# Synthesis of 2-Diazoestrone Sulfate and Use for Affinity Labeling of Steroid Binding Sites*

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ABSTRACT: To extend the application of affinity labeling of steroid binding sites, we have synthesized 2-diazoestrone sulfate and established its structure by infrared and elemental analysis as well as coupling with β-naphthol. Under physiologic conditions, 2-diazoestrone sulfate couples with tryptophan, cysteine, and histidine (and their various esters and peptides) giving a typical spectrum for each. After coupling, absorption maximum for tryptophan is at 297 mμ (ε 7250), for cysteine is at 330 mμ (ε 13,500), and for histidine is at 380 mμ (ε 14,600). In each case, a single coupling product is found on chromatography. Studies with mixtures of these amino acids indicate that coupling with cysteine is most rapid. No evidence for biscoupling with histidine is seen.

When small amounts of 2-diazoestrone sulfate react with excess egg albumin at pH 7.0, it couples predominantly with cysteine residues and the presence of 60 μM 17β-estradiol has no effect on the spectrum. If egg albumin is reacted with excess *p*-mercuribenzoate (which is then removed by dialysis) and subsequently reacted with similar amounts of 2-diazoestrone sulfate, tryptophan, and histidine coupling predominate. When small amounts of 2-diazoestrone sulfate react at pH 7.0 with glutamate dehydrogenase, a protein known to contain allosteric steroid binding sites, the spectrum shows coupling to tryptophan, cysteine, and histidine. Presence of 60 μM 17β-estradiol or 0.60 mM estrone sulfate in the reaction mixture significantly diminishes the coupling to cysteine but has little or no effect on that with tryptophan and histidine. Similar amounts of estriol are without effect. These observations are compatible with a mechanism whereby the steroid moiety of 2-diazoestrone sulfate delivers the reactive diazonium group to the steroid binding site of glutamate dehydrogenase, favoring coupling with a cysteine residue present at that site.

Studies of the molecular mechanism of estrogen action have now proceeded to the point where there is evidence that target organs contain a high-affinity receptor protein which may mediate steroid action (Toft and Gorski, 1966; Jensen *et al.*, 1968). Because affinity labeling has been used to characterize substrate binding sites, we have endeavored to extend this approach to steroid binding sites. Availability of steroid derivatives which will bind covalently to high-affinity steroid binding proteins should ultimately allow: (a) decision as to the obligatory role of receptor proteins and (b) delineation of the specific amino acid residues which are present at steroid binding sites on various protein molecules. We have previously synthesized 4-mercuri-17β-estradiol and shown that it can be used for affinity labeling (Chin and Warren, 1968). Further, the compound has inherent estrogenic activity and displays a persistent effect (as compared with 17β-estradiol which is compatible with covalent bonding to the receptor (Muldoon and Warren, 1969).

Because the use of 4-mercuri-17β-estradiol is restricted to those cases where the steroid binding protein to be studied contains a sulfhydryl group at the binding site, we thought to extend the specificity to include other amino acid residues. Accordingly, we have synthesized 2-diazoestrone sulfate (2-diazo-17-oxo-1,3,5(10)-estrien-3-yl sulfate) and studied its reaction with model compounds and proteins.

## Experimental Section

*Materials.* Reagent grade salts and inorganic acids were purchased from the Mallinckrodt Co. Reagent grade organic solvents were purchased from Fisher Scientific Co. and distilled prior to use. Alumina for chromatography was obtained from the Merck Co. and hydrated by mixing with 5% (w/w) water before use. The β-naphthol was obtained from the Mallinckrodt Co. and recrystallized from benzene. Pyridine sulfur trioxide adduct was obtained from Eastman Organic Chemicals. Palladium (10%) on powdered charcoal was obtained from Matheson, Coleman & Bell. Estrone, estrone sulfate, and 17β-estradiol were obtained from Steraloids, Inc. All amino acids and peptides were obtained from the Sigma Co. Ellman's Reagent (5,5'-dithiobis(2-nitrobenzoic acid)) was obtained from Aldrich Chemical Co. Tyrosine and *N*-acetyltyrosine were crystallized from water. Egg albumin was Sigma (Grade V) with less than 1% extraneous protein present. L-Glutamate dehydrogenase (EC 1.4.1.3) was Sigma (type I) crystalline suspension in ammonium sulfate. Double-distilled (in glass) water was used throughout. Thin-layer chromatography utilized Eastman 6060 silica gel sheets.

