A Filter Assay for Steroid Hormone Receptors†

Daniel V. Santi,* Carol H. Sibley, Evelyne R. Perriard,
Gordon M. Tomkins, and John D. Baxter

ABSTRACT: A filtration assay is described for quantitation of glucocorticoid binding to specific receptor proteins in cytosol fractions from cultured hepatoma cells. Receptor–[^{3}H]-steroid complexes are adsorbed on diethylaminoethylcellulose filter disks under conditions where the unbound steroid is removed by buffers of low ionic strength. The assay is rapid, simple to perform, and applicable over a wide range of protein concentration. The method may also be used for measurements of glucocorticoid receptors in other tissues and for estrogen and mineralocorticoid receptors from their target tissues.

In recent years, extensive investigations of receptor–steroid interactions have provided much new information on the mechanism of these hormones (for recent reviews see Raspe, 1971; Jensen and De Sombre, 1972). It is now apparent that many of these receptors are soluble proteins which should be amenable to standard techniques of purification and characterization. In studies aimed at purification of the glucocorticoid receptor we encountered the need for a method sufficiently sensitive to measure dilute solutions of receptor and rapid enough to permit assay of numerous samples obtained upon fractionation attempts. Measurements of receptor–steroid complexes require separation of the macromolecular bound from unbound steroid and are generally made by density gradient centrifugation, gel chromatography, or adsorption of uncomplexed steroid on activated charcoal (see Raspe, 1971). The former two methods are somewhat tedious to perform, and the charcoal adsorption cannot be used in solutions containing very low concentrations of protein.

A filter assay technique—which depends upon the ability of a filter to retain a protein–ligand complex under wash conditions where unbound ligand may be removed—seems ideally suited for quantitation of receptor–steroid complexes. Unfortunately, it became apparent early in our investigations that free steroids could not be easily washed from nitrocellulose membranes—the filter most commonly used for assays of this nature (Yarus and Berg, 1970). In studies on the behavior of the glucocorticoid receptor on DEAE-cellulose columns, it was observed that free steroid passed through in the void volume at low ionic strengths where the receptor–steroid complex was adsorbed. It occurred to us that this adsorbent might be useful in a filtration assay of the glucocorticoid and other steroid receptors.

We report here a rapid assay for the glucocorticoid receptor protein which involves (a) adsorption of the bound receptor–[^{3}H]steroid complex to DEAE-cellulose filters, and (b) removal of unbound steroid by washing with buffer of low ionic strength. The assay accurately measures low concentrations of the glucocorticoid receptor and is also applicable to the measurement of other steroid receptors investigated.

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Materials and Methods

[1,2-^{3}H]Dexamethasone (22 Ci/mmol) was obtained from Amersham-Searle and [1,2,4-^{3}H]dexamethasone (12 Ci/mmol) was purchased from Schwarz-Mann. [1,2-^{3}H]Progesterone (33.5 Ci/mmol), [1,3-^{3}H]cortisol (42 Ci/mmol), [2,4,6,7-^{3}H]-estradiol (100 Ci/mmol), and [1,2-^{3}H]aldosterone (44 Ci/mmol) were purchased from New England Nuclear. Dexamethasone was a gift from Merck Sharp and Dohme and all other steroids were purchased from Schwarz-Mann. Aqueous solutions of steroids were prepared as described by Baxter and Tomkins (1971); the small amount of nonaqueous solvent introduced into the assay does not affect receptor–steroid interactions. DEAE-cellulose filter paper disks (DE81, 2.4 cm) were purchased from Whatman Co. Buffer solutions used were (A) 20 mM Tricine (pH 7.9)–2 mM $CaCl_2$–1 mM $MgCl_2$ and (B) 20 mM Tricine (pH 7.9)–1.5 mM EDTA. The pH of all solutions was measured at 0–4°.

