$) to give 23.4 mg (87% yield) of a yellow oil which afforded the crude isoindole as a fine powder (dec 142–150 °C) upon addition of petroleum ether and scratching. IR (Nujol) 3559, 3448, 3053, 1701, 1653, 1613, 1322, 759, and 747 cm^{-1}; UV (95% EtOH) 231 nm (ϵ 47.4 × 10^3) and 337 (ϵ 7.66 × 10^3). This is the expected ϵ for the 337-nm isoindole band (Simons & Johnson, 1978b). Thus, the low observed fluorescence of this isoindole (see Results) is not due to a poor yield of the steroidal isoindole.

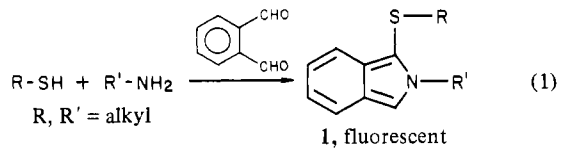
The isoindole from **3** was prepared at 0 °C by adding 5 μL of 1 M OPTA in 95% EtOH to 2.49 mg of **3** (5.02 μmol) in 75 μL of acetone, followed by 5 μL of 1 M *n*-propylamine in 95% EtOH. Mass spectrum (rel intensity) *m/e* 654 (MH^+, 9), 634 (5), and 192 (100) in the CI mode with isobutane. Dilutions for fluorescence measurements were in 95% EtOH with the final dilution into a given solvent being 1:200.

The isoindole from **4** was prepared by adding 10.8 μL of a 95% EtOH solution of OPTA (0.466 M), ethanethiol (0.466 M), and triethylamine (0.489 M) to 2.52 mg of **4**·HCl (5.02 μmol) in 25 μL of dimethylformamide, all at 0 °C. Mass spectrum *m/e* (rel intensity) 626 (MH^+, 36), 409 (42), and 218 (100) in the CI mode with isobutane. Dilutions for fluorescence studies were performed the same as those with the isoindole from **3**.

Fluorescence Measurements. Fluorescence studies were conducted with a Perkin-Elmer MPF-44A with a thermostated cuvette holder (22.3 ± 0.3 °C). The determinations of spectra, kinetics of decay of fluorescence, relative fluorescence intensity (not corrected to constant excitation or emission responses), and the effect of solvent were performed as described elsewhere (Simons & Johnson, 1978b).

Results

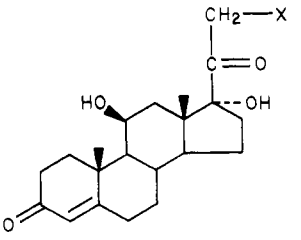
Theory of FCA Labeling of the Glucocorticoid Receptor. The presence of numerous polar functional groups around C_{17} of glucocorticoids, which contribute to glucocorticoid activity (Samuels & Tomkins, 1970; Rousseau et al., 1972; Rousseau & Schmidt, 1977), suggests that those portions of the receptor that interact with this region of the steroid would contain polar, hydrogen-bonding groups such as $-NH_2$ and –SH. In fact, the presence of an –SH group(s) has been implicated since thiol-specific reagents prevent the binding of steroid to receptors but have no effect on preformed receptor–steroid complexes (Simons, 1979). Pyridoxal phosphate at 25 °C will reduce the binding of performed complexes, thus suggesting the importance of an ϵ-NH_2 of lysine in steroid binding to receptor (Cake et al., 1978). The reaction that we have developed for chemoaffinity labeling requires a primary amine, a thiol, and *o*-phthalaldehyde (eq 1) to give a fluorescent



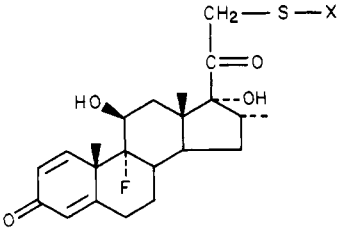
(1)

product **1** detectable at 3 × 10^{-9} M in aqueous solution (Simons & Johnson, 1978b). The generality and properties of this reaction (Simons & Johnson, 1976, 1977a, 1978a), as well as the factors influencing the fluorescence and stability of isoindoles **1** (Simons & Johnson, 1977b, 1978b), have been described elsewhere. Since the steroid binding site of the receptor may contain $-NH_2$ and/or –SH groups, our initial objective was to synthesize glucocorticoid derivatives containing –SH or $-NH_2$ groups. After noncovalent binding of the modified steroid to the receptor, the addition of OPTA could covalently join the steroidal $-NH_2$ and the receptor –SH (or vice versa) as shown in eq 1 to give a covalent, fluorescent receptor–steroid complex.