*Methods.* The synthesis of 2-diazoestrone sulfate involves four steps: (1) nitration of estrone, (2) conversion of 2-nitroestrone into 2-nitroestrone sulfate, (3) reduction to 2-aminoestrone sulfate, and (4) diazotization to yield 2-diazoestrone sulfate (Figure 1).

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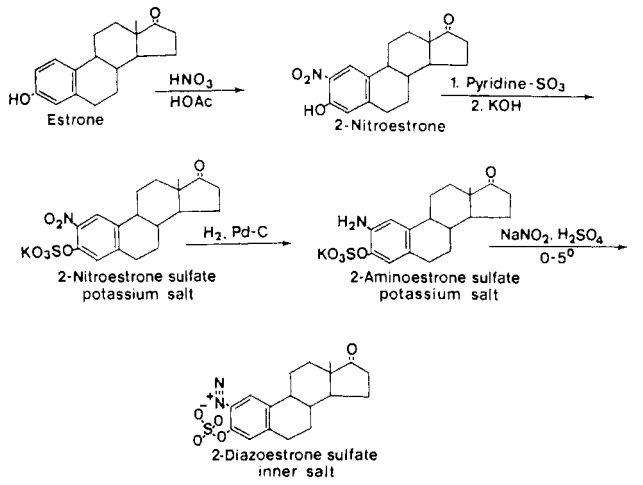



FIGURE 1: Scheme of synthesis of 2-diazoestrone sulfate.

*Synthesis of 2-Nitroestrone.* Estrone (1.0 g) was nitrated in glacial acetic acid as described by Niederl and Vogel (1949) and Werbin and Holoway (1956).

The yellow precipitate (4-nitroestrone) was removed by filtration. The filtrate was poured into 200 ml of water. The resulting yellow precipitate contained mainly 2-nitroestrone. It was collected by filtration, washed with water, and dried in a desiccator.

A 10% (w/v) benzene solution of the steroid mixture was placed on an alumina column; 1 g of the dry yellow powder required 100 g of alumina. On elution with benzene, 2-nitroestrone emerged first, long before the other derivatives. The separation was finished in 3–4 hr. Evaporation of the appropriate solvent fractions with a Rinco evaporator left 2-nitroestrone, which was recrystallized from absolute ethanol to give yellow flakes: mp 178°, yield 54%, $\lambda_{max}^{EtOH}$ 293 mμ (ε 8120) and 362 mμ (ε 3690). Spectral observations are in satisfactory agreement with those of Werbin and Holoway (1956) and completely different from the other nitroestrones. Melting point is 6° lower than they obtained after crystallizing the compound from 80% alcohol but we recrystallized the material several times without change. On thin-layer chromatography developed with chloroform a single spot ($R_F$ 0.67) was seen.

*Synthesis of 2-Nitroestrone Sulfate.* 2-Nitroestrone (1.0 g, 3.2 mmoles) was dissolved in 25 ml of dry pyridine and dry pyridine–sulfurtrioxide (3.0 g, 18.9 mmoles) was added. The mixture was stirred at 55° overnight. The solution was evaporated almost to dryness with a Rinco evaporator and the residue was treated twice with 100 ml of anhydrous ether. After filtering, the solid residue was dissolved in 25 ml of pyridine and partitioned with 50 ml of 12% KOH. The pyridine layer was transferred to a large flask. Ten volumes of ether was added with vigorous shaking. The organic solvent was decanted out and the residue was dried with a Rinco evaporator and dissolved in a minimum volume of methanol (about 100 ml). The golden yellow potassium salt of 2-nitroestrone sulfate was crystallized by adding five to ten volumes of ether. A second recrystallization from methanol–ether was carried out. When partitioned between ether and water, this compound was clearly water soluble: yield 95%; mp 215° dec; $\lambda_{max}$ (0.05 M potassium phosphate, pH 8.0) 280 mμ (ε 5650); infrared spectrum (KBr): 1720 (C=O), 1550, 1355 ($NO_2$), and 1150 $cm^{-1}$ ($OSO_3$).