Cultured hepatoma cells were grown to a density of 2–15 × 10^5 cells/ml and harvested as previously described (Hershko and Tomkins, 1971). Cell pellets were stored at −20° for 1–30 days prior to use. Cytoplasmic extracts (cytosol) were prepared as described by Rousseau *et al.* (1972b). Kidneys and livers were obtained from male rats (250–300 g) which were adrenalectomized and maintained on saline for 3 days prior to sacrifice. Tissues were minced in one volume of ice-cold buffer A, homogenized with a motor-driven tissue grinder in a Teflon-glass homogenizer, and centrifuged at 100,000*g* for 1 hr. Uteri were removed from 21-day-old female rats, minced in one volume of cold buffer B, homogenized with a motor-driven tissue grinder in a ground-glass homogenizer, and centrifuged at 100,000*g* for 1 hr. All extracts were kept at 0–4° and used within 18 hr after preparation.

The standard binding assay for glucocorticoid receptor contained in 0.15 ml, 4 × 10^{-8} M [^{3}H]dexamethasone (*ca.* 6–10 × 10^4 cpm), a limiting amount of receptor sites (<10^{-8} M; 0.075 ml of cell extract containing *ca.* 2 mg of protein), 20 mM tricine (pH 7.9), and 1.5 mM EDTA. After 90–120 min at 4°, 50-μl duplicates were assayed for bound steroid by DEAE-cellulose filtration (described below). To determine nonspecifically bound steroid, parallel reactions were performed in which a 1000-fold excess of unlabeled dexamethasone was included in the mixture as competitor for binding to the glucocorticoid receptor. Only specifically bound counts are diluted since nonspecific binding components are present in large excess (Rousseau *et al.*, 1972b). Steroid specifically bound to the

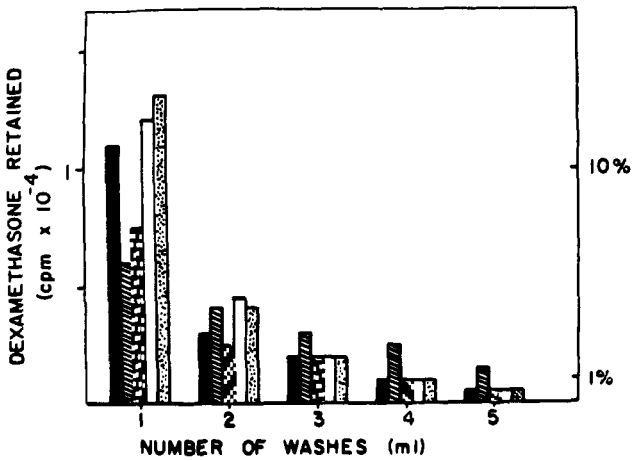

FIGURE 1: [³H]Dexamethasone retained on DEAE filters as a function of volume of wash with: ▣, Tris (pH 8.8); ▦, Tris (pH 8.8)–1.5 mM EDTA; ▩, potassium phosphate (pH 7.2); ■, Tricine (pH 7.50)–1.5 mM EDTA; □, sodium cocodylate (pH 6.1). The total amount of [³H]dexamethasone (22 Ci/mmol) applied to each filter was 90,000 cpm.

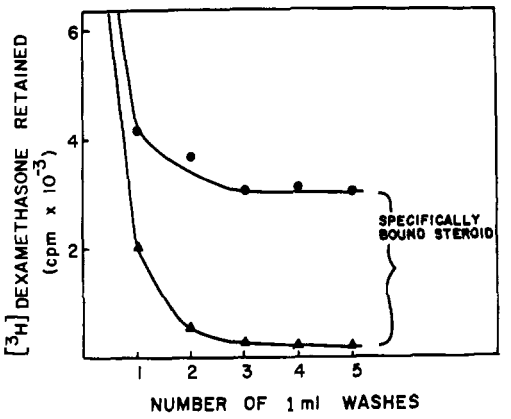

FIGURE 2: [³H]Dexamethasone retained on DEAE filters after incubation of hepatoma cell cytosol (20 mg/ml) with 2×10^{-8} M [³H]-dexamethasone (12 Ci/mmol) (●) and 2×10^{-9} M [³H]dexamethasone (12 mCi/mmol) (▲); each aliquot filtered contained 9960 cpm. Assays were performed as described under Methods using buffer B as wash fluid.

glucocorticoid receptor is calculated by subtracting the nonspecifically bound steroid from that bound in the absence of excess nonradioactive steroid. Rat tissue extracts were treated in a similar manner, incubating kidney with aldosterone, liver with cortisol and dexamethasone, and uterus with estradiol.