The glucocorticoid C_{21} position was chosen as the site of chemical modification due to its ability to accommodate bulk with relatively little loss of biological potency (Samuels & Tomkins, 1970; Rousseau et al., 1972; Failla et al., 1975; Simons et al., 1979b). Three steroids (**2**–**4**) were initially prepared. The –SH, and $-NH_2$, groups of **3** and **4** are both attached by a hydrolytically stable thioether.



2, X = SH



3, X = $-(CH_2)_4SH$
4, X = $-(CH_2)_3NH_2$

Properties of Steroids **2**–**4**. The cortisol derivative **2** was found to be somewhat unstable, probably due to facile oxidation of the relatively acidic –SH group [pK_a of –C(=O)CH_2SH ≃ 7.9 vs. pK_a ≃ 10.5 for *n*-alkylthiol (Crampton, 1974)] to give the disulfide (Capozzi & Modena, 1974). Compound **3** exists as the cyclic hemithioketal in the solid form, as shown by the absence of the C_{20}=O band at

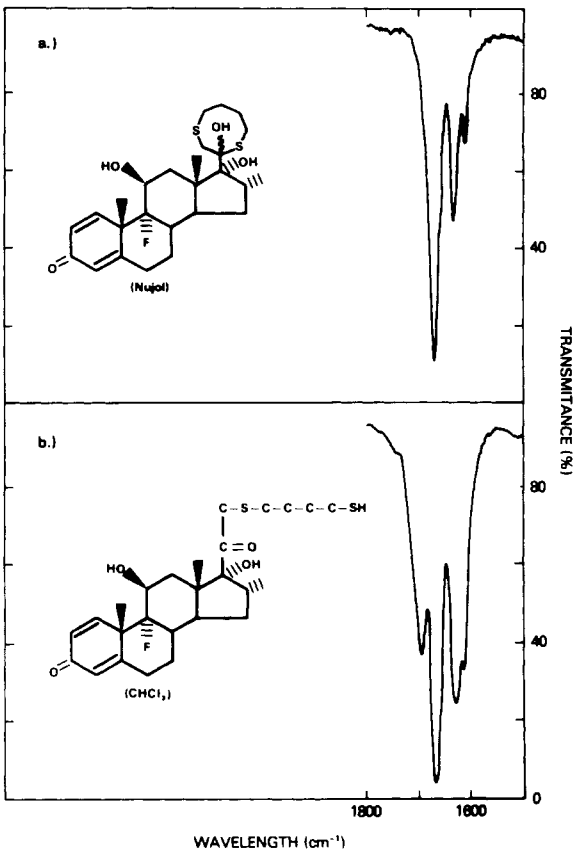


FIGURE 1: Infrared spectrum of **3** (a) in solid form (Nujol mull) and (b) in solution in $CHCl_3$. The hydrogen atoms on the carbon atoms of the C_{17} side chain have been omitted for the sake of simplicity.

$\sim$1700 cm^{-1} in the infrared spectrum (Figure 1a).[1] This was completely unexpected since 8-membered rings are quite difficult to form (House, 1972; Galli et al., 1977). However, the hemithioketal is sufficiently labile that the open-chain thiol ketone is the major, if not exclusive, species in solution as shown by the presence of the usual C_{20}=O band in the solution spectrum (Figure 1b).

In contrast to **3**, the amine **4** did *not* exist as the theoretically easier to form, 7-membered (House, 1972; Galli et al., 1977) hemiaminoketal or imine either in solution or in solid form. Solutions of **4** do slowly give rise to a new species, which probably is the cyclic imine, but this can be avoided by using the HCl salt.

Cell-Free Affinity of Steroids **2**, **3**, *and* **4**·*HCl for Glucocorticoid Receptors.* The well-characterized rat hepatoma tissue culture (HTC) cells (Samuels & Tomkins, 1970; Rousseau et al., 1972, 1973; Simons et al., 1976) were chosen as the source of glucocorticoid receptors. The approximate relative affinities of steroids **2**, **3**, and **4**·HCl for the HTC cell receptor were determined from a cell-free competition assay (Figure 2a). By this criterion, steroid **2** has a slightly higher binding affinity than the parent steroid cortisol. The derivatives **3** and **4**·HCl of the more potent glucocorticoid dexamethasone also show good affinity for HTC cell receptors, albeit considerably less than that of their parent steroid.