*Reduction of 2-Nitroestrone Sulfate to 2-Aminoestrone Sulfate.* Potassium 2-nitroestrone sulfate (1.0 g, 2.3 mmoles) was dissolved in 450 ml of 95% ethanol and 0.5 g of 10% Pd on carbon was then added to the solution as catalyst. Reduction was carried out in a Brown hydrogenator at room temperature for 3 hr. Glacial acetic acid (20 ml) was placed in a 125-ml flask and a 1.0 N solution of $NaBH_4$ in a 0.1 N NaOH was added dropwise from a buret with stirring to generate hydrogen. About 10 ml of the $NaBH_4$ solution had been consumed when the reduction was complete. The resulting colorless solution was filtered and evaporated to dryness with a Rinco evaporator. The white potassium salt of 2-aminoestrone sulfate was recrystallized from water (0.1 g/5 ml): yield 77%; mp 256–259° dec; $\lambda_{max}^{EtOH}$ 238 mμ (ε 2740) and 294 mμ (ε 1115); infrared spectrum (KBr): 3430, 3340 ($NH_2$), 1720 $cm^{-1}$ (C=O), and 1155 $cm^{-1}$ ($OSO_3$); $R_F$: 0.37 for thin layer with MeOH–benzene (20:80) as solvent; 0.40 for paper with butanol–toluene (50:50)–$NH_4OH$–water (20:80) as solvent. Before spraying with Folin reagent, both chromotograms were hung in a glass chamber saturated with dioxane–HCl (80:20) vapor for 3 hr to hydrolyze the sulfate ester. Nuclear magnetic resonance spectrum (δ) ($D_2O$) showed 7.06 ($J$ = 0–1) and 7.15 ($J$ = 0–1), which indicates that the two remaining hydrogens on the A ring are *para*. *Anal.* for $C_{18}H_{22}KNO_5S$: Calcd: C, 53.58; H, 5.51; K, 9.69; N, 3.52; S, 7.95. Found: C, 53.99; H, 5.44; K, 9.53; N, 3.38; S, 7.89.

*Diazotization of 2-Aminoestrone Sulfate to 2-Diazoestrone Sulfate.* 2-Aminoestrone sulfate (100 mg, 0.247 mmole) was dissolved in 8 ml of water. The solution was then cooled to 0–5°, 20 mg of $NaNO_2$ was dissolved in the suspension, and 0.60 ml of 1.0 N $H_2SO_4$ was added dropwise with vigorous stirring. After 2-min reaction at 0–5°, the pale yellow inner salt of 2-diazoestrone sulfate was quickly filtered, washed twice with 4 ml of ice-cold water, and quickly dried over concentrated $H_2SO_4$ under reduced pressure. The compound, stored dry and dark at −20°, is stable for 2–3 months: yield 72%; mp 129–130° dec; infrared spectrum (KBr): 2250 (diazonium), 1720 (C=O), and 1155 ($OSO_3$). The bands at 3430 and 3340 $cm^{-1}$ ($NH_2$) were now absent. *Anal.* for $C_{18}H_{20}N_2O_5S$: Calcd: C, 57.27; H, 5.61; N, 7.42; S, 8.49. Found: C, 56.45; H, 5.30; N, 7.10; S, 8.58.

Throughout this study, 2-diazoestrone sulfate was always dissolved in a small volume of cold 2.0 N KOH for use. If frozen each night this solution is stable for 2–3 days. Further, coupling reactivity was checked frequently with β-naphthol.

*Coupling Reaction of 2-Diazoestrone Sulfate with Amino Acids, Egg Albumin, and Glutamate Dehydrogenase.* Three milliliters of $5 \times 10^{-4}$–$1 \times 10^{-2}$ M amino acids in 0.2 M potassium phosphate buffer (pH 7.0) was mixed with 5–75 μl of 2-diazoestrone sulfate in 2.0 N KOH in a 3-ml cuvet. All reactions were at 25°. In the reference cuvet, which contained an equal amount of amino acid, peptide, or protein, an equal volume of 2.0 N KOH was always used in place of the 2-diazoestrone sulfate solution. After 10 min at room temperature all spectra were recorded with a Cary 14 spectrophotometer.

To determine if biscoupling of 2-diazoestrone sulfate occurs with histidine, 0.5 ml of 1.0 mM histidine in 0.2 M

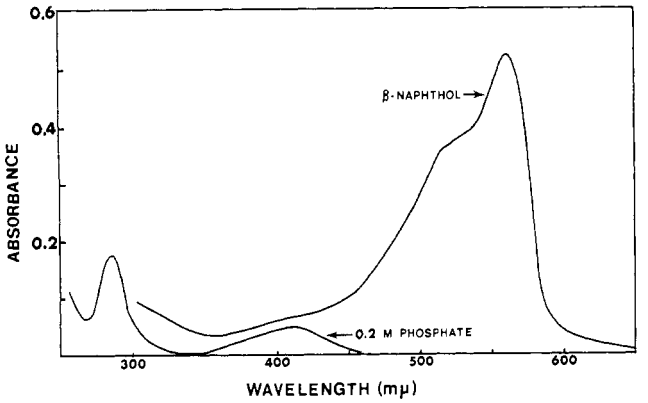



FIGURE 2: Absorbance of 25 μM 2-diazoestrone sulfate and its coupling product with 0.5 mM β-naphthol in 0.2 M potassium phosphate buffer, pH 7.0, 25°. Light path was 1.0 cm. This light path, buffer, pH, and temperature apply to all the following figures.