Filtration assays were performed at 0–4° under the following conditions: DEAE-cellulose filters were soaked in buffer B prior to use, placed in a ten-place filter manifold (Hoeffer Scientific Instruments), and freed of excess moisture by application of a vacuum. The vacuum was removed and 50-μl samples were uniformly applied to each of the filters. After at least 1 min, a gentle vacuum was applied and the filters were washed with five 1.0-ml portions of buffer A or B[1] (Cornwall syringe or Eppendorf pipet). Suction was increased to remove excess moisture and the damp filters were placed in 5 ml of toluene containing 0.4% (w/v) Omnifluor (New England Nuclear), 25% Triton X-100, and 4% H_2O. After allowing sufficient time for dissolution of the steroid in the scintillation cocktail (*ca.* 6 hr), the vials were counted in a Beckman liquid scintillation counter with 34% efficiency.

Results

Figure 1 shows that when [³H]dexamethasone is applied to DEAE-cellulose filter disks, washing with 3–5 ml of various buffers at 20 mM concentration is sufficient to remove almost all radioactivity. This is independent of pH within the range of 6.4–8.7 and, with the exception of phosphate, not a function of the buffer used; for undetermined reasons, phosphate buffer is somewhat less effective but the amount of radioactivity retained does not prohibit its use in the assay described below. On the other hand, when hepatoma cell cytosol is equilibrated with [³H]dexamethasone before application, a substantial amount of radioactivity remains on the filters, even when they are extensively washed with buffer B (Figure 2). We note that up to 20 ml of this wash has been used with little effect upon the amount of retained complex. The retained radioactivity is reduced when the equilibration of [³H]steroid with cytoplasm is performed with a 1000-fold excess of nonradioactive dexamethasone as a competitor for specific glucocorticoid binding (Rousseau *et al.*, 1972b). The difference between the filter-bound steroid in the presence of competing unlabeled steroid is attributable to specific receptor–steroid complexes adsorbed on the filter. After incubation of cytosol which was pre-bound with [³H]dexamethasone at 37° for 30 min—conditions which denature the glucocorticoid receptor (G. Rousseau, unpublished observations)—specifically bound steroid is undetectable by DEAE adsorption but the amount of nonspecifically bound steroid retained is unchanged.

A number of experiments were performed which define minimal conditions required for reproducibility of the assay; in these, a saturating amount of [³H]dexamethasone was incubated with cytoplasmic extract and assayed under the specified conditions. Optimal binding is obtained if at least 1 min is permitted for the extract to adsorb to the filters; longer periods (up to 5 min) produce a slight decrease in specifically bound steroid, although not significant to warrant concern. Using the buffers described in Figure 1, it was shown that the amount of specifically bound complex retained is independent of pH over the range 7.2–8.6. Less specifically bound steroid is retained with lower pH washes (cacodylate, pH 6.3) and the results were more erratic using washes of higher pH (Tris, pH 8.9). For this reason we use buffers in the pH range of 7.2–8.3, and favor Tris or Tricine at pH 7.9. Under standard conditions the range of values for nine determinations differed by less than 6% of the mean (Table I). Washing the filter-bound complex with buffers of higher ionic strength ($\mu > 0.09$) results in loss of filter-bound counts. Analogous experiments with DEAE columns (D. Gelfand, unpublished results) suggest that this is for the most part due to elution of the receptor–steroid complex rather than disruption of the complex on the filter.

Figure 3 shows that the amount of specifically bound steroid retained on the filters is linearly related to the protein applied up to about 4 mg per filter; apparent saturation of the filter is observed at higher levels. This is true when varying volumes of cytosol are applied, or when varying amounts of cytosol are diluted to a constant volume prior to application. The lower limits of sensitivity were ascertained by both diluting bound cytosol before application to the filters, and by diluting cytosol before equilibration with [³H]dexamethasone.

[1] We have found that either of these buffers may be used with identical results; we now routinely use buffer B for all washing procedures.