Whole Cell Biological Activity of Steroids **2**, **3**, *and* **4**·*HCl.* The affinity of glucocorticoids for HTC cell receptors usually correlates well with the whole cell induction of the enzyme tyrosine aminotransferase (TAT) (Granner et al., 1968; Samuels & Tomkins, 1970; Rousseau et al., 1972, 1973). In a determination of the biological activity of **2**, **3**, and **4**·HCl, each new steroid induced TAT (Figure 2b). The biological activities of **2**, **3**, and **4**·HCl were less than that of their parent compounds and both of the –SH-containing steroids exhibited a lower potency than would be expected from their relative affinities (Figure 2a). This may be due to oxidation (Capozzi & Modena, 1974), perhaps by microsomes, to give less potent steroids. In contrast, **4**·HCl was more potent than predicted from its relative affinity. One explanation is that the potentially labile **4**·HCl experiences a different fate in whole cells as compared to cell-free extracts. Alternatively, this may be another situation where the affinity of steroid for its receptor is not the limiting determinant for biological activity (Simons et al., 1979a).

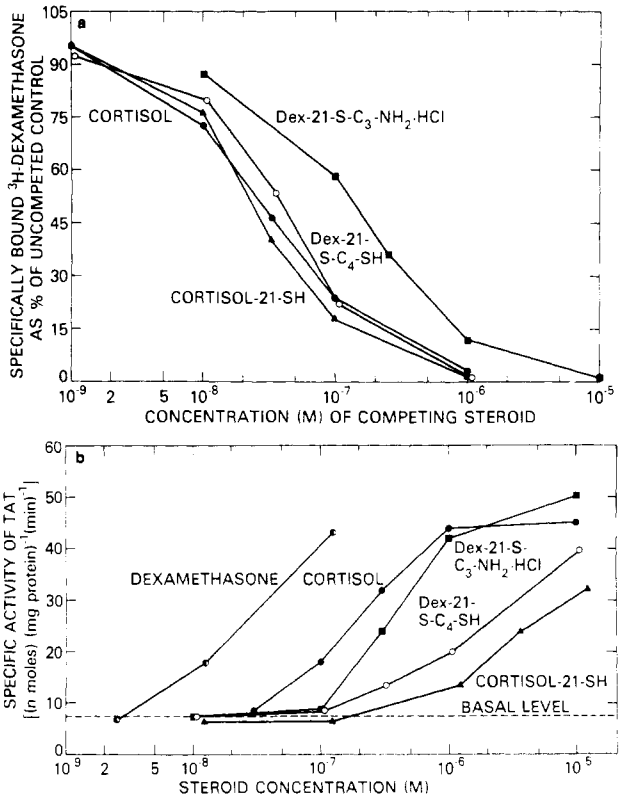
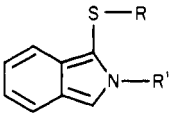


FIGURE 2: (a) Determination of relative affinities of **2**, **3**, and **4**·HCl for glucocorticoid receptors in a cell-free competition assay. Cytosol (75 μL of a 200000*g* supernatant of homogenized HTC cells containing 9.8 mg of protein per mL) was added to 150 μL of 8.54 × 10^{-9} M [^{3}H]dexamethasone in homogenization buffer [20 mM *N*-[tris(hydroxymethyl)]glycine, 2 mM $CaCl_2$ and 1 mM $MgCl_2$ (pH 7.8 at 0 °C)] and 25 μL of 10% EtOH in homogenization buffer ± nonradioactive steroid (Simons et al., 1979a). After incubation at 0 °C for 2.8 h and the usual workup with dextran-treated activated charcoal, followed by counting the macromolecularly bound [^{3}H]steroid at about 54% efficiency, the specifically bound [^{3}H]dexamethasone in the presence of cortisol (●), **2** (▲), **3** (○), or **4**·HCl (■) was expressed as % of the uncompeted control (i.e., 15 600 dpm/250 μL of assay volume). Each point represents the average of a duplicate determination after subtracting the nonspecific binding of [^{3}H]dexamethasone in the presence of 3.0 × 10^{-6} M [^{1}H]dexamethasone. (b) Biological activity of dexamethasone, cortisol, and **2**, **3**, and **4**·HCl in whole HTC cells. The ability of each steroid to induce the synthesis of TAT was determined by incubating monolayer cultures of HTC cells in growth medium containing 1% EtOH ± steroid for 18 h at 37 °C. The specific enzyme activity of TAT in each dish was determined, and the average value for duplicate dishes is plotted against the steroid concentration of dexamethasone (◐), cortisol (●), **2** (▲), **3** (○), or **4**·HCl (■) present during the 18 h induction. Basal level enzyme activity is indicated by the dashed line (---).

