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The Nonenzymatic Modification of Proteins by Steroids
and DNA by Reducing Sugars; In Vitro and In Vivo Studies

A thesis submitted to the Faculty of The Rockefeller University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

by

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PREFACE

The studies described in this thesis were performed in the Laboratory of Medical Biochemistry during the years 1981-84. Several members of the laboratory assisted in these studies. Peter Ulrich was invaluable in the chemical synthesis and structural analysis of steroid-lysine adducts. The lens studies were done together with Dr. Shigeo Manabe. Vincent Monnier, Nguyen Le Trang, Helen Vlassara, and Michael Brownlee provided many helpful discussions.

The 16 α -hydroxyestrone investigations were performed in collaboration with Dr. Jack Fishman. I am grateful for his insight and encouragement throughout these studies. David Cowburn, Francis Picart, Brian Chait, and Aladar Bencsath assisted in performing the NMR and mass spectroscopic work. Thanks are also due to Carl Monder and Leon Bradlow for their interest and helpful discussions concerning the glucocorticoid structural studies.

Ed Cotlier and Michelle Gallati (Cornell University Medical College) were instrumental in developing the rabbit model of the steroid-induced cataract.

The clinical studies were performed together with Bob Lahita at the Rockefeller Hospital. I am thankful for his many insights and observations about SLE and autoimmunity. I am particularly grateful to Charles Christian (Hospital for Special Surgery) for acting as a clinical tutor during my graduate studies and allowing me to learn, firsthand, many aspects of clinical medicine and rheumatic disease.

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I am also indebted to Peter Model, in whose laboratory and under whose guidance the DNA modification experiments were performed. I am thankful to Marjorie Russell for teaching me the practical aspects of genetic analysis and to Norton Zinder for his insights.

I am also grateful to my wife, Anne, for her support and encouragement during my graduate years.

Finally, I am most indebted to my thesis advisor Dr. Anthony Cerami, not only for his guidance and enthusiasm throughout these investigations, but also for his friendship and his dedication to my development as a scientist.

ABSTRACT

A number of studies have demonstrated that small metabolites can react nonenzymatically with proteins to form covalent, addition products. An example of this type of reaction is the modification of hemoglobin A by glucose to form the minor species hemoglobin A_{1c}. In this case, glucose reacts by forming a reversible Schiff base adduct with the N-terminal valine of the β -chain of hemoglobin. The Schiff base intermediate then slowly undergoes an Amadori rearrangement to produce a stable ketoamine addition product.

The following studies investigate two novel aspects of nonenzymatic addition reactions. The first is the demonstration that ketolic steroids, i.e. steroids bearing a carbonyl function adjacent to a hydroxyl group, can react with protein amino groups to form stable Heyns rearrangement products. The second is the observation that reducing sugars can react with the nucleotides of DNA to form covalent adducts. The steroids which react with proteins include the glucocorticoids; such as cortisol and prednisolone, which contain side-chain ketols, and the estrogen metabolite 16 α -hydroxyestrone (16 α OHE), which contains a ring ketol. The long-term therapeutic administration of glucocorticoids is associated with many toxic manifestations and it was reasoned that one of these effects, cataract formation, might be the result of the progressive accumulation of protein bound glucocorticoid adducts. 16 α -hydroxyestrone on the other hand, has been reported to be present in elevated amounts in the autoimmune disease Systemic Lupus Erythematosus (SLE). Protein modification by this steroid might induce immune dysfunction by either hapten formation or by covalently modifying immunoregulatory proteins.

Both cortisol and 16 α OHE react with albumin in vitro, modifying 2-3 discrete lysine residues. Structural analysis of model steroid-lysine adducts showed that these products form through a Schiff base addition reaction between the steroid carbonyl and the ϵ -amino group of lysine.

The role of glucocorticoid-protein adducts in cataract formation was investigated first in two experimental models of the steroid-induced cataract. The addition of the glucocorticoid, prednisolone, to the rat

lens in culture resulted in a time and concentration dependent lens opacification that correlated with the incorporation of [^3H]prednisolone into lens protein. The most extensively modified proteins were two subunits of the lens crystallins, the major structural component of the lens. In the rabbit, lens opacities similar in morphology to human steroid-induced cataracts were induced by the intravitreal injection of glucocorticoids. These lesions also were associated with steroid incorporation into lens protein.