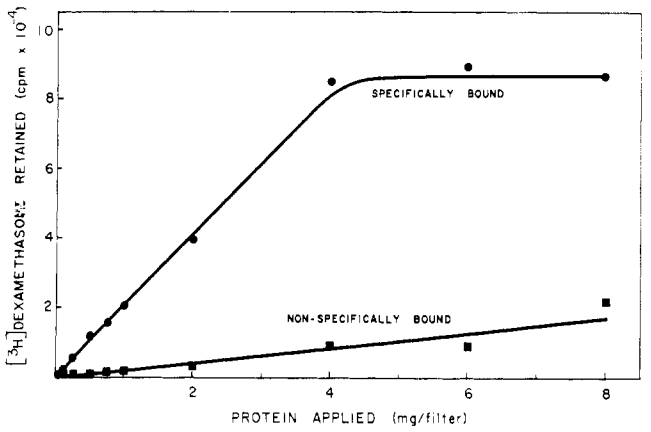

FIGURE 3: [^{3}H]Dexamethasone retained on DEAE filters as a function of amount of receptor–steroid complex applied. Cytosol (20 mg/ml) was incubated with [^{3}H]dexamethasone (22 Ci/mmol) and varying volumes (5–400 μl) were filtered through DEAE-cellulose disks using buffer A as described under Materials and Methods. Specifically bound [^{3}H]dexamethasone (●) was calculated by subtracting the nonspecifically bound steroid (▲) from the total retained (not shown).

The latter simulates situations where only low levels of receptor are present in cytoplasmic extracts. As shown in Figure 4, the assay is linear under both circumstances down to *ca.* 20 μg of protein. At the lower concentrations used the amount of radioactivity bound approaches background and the sensitivity of the assay is limited by the available specific activity of the steroid. It is noted with this assay the glucocorticoid receptor can be detected in cytosol which has been diluted 200-fold.

Figure 5 shows the titration of a constant amount of hepatoma cell cytosol with varying [^{3}H]dexamethasone concentration. At high steroid concentration, saturation of specific binding sites is obtained, and a dissociation constant of $K = 2.5 \times 10^{-9}$ M may be calculated by Scatchard (1949) analysis. This value is in excellent agreement with that obtained ($K = 3.1 \times 10^{-9}$ M) by charcoal adsorption of unbound steroid

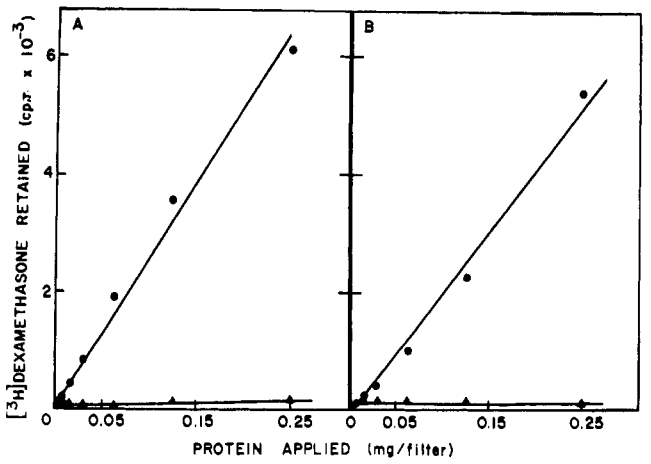

FIGURE 4: Linearity of receptor-[^{3}H]dexamethasone complex retained on DEAE filters at low concentrations. (A) Incubation mixtures were serially diluted in buffer B and 50-μl aliquots were filtered after 10 min; (●) specifically bound steroid, (▲) nonspecifically bound steroid. (B) Cytosol was serially diluted into buffer B containing 4×10^{-8} M [^{3}H]dexamethasone (22 Ci/mmol) or 4×10^{-6} M [^{3}H]dexamethasone (22 mCi/mmol), incubated, and assayed (50 μl) in duplicate; (●) specifically bound steroid, (▲) nonspecifically bound steroid. Buffer A was used as the wash fluid.

TABLE I: Comparison of DEAE and Charcoal Assays for Measurement of Specific Binding of Dexamethasone.[a]

Expt	Specific Binding (cpm) DEAE	Charcoal
1	4100	4100
2	6422	6148
3	5770	5777
4	5350	5400
5	6740 ± 345[b]	6400 ± 320[b]

[a] Hepatoma cell cytosol was equilibrated with [^{3}H]dexamethasone as described under Materials and Methods and aliquots of equal volume were assayed by charcoal adsorption of unbound steroid and by filtration through DEAE. Parallel reactions were performed in the presence of a 1000-fold excess of unlabeled competitor to obtain values for nonspecifically bound steroid. The experiments listed utilized extracts prepared on different days. [b] Values given are the mean ± the range of nine determinations.