[1] We cannot rule out the existence of a polymeric hemithioketal, but the sharp melting point of the crystalline solid (see Experimental Procedures) appears to argue against this interpretation. However, even if **3** does exist in solid form as a polymer, the following arguments regarding the special properties of C_{20}-carbonyl–thiol interactions would still apply.

Table I: Fluorescence Spectral Properties of Isoindoles[a]



isoindole	R	R'	95% EtOH λ_{ex} (nm)	95% EtOH λ_{em} (nm)	95% EtOH rel fluorescence intensity	38% EtOH in pH 9.2 borate buffer λ_{ex} (nm)	38% EtOH in pH 9.2 borate buffer λ_{em} (nm)	38% EtOH in pH 9.2 borate buffer rel fluorescence intensity
ET adduct	-Et	-*n*-Pr	337, 348	431	1	~344	451	1
cortisol-21-S-isoindole	**2**, X = -	-*n*-Pr	~336, ~348	~424	≤0.003			
dexamethasone-21-S-C_4-S-isoindole	**3**, X = $-(CH_2)_4-$	-*n*-Pr	336, 348	430	0.17	~344	445	0.072
dexamethasone-21-S-C_3-N-isoindole	-Et	**4**, X = $-(CH_2)_3-$	336, 347	431	0.13	~344	446	0.049

[a] The λ_{max} values were taken directly from the uncorrected spectra, and no other major peaks were ever observed between 340 and 800 nm. For relative fluorescence intensity measurements, all readings in a given experiment were obtained by using identical slit (excitation slit = 1.5 nm; emission slit = 14 nm) and sample sensitivity settings (i.e., 1) and with an internal ET adduct control. Except for the λ_{max}'s, these settings were also the same when different solvents were used in the same experiment and when the intensity of the solvent Raman band was determined (except that the sample sensitivity would be increased). The excitation and emission λ_{max}'s to be used were determined from the previously obtained spectra. In those cases where the adduct was unstable, the extrapolation of linear semilog plots was used to determine the fluorescence intensity at zero time.

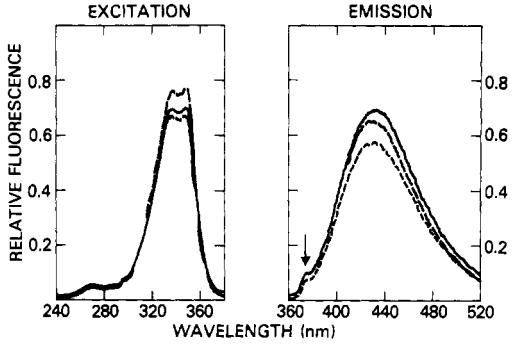


FIGURE 3: Fluorescence spectra of isoindoles in 95% EtOH at 22.4 °C. (a) The excitation spectra of 95% EtOH solutions containing 6.67×10^{-7} M ET adduct (—), 5.91×10^{-6} M dexamethasone-S-isoindole (– – –), or 7.00×10^{-6} M dexamethasone-N-isoindole (---) were obtained under slightly different conditions (excitation slit = 1.25–1.5 nm; λ_{em} = 428 or 430 nm; emission slit = 8 or 13 nm; sensitivity = 3 or 1) so that only the shapes, and not the magnitude, of the spectra are directly comparable. (b) The emission spectra of above solutions were recorded with slightly different settings (λ_{ex} = 336 nm; excitation slit = 2.5 or 3.0 nm; emission slit = 3.5 nm; sensitivity ≃ 3) so that only the shapes, and not the magnitude, of the spectra are directly comparable. The arrow (↓) indicates the position of solvent Raman bands which are, in each case, <6% of the peak intensity.