potassium phosphate buffer (pH 7.0) was mixed with increasing amounts of 6 mM 2-diazoestrone sulfate until the mole ratio of 2-diazoestrone sulfate to amino acid in the solution reached 2. After 10 min, the mixture was diluted to 5 ml with 0.2 M potassium phosphate buffer at pH 7.0 and the spectrum was recorded in 3-ml cuvets.

The reaction with egg albumin or glutamate dehydrogenase was carried out in 3-ml cuvets. Egg albumin was dissolved in 0.2 M potassium phosphate buffer at pH 7.0. Glutamate dehydrogenase in ammonium sulfate was centrifuged at 20,000*g* for 30 min and the pellet dissolved in 0.2 M potassium phosphate buffer (pH 7.0) to make the desired concentration. The solution was centrifuged at 20,000*g* for another 30 min to separate any undissolved matter. The final protein concentration was determined spectrophotometrically at 280 mμ with an $\epsilon$ value of $1 \times 10^6$ (Olson and Anfinsen, 1952). The 2-diazoestrone sulfate solution was then added in small amounts. After 10 min, spectra were recorded. (This time allowed completion of the reaction in all cases.) In the reference cuvet, which contained an equal amount of protein, an equal volume of 2.0 N KOH was used in place of the 2-diazoestrone sulfate solution. The pH values were checked after each reaction and found to be essentially unaltered (<0.1 pH unit) by the small amounts of base added. When 17β-estradiol or estrone sulfate was added it was in 0.1 ml of 1.0 N KOH, placed in both reference and sample cuvets.

## Results

The spectrum of 2-diazoestrone sulfate in 0.2 M potassium phosphate buffer at pH 7.0 is shown in Figure 2. Absorption maxima at 285 mμ ($\epsilon$ 6720) and 415 mμ ($\epsilon$ 2030) are noted. A similar spectrum was obtained in 0.01 M phosphate buffer with pH maintained at 7.0. The spectrum of 2-diazoestrone sulfate added to a 20-fold excess of β-naphthol is shown in the same figure. A spectrum obtained with β-naphthol in 15-fold excess was identical indicating that with these quantities of β-naphthol, essentially all of the 2-diazoestrone sulfate is coupled to it. Thus, $\epsilon_{562}$ of the reaction product is shown to be 20,500. These spectra were obtained 10 min after addition of the steroid and no subsequent change occurred indicating that the reactions were complete.

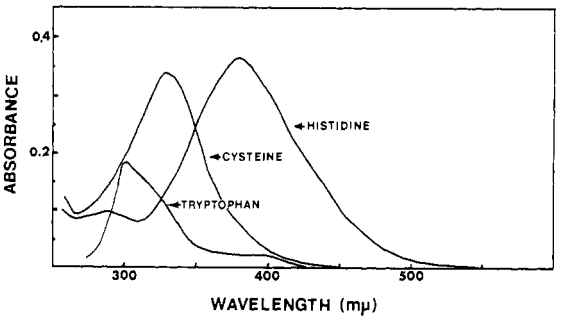



FIGURE 3: Absorbance of coupling product of 25 μM 2-diazoestrone sulfate with 10.0 mM cysteine, tryptophan, and histidine.

When the reaction was carried out by placing 2-diazoestrone sulfate in the neutral buffer solution first and adding a 20-fold excess of β-naphthol at given time intervals, spectra indicated clearly that the compound decomposes or reacts with the buffer solution such that it loses 50% of its coupling reactivity in 1–2 min.

The spectra of 2-diazoestrone sulfate coupled with 0.01 M cysteine, tryptophan, and histidine are shown in Figure 3. Spectral data on these compounds and some *N*-acetyl derivatives and simple peptides are given in Table I. It will be noted that the amino acids and their derivatives are present in great excess and it was shown that further increase in their concentrations did not enhance coupling to any significant degree. This excess was necessary to calculate absolute extinction coefficients because 2-diazoestrone sulfate decomposes throughout the reaction.