(Baxter and Tomkins, 1971). Additional comparisons between the DEAE filter and charcoal assays (Table I) lead us to conclude that the assays yield equivalent results.

The method described should be generally applicable to assay of steroid–receptor complexes provided that the free steroid is readily washed from the filter under conditions which retain the steroid–protein complex. Figure 6 shows that a variety of [^{3}H]steroids may be washed from DEAE filter disks under the conditions described above. Preliminary results showing titration curves (Figure 7) obtained with glucocorticoid, estrogen, and aldosterone receptors contained in the cytoplasmic fluid of appropriate tissues indicate that this assay is of general applicability; Table II gives comparisons of dissociation constants derived from these data with those reported by other methods.

The "efficiency" of a filter assay refers to the probability that a protein–ligand complex will survive the filtration and washing procedure (Yarus and Berg, 1970); this is determined

TABLE II: Comparison of Dissociation Constants for Steroid–Receptor Complexes as Determined by DEAE Filter Assay and Other Methods.

Tissue	Steroid	K_{diss} (M) DEAE Assay	Published
Rat hepatoma	Dexamethasone	2.5×10^{-9}	3.1×10^{-9} [a]
Rat liver	Dexamethasone	1.6×10^{-8}	7.4×10^{-9} [b]
Mouse lymphoma	Dexamethasone	2.0×10^{-8}	2.0×10^{-8} [c]
Rat uterus	Estradiol	2.5×10^{-9}	$0.7\text{–}7 \times 10^{-10}$ [d]
Rat kidney	Aldosterone	4.0×10^{-9}	5.0×10^{-9} [e]

[a] Baxter and Tomkins, 1971. [b] Rousseau *et al.*, 1972b. [c] Rosenau *et al.*, 1972. [d] Erdos *et al.*, 1971; Alberga *et al.*, 1971; Puca and Bresciani, 1969; Puca *et al.*, 1971. [e] Rousseau *et al.*, 1972a.

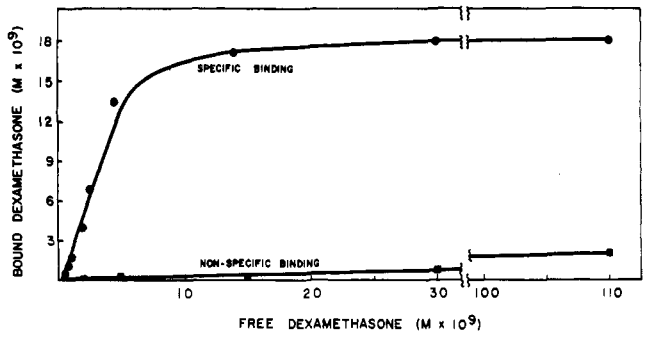

FIGURE 5: Titration of glucocorticoid receptor with [^{3}H]dexamethasone. The receptor–steroid complex was formed with the specified amount of [^{3}H]dexamethasone and filtered through DEAE disks as described under Materials and Methods; (●) specifically bound steroid, (■) nonspecifically bound steroid.

as the fraction of bound ligand retained under conditions of saturation with the protein. The initial slope of the curve presented in Figure 5, where steroid is limiting, indicates the efficiency of the DEAE filter assay to be *ca.* 86%. The equivalent experiment of measuring specific binding at increasing amounts of protein in the presence of a constant amount of steroid (not shown) gives a value of 88% efficiency when data are plotted in double reciprocal form and extrapolated to infinite receptor concentration.

Discussion

In this report, a simple filtration assay is described for quantitation of the glucocorticoid receptor protein. To date, filtration assays for steroid receptors have been unsuccessful because of the tendency of free steroid to bind to most filters. In the present case, the cytoplasmic solution containing the receptor is incubated with [^{3}H]dexamethasone to form receptor–steroid complexes, adsorbed on DEAE-cellulose disks, and the filter washed free of unbound steroid. The filters are then counted, the data corrected for nonspecifically bound steroid and used as a measure of the receptor. As with any assay of high sensitivity, uniform and meticulous technique is required for reproducibility and some experience is usually required before the accuracy described here is achieved. The results obtained are as accurate as those with previously described methods.