Preparation of Steroidal Isoindoles. In preparation for FCA labeling of the receptor, a number of model studies were conducted using the steroids **2**, **3**, and **4**·HCl, OPTA, and ethanethiol or *n*-propylamine in place of the receptor. Each steroid gave a good yield of the isoindole **1** (≥80% as determined by TLC) which was initially identified on the basis of mass spectral evidence and the differential I_2 staining of isoindoles on TLC plates (Simons & Johnson, 1978a). Confirmation of the isoindole structure was obtained from the fluorescence spectra. Not only was the general appearance of the fluorescence spectra the same as that of an authentic isoindole formed from OPTA, ethanethiol, and *n*-propylamine, i.e., the ET adduct (Figure 3), but also the λ_{max} values are similar (Table I). The fluorescence intensity of the cortisol–isoindole (Table I) was very low. However, another thiol with a β-carbonyl, i.e., methyl mercaptoacetate, also gives an essentially nonfluorescent isoindole for reasons which presently are not understood (Simons & Johnson, 1978b). The long chains in steroids **3** and **4** serve to remove the offending β-carbonyl and give isoindoles which have close to the expected fluorescence intensity.

Effect of Solvent on Isoindole Fluorescence. One of the novel aspects of FCAL is that the fluorescent molecule joining the steroid to the receptor can in theory also probe the local environment of the binding cavity; thus, fluorescence depolarization,[2] energy transfer (see below), and changes in the λ_{max} and fluorescence intensity should yield information about the size and polarity of the binding cavity (Guilbault, 1973). As shown in Figure 4A, the emission λ_{max} for the model isoindole, i.e., the ET adduct, decreases with the EtOH concentration in aqueous solutions. The long straight chains joining the steroid to the isoindole allow the steroidal isoindoles to be almost as responsive to solvent polarity as the simpler ET adduct. The steroidal isoindoles were too insoluble to allow measurements in pH 9.2 borate buffer.

The effect of solvent polarity on the fluorescence intensity was not uniform (Figure 4B). While the intensity of the ET adduct was highest in 38% EtOH, the intensity of the steroidal isoindoles increased by almost 100% in going to 95% EtOH.

Stability of Isoindoles. The steroids **2**–**4** were designed to maximize isoindole stability (Simons & Johnson, 1977b, 1978b). In 95% EtOH, each adduct examined was very stable (i.e., <4% decay after 82 h at 25 °C) while the pseudo-first-order decay rates of both steroidal isoindoles and the ET adduct in 38% EtOH in pH 9.2 borate buffer were identically slow ($T_{1/2} \simeq 200$ h at 25 °C; data not shown). In pH 9.2 aqueous solution, simple isoindoles are generally less stable [$T_{1/2} \simeq 35$ h at 25 °C (Simons & Johnson, 1977b, 1978b)]. However, aqueous solutions of protein–isoindole derivatives are quite stable. For example, receptor-containing cytosol preparations that have been somewhat purified by chromatography over Sephadex G-25 (to remove all low molecular weight compounds), followed by step elution off a DEAE-cellulose column, are cross-linked by the addition of 10^{-4}–10^{-5} M OPTA to give protein–isoindole derivatives. The decay of these derivatives is nonlinear on semilog plots, but 50% of the original fluorescence remains after 13 days at 0 °C in pH 8.0

[2] The fluorescence lifetimes of isoindoles **1** are reported to be 17–20 ns (Chen et al., 1979).