Glycine, alanine, lysine, arginine, cystine, aspartic acid, and serine (0.01 M) give no spectral evidence of coupling with 2-diazoestrone sulfate (final concentration 25 μM) in 0.2 M potassium phosphate buffer at pH 7.0. Despite the reports (Higgins and Fraser, 1952; Tabachnick and Sobotka, 1959) that tyrosine and its *N*-acetyl derivatives

TABLE I: Spectral Data of the Coupling Products of 2-Diazoestrone Sulfate.[a]

| Compounds | max (mμ) | Obsd *A* | $\epsilon$ at $\lambda_{max}$ |
|---|---|---|---|
| β-Naphthol (0.5 mM) | 562 | 0.520 | $2.05 \times 10^4$ |
| Cysteine | 330 | 0.336 | $1.35 \times 10^4$ |
| Glutathione | 330 | 0.336 | $1.35 \times 10^4$ |
| Tryptophan | 297 | 0.180 | $7.25 \times 10^3$ |
| *N*-Acetyltryptophan | 297 | 0.112 | $4.52 \times 10^3$ |
| Tryptophanylglycine | 297 | 0.182 | $7.26 \times 10^3$ |
| Glycyltryptophan | 297 | 0.130 | $5.01 \times 10^3$ |
| Histidine | 380 | 0.360 | $1.46 \times 10^4$ |
| *N*-Acetylhistidine | 380 | 0.360 | $1.46 \times 10^4$ |

[a] Data collected with 25 μM 2-diazoestrone sulfate and compounds above at 10 mM concentrations (except β-naphthol) in 0.2 M potassium phosphate buffer, pH 7.0, 25°. Light path was 1.0 cm. Spectra recorded after 10-min reaction and they did not subsequently change.

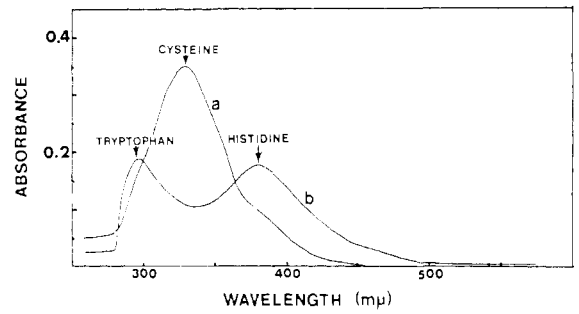



FIGURE 4: Absorbance of coupling products of 25 μM 2-diazoestrone sulfate: (a) with a mixture of cysteine, tryptophan, and histidine (each at 2.5 mM) and, (b) with a mixture of tryptophan and histidine at the same concentrations.

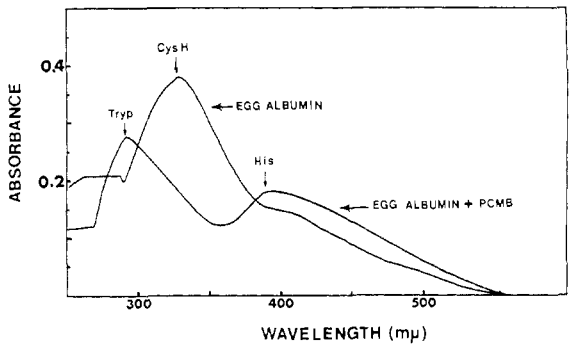



FIGURE 5: Absorbance of coupling product of 50 μM 2-diazoestrone sulfate with native egg albumin (0.5 mM) compared with that where albumin had been pretreated with (2.0 mM) *p*-mercuribenzoate and dialyzed prior to coupling.

are good coupling compounds with many single diazonium salts at high pH, this amino acid gave no spectral evidence of coupling with 2-diazoestrone sulfate under the conditions above. Proline does react with 2-diazoestrone sulfate. In a protein, this amino acid in a nonterminal position would be nonreactive as alanylproline fails to display spectral evidence of coupling.

While it has been shown (Higgins and Fraser, 1952; Tabachnick and Sobotka, 1959) that when the concentration of some simple diazonium salts approaches or exceeds that of histidine, biscoupling on one molecule of amino acid may occur with a shift of absorption to considerably longer wavelength, this phenomenon was not observed with 2-diazoestrone sulfate under the conditions used in the present study. The absorption maximum at 380 mμ persisted and no new absorption bands were seen.