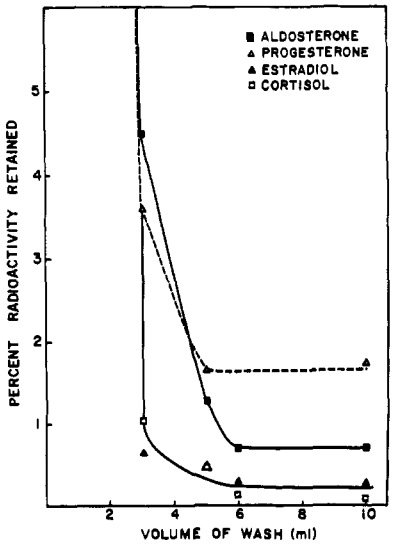

FIGURE 6: Retention of various [^{3}H]steroids on DEAE filters as a function of the volume of wash. The wash fluid used was buffer A except for estradiol, where buffer B was used.

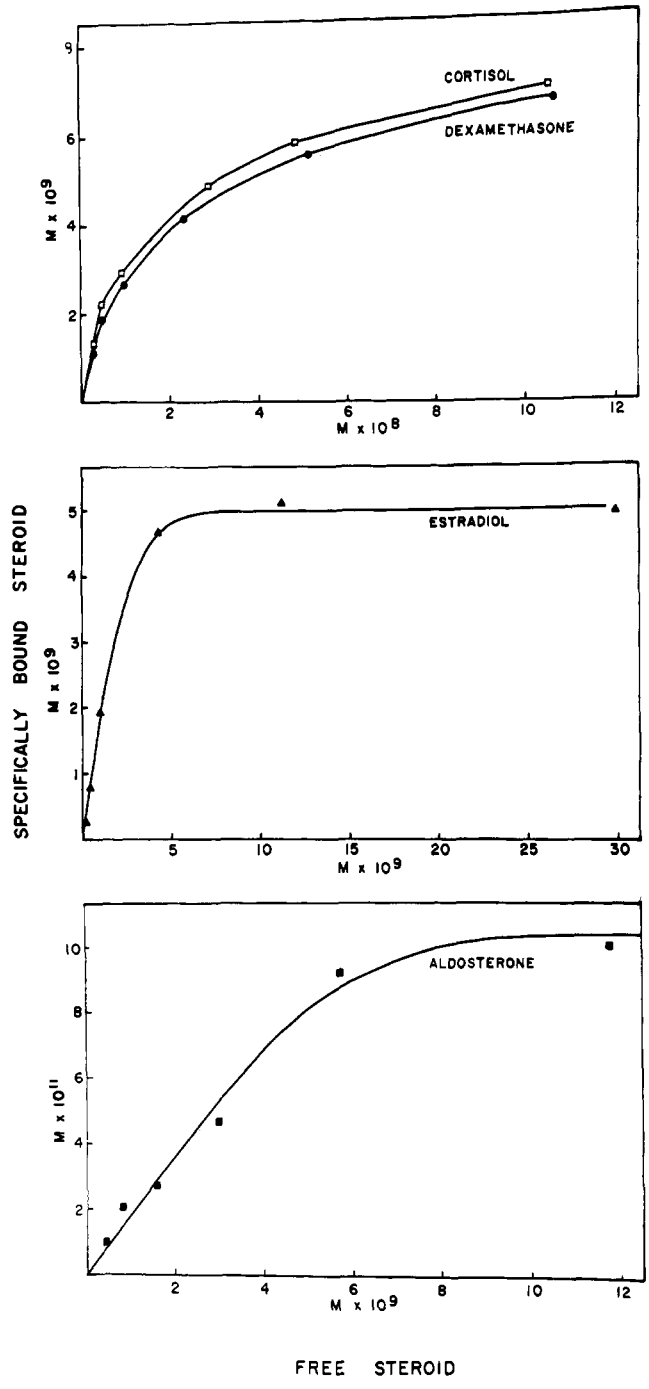

FIGURE 7: Binding curves obtained for various cytoplasmic steroid receptors. [^{3}H]Cortisol and [^{3}H]dexamethasone were incubated with liver cytosol, [^{3}H]estradiol with uterine cytosol, and [^{3}H]-aldosterone with kidney cytosol, and then filtered through DEAE filter disks as described under Materials and Methods. Buffer A was used in all cases except for the estradiol receptor where buffer B was used. Dissociation constants for the receptor–steroid complexes are presented in Table II.