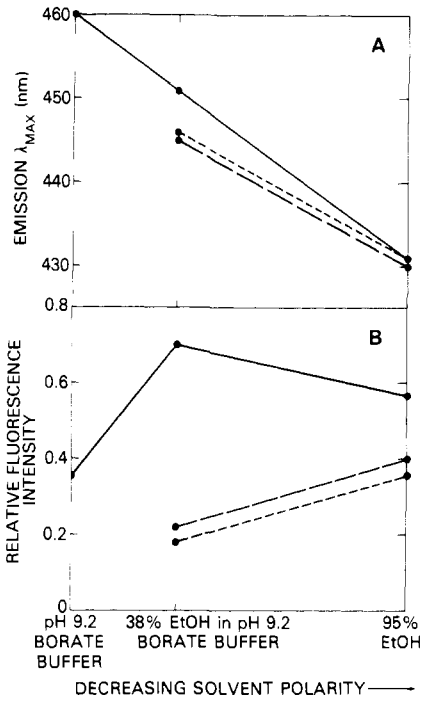


FIGURE 4: Effect of solvent on isoindole fluorescence. (A) Effect of solvent on fluorescence λ_{em}. The λ_{em} data of Table I plus the data for ET adduct in pH 9.2 borate buffer (not shown) were plotted against the percent EtOH in the solvent for ET adduct (—), dexamethasone-S-isoindole (– – –) and dexamethasone-N-isoindole (---). Since $Na_2B_4O_7 \cdot 10H_2O$ is only sparingly soluble in 95% EtOH, this solution was prepared without any sodium borate. (B) Effect of solvent on fluorescence intensity. The relative fluorescence intensities [not corrected relative fluorescence intensity as described in Simons & Johnson (1978b)] of 6.67×10^{-7} M ET adduct (—), 2.97×10^{-6} M dexamethasone-S-isoindole (– – –), or 3.50×10^{-6} M dexamethasone-N-isoindole (---) in the different solvents were normalized to the intensities of the solvent Raman bands (λ_{ex} = 340 nm; slit widths identical with those used with the isoindoles), as described in Simons & Johnson (1978b). This procedure also corrects for fluctuations in machine response during long-term measurements. In 95% EtOH (see Table I for λ_{ex}, λ_{em}, excitation and emission slits, and machine sensitivity), the relative intensity was determined within 3 h of mixing. In 38% EtOH in borate buffer (see Table I for settings), the relative fluorescence intensity at the time of mixing was determined by extrapolation of the kinetic data (data not shown). The value for the ET adduct in borate buffer was taken from Simons & Johnson (1978b).

buffer (data not shown). Furthermore, Chen et al. (1979) have found that aqueous solutions of protein–isoindole derivatives are just as stable as solutions of dansylated proteins. Thus, the isoindole adducts of **2**–**4** would be expected to be quite stable when bound to receptor.

Stability of Receptor–Steroid Complexes under FCAL Conditions. The FCAL reaction requires free –SH and $-NH_2$ groups. Since it is known that protection of –SH groups in receptor-containing cytosol stabilizes functional receptors, disulfide reducing agents [e.g., dithiothreitol (DTT)] are often added to cell extracts for receptor assays (Simons, 1979). However, a large excess of –SH groups would interfere with the FCAL reaction. Thus, we reinvestigated the stability of HTC cell glucocorticoid receptors. Under our conditions, omission of DTT did not affect HTC cell receptor activity (data not shown). We then examined the ability of the disulfide cystamine to block nonreceptor –SH groups (Sweet & Szabados, 1977). At concentrations of $\leq 10^{-2}$ M, cystamine had little effect on preformed complexes; however, free receptors were completely inactivated by pretreatment with as little as 10^{-3} M cystamine (Table II), in marked contrast to the increased binding caused by cystamine treatment of free

Table II: Effect of Cystamine and OPTA on Cell-Free Binding of [^{3}H]Dexamethasone to HTC Cell Receptors[a]

chemical	final concn for chemical (M)	specifically bound [^{3}H]dexamethasone as % of control: for chemical added 2 h before [^{3}H]-dexamethasone	specifically bound [^{3}H]dexamethasone as % of control: for chemical added 2 h after [^{3}H]-dexamethasone
cytamine	10^{-1}	*b*	41
cytamine	10^{-2}	*b*	80
cytamine	10^{-3}	1	87
OPTA	10^{-2}		27
OPTA	10^{-3}		91
OPTA	10^{-4}		105

[a] The effect of cystamine was determined by adding 10 μL of 1.35×10^{-7} M [^{3}H]dexamethasone ± 7.5×10^{-5} M [^{1}H]dexamethasone to 75 μL of HTC cell cytosol (150000*g* supernatant; 19.1 mg of protein per mL) in 155 μL of homogenization buffer containing 1.6% EtOH either 2 h before or 2 h after the addition of 10 μL of 25× cystamine solutions. After an additional 2-h incubation, all at 0 °C, the amount of charcoal-nonextractable [^{3}H]-dexamethasone was determined in the usual manner and described as percent of control binding (i.e., 40 600 dpm/250 μL). The effect of OPTA was determined by adding 5 μL of 50× solutions of OPTA in 50% EtOH–homogenization buffer to a preincubated (2 h at 0 °C) solution of 75 μL of the above cytosol and 170 μL of 8.1×10^{-9} M [^{3}H]dexamethasone ± 4.4×10^{-6} M [^{1}H]dexamethasone in homogenization buffer. Subsequent incubation and workup were performed as described above. In all cases, the data represent the average of duplicate determinations whose variation was ≤2% of control binding. [b] The noncompeted samples gave less binding than did the competed samples by an amount equal to 1% of control binding.

uterine estradiol receptors (Sweet & Szabados, 1977).