*Coupling with Mixed Amino Acids.* When 2.5 mM cysteine, tryptophan, and histidine are reacted with 2-diazoestrone sulfate (final concentration 25 μM), cysteine is clearly the most reactive as shown in Figure 4. If absorbance of the coupling mixture is compared with that of cysteine, it will be seen that more than 90% of the 2-diazoestrone sulfate is coupled to cysteine. When only tryptophan and histidine are present, absorption maxima are seen at 297 and 380 mμ representing coupling with these two amino acids. The coupling reactivity of tryptophan to histidine is 55:45 according to

$$[C]_{Trp\text{-}D}\,(\epsilon_{297}) + [C]_{His\text{-}D}\,(\epsilon_{297}) = A_{297}$$

$$[C]_{Trp\text{-}D}\,(\epsilon_{380}) + [C]_{His\text{-}D}\,(\epsilon_{380}) = A_{380}$$

where $[C]_{Trp\text{-}D}$ and $[C]_{His\text{-}D}$ are molar concentrations of reaction products of tryptophan and histidine, respectively, with 2-diazoestrone sulfate, $\epsilon$ is the molar extinction coefficient of that product at the given wavelength, and $A$ is the absorbance observed. Presence of 2.5 mM *N*-chloracetyltyrosine or alanine had no effect on these spectra.

*Analysis of Coupling Products.* Separate solutions of cysteine, tryptophan, and histidine ($10^{-2}$ M in 5.0 ml of 0.2 M potassium phosphate buffer, pH 7.0) were prepared. To each, 0.80 ml of 5 mM 2-diazoestrone sulfate in 2.0 N KOH was slowly added. Simultaneous additions of 0.2 M $KH_2PO_4$ was carried out to keep the pH at 7.0. After 10-min reaction, solutions were lyophilized. The residues were extracted with 1.0 ml of methanol. One-tenth of each extract was added to 3.0 ml of 0.2 M potassium phosphate buffer (pH 7.0) and shown to display the typical absorbance spectrum. The remainder of each extract was taken almost to dryness and submitted to thin-layer chromatography using butanol–acetic acid–$H_2O$ (6:1:2) for development. As controls, similar extracts prepared from solutions where 2.0 N KOH replaced the diazosteroid were used. Ninhydrin and Folin reagents (the latter after hydrolysis with dioxane–HCl as described in methods) were applied to the dried chromatograms. In the case of tryptophan ($R_F$ 0.56), a single, new, Folin- and ninhydrin-positive spot ($R_F$ 0.70) representing the reaction product was seen. In the case of cysteine ($R_F$ 0.28), a single, new, Folin- and ninhydrin-positive spot ($R_F$ 0.61) was seen. In the case of histidine ($R_F$ 0.13) a single, new yellow, Folin- and ninhydrin-positive spot ($R_F$ 0.39) was seen.

When 2-diazoestrone sulfate was added to a buffer solution lacking amino acid and similarly chromatographed and treated, a Folin-positive, ninhydrin-negative spot ($R_F$ 0.50) was seen. Treatment of a $10^{-2}$ M *N*-chloroacetyltyrosine solution, after reaction with 2-diazoestrone sulfate gave only the same spot, indicating that no coupling product resulted.

*Reaction with Egg Albumin.* When 1.5 μmoles of egg albumin (mol wt 44,000) reacted with 0.15 μmole of 2-diazoestrone sulfate in 3.0 ml of 0.2 M potassium phosphate buffer (pH 7.0), cysteine was the most reactive amino acid residue in the protein molecule as shown in Figure 5. When egg albumin was first treated with 6.0 μmoles of *p*-mercuribenzoate, dialyzed, and then reacted with a similar amount of 2-diazoestrone sulfate, the cysteine peak at 330 mμ disappeared while the tryptophan peak at 297 mμ and the histidine peak at 380 mμ were clearly enhanced.

The effects of 17β-estradiol on the reaction of 2-diazoestrone sulfate with egg albumin were studied under conditions described above except that 2-diazoestrone sulfate was added in amounts of 0.05, 0.1, 0.15, and 0.25 μmole. In all cases, spectra recorded in the presence and absence of 60 μM 17β-estradiol were identical indicating that the natural estrogen had no effect on coupling. Further, studies conducted by adding similar amounts of 2-diazoestrone sulfate to 60 μM 17β-estradiol revealed no spectral evidence of reaction.