A major advantage of this assay is the ease and rapidity with which it may be performed. Although low temperatures are required to prevent denaturation and/or dissociation, all manipulations may be performed on a bench top provided solutions are kept on ice. Using a filter manifold we routinely perform over 100 assays in an afternoon. In addition, the

assay is usable for low concentrations of receptor in solutions too dilute to be accurately quantitated by the charcoal method.

Preliminary experiments demonstrate that this assay may also be used for estrogen and aldosterone receptors. Although optimal conditions have not been established for these, it is clear that, at most, only minor modification of the conditions used here will be necessary. Further, utilization of a calibration curve as given in Figure 5, and fluids containing appropriate receptors, would permit a rapid isotope-dilution assay for quantitation of steroids in biological fluids.

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Model Studies of Thymidylate Synthetase. Intramolecular Catalysis of 5-Hydrogen Exchange and 5-Hydroxymethylation of 1-Substituted Uracils†

Daniel V. Santi* and Curtis F. Brewer‡

ABSTRACT: The mechanisms of base-catalyzed 5-hydrogen exchange and 5-hydroxymethylation of 1-substituted uracils have been examined to provide insight into the mechanism of the analogous reactions catalyzed by thymidylate synthetase. Kinetic studies of 5-H exchange demonstrate that the reaction requires attack of a nucleophile at the 6 position of the heterocycle to form carbanionic intermediates, as in classical addition reactions of α,β-unsaturated carbonyl compounds. The intermediate then accepts a proton from water to form the corresponding carbon acid; reversal of these steps results in 5-H exchange. Proton transfers at the 5 position of the saturated pyrimidine intermediates have been found to be susceptible to general acid–base catalysis and may be rate determining. The exchange reaction and 5-hydroxymethylation are greatly facilitated by intramolecular catalysis involving addition of a nucleophile attached to the 1 substituent to the 6 position of the heterocycle; in fact, the rate of intramolecular catalyzed exchange of one of the reactive ionic species of 2′,3′-*O*-isopropylideneuridine is comparable to that of the enzyme-catalyzed reaction. Depending upon the efficacy of the intramolecular catalyst the rate-determining step of the exchange may be either nucleophilic attack at the 6 position of the heterocycle or proton transfer reactions at the 5 position. From these results, a minimal mechanism for the thymidylate synthetase catalyzed 5-H exchange of 2′-deoxyuridylate and its condensation with 5,10-methylenetetrahydrofolic acid is proposed.

Thymidylate synthetase catalyzes the reductive methylation of dUMP[1] to TMP with the concomitant conversion of 5,10-CH_2H_4folate to 7,8-H_2folate (for a recent review, see Blakley, 1969). One of our interests has been the elucidation of the mechanism of catalysis of thymidylate synthetase and, as a prelude to enzymological studies, we sought to develop congruent model systems which would provide a basic understanding of underlying mechanistic features of this reaction (Scheme I).

The complexity of the reaction *in toto* requires that certain simplifying assumptions be made before approaching detailed model studies. Segregation of the overall reaction into two discrete steps permits it to be defined in terms of well-known chemical reaction types: (1) electrophilic substitution

† From Department of Biochemistry and Biophysics and Department of Pharmaceutical Chemistry, University of California, San Francisco, California 94122. *Received January 31, 1973.* This work was supported by U. S. Public Health Service Grant No. CA-14394 from the National Cancer Institute and a National Science Foundation trainee award to C. F. B. A portion of this work has been described in a preliminary communication (Santi and Brewer, 1968).

‡ Present address: Department of Pharmacology, Albert Einstein College of Medicine, Bronx, N. Y. 10461.

[1] Abbreviations used are: dUMP, 2′-deoxyuridylic acid; TMP, thymidylic acid; 5,10-CH_2H_4folate, 5,10-methylenetetrahydrofolic acid; 7,8-H_2folate, 7,8-dihydrofolic acid; H_4folate, tetrahydrofolic acid; $(EtOH)_3N$, triethanolamine; Et_3N, triethylamine.