In order to detect glucocorticoid adducts formed in vivo, a radioimmunoassay was developed for these products utilizing antisera produced against prednisolone which had been nonenzymatically attached to albumin. Lens proteins from 33 normal and cataractous human lenses were fractionated and assayed for prednisolone adducts by competitive radioimmunoassay. Steroid adducts were detected only in lens proteins obtained from the steroid cataracts, and at levels similar to those observed in the rat and rabbit experimental models.

In vitro, prednisolone attaches to the amino groups of the lens crystallins. This modification imparts on the crystallins an increased susceptibility to sulfhydryl oxidation, leading with time to disulfide bond exchange and the formation of high-molecular weight aggregates which refract light. This effect was blocked by the pretreatment of lens crystallins with the mild acetylating agent, acetylsalicylic acid. Acetylation itself did not induce aggregation, but did prevent prednisolone incorporation and the subsequent formation of high-molecular weight aggregates.

The estrogen metabolite, $16\alpha\text{OHE}$, reacts in vitro with a number of proteins, including albumin, hemoglobin, and erythrocyte membrane proteins. When added to whole blood however, $16\alpha\text{OHE}$ reacts preferentially with membrane proteins; for example, erythrocyte membrane proteins are labeled to a greater than 15 fold higher specific activity than albumin. A radioimmunoassay for the $16\alpha\text{OHE}$ -lysine product was developed and a number of proteins from both normal and SLE individuals were assayed for the presence of these adducts. Women with SLE were found to have higher than normal levels of $16\alpha\text{OHE}$ modified proteins in erythrocyte and lymphocyte

membranes. In the case of the erythrocyte there was a correlation between the protein 16 α OHE-lysine level and the circulating level of free 16 α OHE. Glomerular basement membrane collagen also was analyzed and found to contain high amounts of 16 α OHE-lysine. It appears that the level of 16 α OHE modification of a given protein is proportional to its biological lifespan and that, in relative terms, this level of modification is increased in SLE patients. This is consistent with the idea that 16 α OHE adducts accumulate over a long time period, and that the degree of protein modification is a time integral of the free 16 α OHE level.

The increased level of 16 α OHE-protein adducts in patients with an autoimmune disease led to the possibility that these products might be antigenic in some individuals. An enzyme-linked immunoassay for anti-estrogen autoantibodies was developed and used to screen human plasma. Unexpectedly, a number of normal, disease-free women were found to display this anti-estrogen activity. On further analysis, it was found that approximately 30% of normal women who had ever used oral contraceptive medication had sera which gave positive results on this assay. Oral contraceptives contain the synthetic estrogen 17 α -ethinylestradiol, and this derivative has been observed previously to undergo enzymatic conversion to products which react with proteins to form covalently bound ligands. Presumably, in some women, these protein-bound estrogens are antigenic and induce antibody formation. On the other hand, a few male and female patients with SLE displayed this anti-estrogen antibody activity without ever having been on oral contraceptives. This anti-estrogen activity may represent an antibody formed to the endogenous 16 α OHE-lysine adduct or alternatively, an autoantibody elicited in response to another cross-reactive antigen. The role of these anti-estrogen antibodies in the pathological sequelae of long-term contraceptive use or SLE is worthy of further investigation.

The second aspect of nonenzymatic reactions which were investigated was the nonenzymatic modification of DNA by reducing sugars. In the case of proteins, glucose derived Amadori products undergo further rearrangements, oxidations, and dehydrations to form fluorescent, cross-linking

chromophores. This process, called nonenzymatic browning or the Maillard reaction occurs in vivo. Long-lived structural proteins, such as collagen and the lens crystallins, accumulate enough of these products that they acquire many of the chemical properties characteristic of aged proteins. Nucleic acids also are long-lived molecules in the resting cell, and the accumulation of glucose-derived DNA adducts with time would be expected to adversely affect DNA function.