*Reaction with Glutamate Dehydrogenase.* Glutamate dehydrogenase was chosen as a model protein for affinity labeling because it has allosteric binding sites of moderate affinity for 17$\beta$-estradiol and estrone (Yielding and Tomkins, 1960; Warren *et al.*, 1964; Douville and Warren, 1968). The reaction was carried out by adding 0.09 $\mu$mole of 2-diazoestrone sulfate (final concentration 30 $\mu$M) to 3.0 ml of 0.2 M potassium phosphate buffer (pH 7.0, 25°) containing 4.7 $\mu$M glutamate dehydrogenase. The spectrum obtained, which was unchanged after 10 min, is shown in Figure 6. It will be noted that absorbance at 330 m$\mu$ (cysteine) exceeds that at 297 m$\mu$ (tryptophan) only slightly. When the final concentration of 2-diazoestrone sulfate in the reaction solution is only 6 $\mu$M (not shown), absorbance at 330 m$\mu$ markedly exceeds that at 297 m$\mu$ showing that when 2-diazoestrone sulfate is extremely limited, it reacts preferentially with cysteine.

When the reaction was repeated in the presence of 60 $\mu$M 17$\beta$-estradiol, the cysteine spectrum was markedly diminished while the tryptophan peak is about the same and the histidine peak is somewhat enhanced (Figure 6). Calculations were made using

$$[C]_{Gly\text{-}Trp\text{-}D}(\epsilon_{297}) + [C]_{Cys\text{-}D}(\epsilon_{297}) + [C]_{His\text{-}D}(\epsilon_{297}) = A_{297}$$

$$[C]_{Gly\text{-}Trp\text{-}D}(\epsilon_{330}) + [C]_{Cys\text{-}D}(\epsilon_{330}) + [C]_{His\text{-}D}(\epsilon_{330}) = A_{330}$$

$$[C]_{Gly\text{-}Trp\text{-}D}(\epsilon_{380}) + [C]_{Cys\text{-}D}(\epsilon_{380}) + [C]_{His\text{-}D}(\epsilon_{380}) = A_{380}$$

where $[C]_{Gly\text{-}Trp\text{-}D}$, $[C]_{Cys\text{-}D}$ and $[C]_{His\text{-}D}$ are molar concentrations of coupled glycyltryptophan, cysteine, and histidine, respectively, $\epsilon$ is the molar extinction coefficient of that product at the given wavelength (for glycyltryptophan: $\epsilon_{297}$ 5010, $\epsilon_{330}$ 2120, and $\epsilon_{380}$ 2700), and $A$ is the absorbance observed. It was found that in the absence of 17$\beta$-estradiol, 3.8 sulfhydryl residues/molecule (molecular weight taken at $10^6$) are coupled with 2-diazoestrone sulfate while in its presence, only 0.5 sulfhydryl group is coupled. When the reaction is repeated in the presence of 0.6 mM estrone sulfate, the cysteine spectrum was again diminished (Figure 6) and calculations as above revealed that only 0.7 sulfhydryl residue/molecule is coupled.

These observations indicate that 17$\beta$-estradiol and estrone sulfate exclude 2-diazoestrone sulfate from sulfhydryl residues of glutamate dehydrogenase. Estriol at a concentration similar to that of 17$\beta$-estradiol had no significant effect on the coupling spectrum.

*Reaction of Glutamate Dehydrogenase with Ellman's Reagent.* Ellman's reagent and glutamate dehydrogenase (at final concentrations of 0.66 mM and 4.7 $\mu$M, respectively) were allowed to react in 0.2 M potassium phosphate buffer (pH 7.0). The reaction was carried out after addition of 25 $\mu$l of 1.0 N KOH. It was repeated after addition of a similar amount of KOH containing 17$\beta$-estradiol (final concentration 60 $\mu$M) or estrone sulfate (final concentration of 0.60 mM). It was followed spectrophotometrically at 412 m$\mu$, where for the nitrobenzenethiol ion, the value for $\epsilon$ is 13,600. The number of sulfhydryl groups per mole of glutamate dehydrogenase reacting in 15 min were: 0.1 in the absence of steroid, 0.7 in the presence of estrone sulfate, and 1.3 in the presence of 17$\beta$-estradiol. Thus, it is seen that when a non-steroidal sulfhydryl reagent is used, the steroids do not mask sulfhydryl groups but expose them.

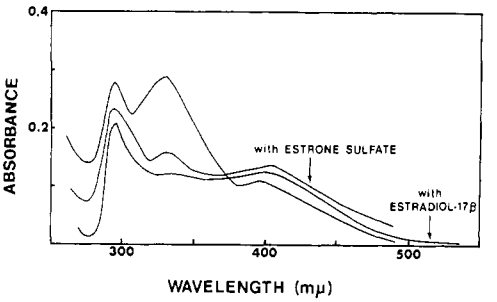


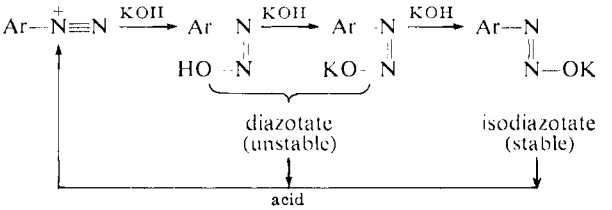

FIGURE 6: Coupling spectra of 30 $\mu$M 2-diazoestrone sulfate with 4.7 $\mu$M glutamate dehydrogenase in the presence and absence of 60 $\mu$M 17$\beta$-estradiol and 0.60 mM estrone sulfate.