Other components of the FCAL reaction were also studied for their effects on steroid–receptor binding. Preformed complexes are not affected by $\leq 10^{-3}$ M OPTA (Table II). This concentration of OPTA should be more than sufficient for FCAL since the presumed high local concentration of $-NH_2$ and –SH in the receptor–steroid complex would reduce the usual trimolecular reaction to a less concentration-dependent pseudo bimolecular reaction. Our buffers contain phosphate or Tricine which, being a quaternary ammonium salt, will not interfere with the reaction of eq 1 (Simons & Johnson, 1978b). Dilute solutions of numerous organic solvents for the steroids **2**, **3**, and **4**·HCl, i.e., 1% acetonitrile, tetrahydrofuran, or dimethylformamide, were found to have no effect on the formation of receptor–steriod complexes (data not shown).

Background Fluorescence. The usefulness of FCAL depends on the relative fluorescence intensity of the desired protein–isoindole–ligand vs. that of background. Accordingly, we tried to determine the magnitude of background fluorescence. The fluorescence of Sephadex G-25 pretreated, DEAE-cellulose-fractionated receptor solutions at 3 °C was 40% above that of buffer alone at the wavelengths of isoindole fluorescence in aqueous solution [i.e., λ_{ex} = 340 nm and λ_{em} = 450 nm (Simons & Johnson, 1978b)]. Addition of 10^{-5}–10^{-4} M OPTA, followed by incubation at 0 °C for 40 min, increased the fluorescence to 120–680 times that of buffer alone. For comparison, the fluorescence of 1.3×10^{-9} M ET adduct, or 8×10^{-9} M Dex-S-C_4-S-isoindole, in 95% EtOH at 2.8 °C is calculated to be 2 times that of 95% EtOH, which is 70% as fluorescent as the above aqueous buffer (data not shown). Numerous approaches should reduce the high backgrounds to much lower levels (see Discussion).

Experiments with fractionated receptor solutions also re-

vealed that, in the absence of OPTA, the observed fluorescence is predominantly due to tryptophan. When OPTA was added to give protein–isoindole derivatives, an isoindole concentration-dependent energy transfer from tryptophan to isoindole was observed (data not shown).

Discussion

We have described the methodology for a new, general approach to affinity labeling, i.e., chemoaffinity labeling (CAL), which appears to combine some of the desirable features previously associated with either photo- or electrophilic affinity labeling. The reaction that we have developed for CAL (eq 1; Simons & Johnson, 1978a,b) has the following advantages: (1) there is an absolute specificity for an $-NH_2$ and an –SH group, thus allowing very precise localization of the covalent linkages, (2) the reaction is very fast and goes in high yield, (3) the reaction proceeds optimally in dilute aqueous solutions at pH 8–10 (Roth, 1971; Benson & Hare, 1975), conditions that are well suited for reaction with most biological macromolecules, (4) the product of the reaction is usually intensely fluorescent, thus permitting nonradioactively labeled complexes to be followed and enabling the local environment of the covalent linkage to be studied, (5) the fluorescent protein–isoindole derivatives possess good stability in aqueous solutions at 0 °C, and (6) protein–isoindole derivatives display very efficient energy transfer from tryptophan to isoindole which, due to the $1/r^6$ dependence (Guilbault, 1973), offers a sensitive probe for conformational changes. On account of the fluorescent products, we call this particular example of chemoaffinity labeling FCAL, or fluorescent chemoaffinity labeling. In those cases where the covalently linked product is not fluorescent, the use of radioactively labeled ligand, *or OPTA*, would extend the utility of the FCA procedure.

While CAL and FCAL should be generally useful methods for affinity labeling, we have concentrated on using these methods for affinity labeling glucocorticoid receptors. There is good reason to suspect the presence of one, or both, of the –SH and $-NH_2$ groups required for FCAL in the steroid binding cavity. In order to try to obtain a fluorescent, covalent steroid–isoindole–receptor complex, we have prepared three glucocorticoid derivatives (**2**, **3**, and **4**·HCl), each containing an $-NH_2$ or –SH group. These steroids show excellent promise for being effective FCA labels of the HTC cell glucocorticoid receptor for the following reasons. (1) The steroidal $-NH_2$ or –SH groups are attached by hydrolytically stable linkages. (2) The steroids **2**, **3**, and **4**·HCl have a high affinity for receptors. (3) Each steroid demonstrates some ability to induce TAT, and **4**·HCl gives good whole cell induction; thus, **4**·HCl may be useful in other whole cell studies. (4) Each steroid affords a good yield of the isoindole in a model system. (5) Two of the steroids (**3** and **4**·HCl) give isoindoles which are highly fluorescent. (6) The two fluorescent steroidal isoindoles are very stable. (7) The two fluorescent isoindoles exhibit large responses to changes in the polarity of the environment.

Experiments aimed at FCA labeling the glucocorticoid receptor, instead of ethanethiol or *n*-propylamine, are currently in progress. Should a fluorescent covalent complex be formed, we would be in a unique position to examine activation and nuclear binding of receptor–glucocorticoid complexes. The backgrounds for FCA labeling of DEAE-fractionated cytosol appear too high to obtain much data with these preparations. Backgrounds could be reduced by employing lower concentrations of OPTA or by further purification of the receptor–steroid complex. While concentrations of OPTA less than 10^{-5} M may give complete FCA linking of the steroid and receptor, it is likely that FCAL (as with EAL and PAL) will be most useful with partially purified proteins. In particular, we could purify the receptor–steroid complex before or after FCA labeling and, if necessary, use ^{3}H-labeled **3** or **4** to follow the purification until the background fluorescence was sufficiently reduced to allow one to utilize the unique aspects of FCAL (i.e., polarization, energy transfer, and solvent effect studies). It should be realized that use of OPTA and ^{3}H-labeled steroid to obtain a covalent ^{3}H-labeled steroid–isoindole–receptor complex via CAL will give much lower backgrounds than FCAL of the same protein solution.

While our use of the reaction of eq 1 has been for fluorescent affinity labeling, this reaction should find broad use where chemically specific cross-linking is desired. OPTA does cross-link HTC cell proteins and reinterpretation of published data (Weidekamm et al., 1973) shows that other examples of OPTA as a fluorescent cross-linker exist (Simons & Johnson, 1978b). Thus, OPTA will complement the existing bifunctional reagents (Martinson & McCarthy, 1975; Peters & Richards, 1977; Smith et al., 1978). The presence of a fluorescent, OPTA cross-linked product would be especially useful for studying intramolecularly cross-linked proteins, thiol-substituted nucleic acids, and intermolecularly cross-linked molecules such as ribosomes and enzyme–substrate and protein–nucleic acid complexes.

The observation that steroid **3** exists in the solid form as a cyclic hemithioketal but is present as the open thiol ketone in solution is significant. In general, 8-membered rings are quite difficult to form and do so less readily than 7-membered rings (House, 1972; Galli et al., 1977). The fact that **3** affords an 8-membered ring under conditions that do not give a similar 7-membered ring with **4** or **4**·HCl suggests that the interaction of the C_{20}-carbonyl with a thiol is very strong and in some ways unique. We have recently prepared the thiol analogue of **4** (i.e., X = $(CH_2)_3SH$) and it too exists as the cyclic hemithioketal in solid form (data not shown). It is therefore an intriguing possibility that the C_{20}-carbonyl of glucocorticoids may react with an –SH group in the binding cavity of the receptor to yield a steroid–receptor hemithioketal. Such a covalent linkage of steroid and receptor would be readily reversible (Field & Sweetman, 1969; Simons & Johnson, 1978a) but strong enough to help explain the high-affinity binding of glucocorticoids to their specific receptors and the absolute requirement that glucocorticoids contain a C_{20}-carbonyl (Steelman & Hirschmann, 1967; Samuels & Tomkins, 1970; Rousseau et al., 1972; Rousseau & Schmidt, 1977). This hypothesis is also consistent with the known data concerning the reactivity of –SH groups in crude receptor preparations (Simons, 1979).

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