## Discussion

The mechanism of affinity labeling depends upon concentration of the reagent by the steroid portion of the molecule in the act of reversible binding at the binding site so that covalent bond formation will be relatively favored at that site. To achieve success, the derivative must resemble the parent steroid and the protein involved must contain, at or near the binding site, a residue capable of reacting with the reagent. The synthesis of a diazonium compound which is capable of coupling to cysteine, histidine, and tryptophan extends its applications as compared to the previously reported 4-mercuri-17$\beta$-estradiol.

We originally carried out the synthesis of 2-diazoestrone for this purpose. Although the compound could be synthesized, it decomposed within a few minutes regardless of every attempt we made to store it. This decomposition was characterized by a deepening yellow color and inability to couple with $\beta$-naphthol. We presumed it was due to quinone formation and next synthesized 2-diazoestrone-3-methyl ether. This compound was stable and coupled with $\beta$-naphthol. Unfortunately, its reaction with amino acids was so slow that it was deemed undesirable for use as an affinity-labeling compound. Ultimately, 2-diazoestrone sulfate was chosen because steroid sulfates are natural products which have been shown, in some cases, to bind at the site utilized by the free steroid (Warren and Crist, 1967; Betz and Warren, 1968).

2-Diazoestrone sulfate is a reactive compound, unstable in neutral aqueous solution and most organic solvents. It is unlike the common diazonium salts which are stable in dilute acid. Only certain inert solvents (ether, petroleum ether (bp 30–60°), and benzene) do not react with it. However, 2-diazoestrone sulfate appears to be converted into a stable isodiazotate by concentrated alkali and can be used in this form according to (Hantzsch and Lifschitz, 1912)




It reacts with histidine, tryptophan, and cysteine, giving a typical spectrum for each.

Glutamate dehydrogenase binds 17β-estradiol and estrone at an allosteric site where the $K_d$ approximates $10^{-5}$ M (Douville and Warren, 1968). When it reacts with 2-diazoestrone sulfate, the presence of 60 μM 17β-estradiol or 0.60 mM estrone sulfate significantly decreases the coupling with cysteine. Coupling to tryptophan and histidine is not decreased and histidine coupling is actually somewhat enhanced. One would presume that 2-diazoestrone sulfate, if excluded from the steroid binding site, would be available to couple with exposed nonspecific residues as well as decompose in solution. That the spectrally observed cysteine coupling is, in fact, affinity labeling is supported by several factors. As the amounts of 2-diazoestrone sulfate added are decreased, the cysteine coupling peak becomes relatively more prominent. 17β-Estradiol and estrone sulfate exclude 2-diazoestrone sulfate, apparently by occupying the binding site, while estriol which has little or no affinity for the binding site, has no effect. With egg albumin, which has no high-affinity steroid binding site, no affinity labeling is to be expected. This is the case, as the coupling of cysteine residues with 2-diazoestrone sulfate is not altered by the presence of 17β-estradiol.

It must be considered that 17β-estradiol and estrone sulfate might block appearance of the cysteine coupling peak seen on reaction of 2-diazoestrone sulfate and glutamate dehydrogenase by an allosteric mechanism, masking cysteine side chains distant from the steroid binding site. This possibility was evaluated using a nonsteroid sulfhydryl reagent (Ellman's). The results clearly indicate that the general effect of 17β-estradiol and estrone sulfate on sulfhydryl groups is one of exposure rather than masking and militate against an allosteric mechanism.

These observations are compatible with a mechanism whereby 2-diazoestrone sulfate first forms a reversible complex with the 17β-estradiol binding site, dependent on the gross structure of the steroid molecule. They also indicate a sulfhydryl group to be present at or very near that site, supporting the observation made with 4-mercuri-17β-estradiol (Chin and Warren, 1968).

When 2-diazoestrone sulfate is used with other steroid binding proteins which contain tryptophan or histidine at the steroid binding site, similar inhibition of coupling by 17β-estradiol is to be expected. Therefore, the compound should be useful in characterizing steroid binding sites containing any of these three reactive amino acid residues.

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