The incubation of DNA or nucleotides with glucose-6-phosphate (G6P) produced spectral changes similar to those described for the nonenzymatic browning of proteins. The occurrence of chemical modification was verified by measuring the transfection efficiency of viral DNA after incubation with glucose or G6P. A loss of transfection potential occurred that was first order with respect to time and sugar concentration. Glucose-6-phosphate also produced DNA strand scission in a time and concentration manner, but at a rate which was significantly slower than the rate of transfection inhibition. It appears that reducing sugars form adducts with the primary amino groups of nucleotides which inhibit template function. With time, these adducts undergo chemical rearrangement which labilizes the glycosidic bond between the base and the deoxyribose, causing depurination and DNA strand-scission.

Plasmid DNA modified by incubation with G6P was mutagenized when assayed in a prokaryotic host. Structural analysis of these mutants showed that many of the plasmids had undergone gross DNA alterations, including insertions and deletions of several hundred base-pairs. The ability of an endogenous sugar to induce such extensive DNA rearrangements suggests that nonenzymatic reactions of this type may represent an important source of cellular mutations. The accumulation of glucose-derived DNA adducts with time might be a mechanism for the decreased genetic viability and increased tumorigenesis observed in the aged organism.

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Abbreviations

dsDNA: double-stranded DNA.

ssDNA: single-stranded DNA.

DTT: dithiothreitol.

EDTA: ethylenediaminetetraacetate.

EtOH: ethanol.

G6P: glucose-6-phosphate.

HPLC: high-pressure liquid chromatography.

HSA: human serum albumin.

Boc-lysine: N- α -tert-butoxycarbonyl-lysine.

Fmoc-lysine: N- α -fluorenylmethyloxycarbonyl-lysine.

MeOH: methanol.

16 α OHE: 16 α -hydroxyestrone.

SDS: sodium dodecyl sulphate.

SLE: Systemic Lupus Erythematosus

1. INTRODUCTION.

Within the last ten years, it has become apparent that a number of endogenous, low-molecular weight substrates can react nonenzymatically with macromolecules to form covalent addition products. These reactions are slow and since the rate is first order with respect to the reactive substrate, the level of modification depends only on the substrate concentration and the biological half-life of the affected macromolecule. Table 1 lists molecules which have been shown to react nonenzymatically. They include metabolites, such as cyanate, glucose, and acetaldehyde and enzyme cofactors such as acetyl-CoA and S-adenosyl-methionine. The first class of compounds contain carbonyl groups which form addition products with the primary amino groups of proteins. The second class of reactive substrates are enzymatic cofactors which serve to transfer an activated acetyl or methyl group. At a very low frequency however, these groups can add directly to an amino acid or nucleotide residue without the aid of an enzyme.

The first metabolite shown to react nonenzymatically with proteins was cyanate. In 1960, Stark, Stein, and Moore (1) observed that cyanate, which exists in equilibrium with urea, readily combined with and inactivated ribonuclease. Interest in carbamylation reactions increased in the early 1970's when Cerami and Manning (2) provided the rationale for the purported anti-sickling activity of urea. At that time, the administration of high concentrations of urea was reported to alleviate the crisis phase of sickle-cell anemia. Cerami and Manning found that sodium cyanate could specifically carbamylate the N-terminal valine of hemoglobin S and prevent red cell sickling. Carbamylated proteins have been detected in states associated with high blood urea, such as renal failure and chronic diarrhea (3-4). Attempts have been made recently to

Nonenzymatic Addition Reactions

Endogenous Substrates		Target Molecules	Reactive Residues
Cyanate	$\ominus \text{N}=\text{C}=\text{O}$	Protein	α -amino acids, lysine
Glucose, reducing sugars	$\begin{array}{c} \text{HC}=\text{O} \\ \\ \text{HC}-\text{OH} \\ \\ \text{R} \end{array}$	Protein DNA	α -amino acids, lysine A,G,C
Acetaldehyde	$\begin{array}{c} \text{O} \\ \\ \text{CH}_3\text{CH} \end{array}$	Protein	α -amino acids lysine, tyrosine
Acetyl- coenzyme A	$\text{CoA}-\text{S}-\begin{array}{c} \text{O} \\ \\ \text{C} \end{array}-\text{CH}_3$	Protein	lysine
S-adenosyl- methionine	$\begin{array}{c} \text{CH}_3 \\ \\ \text{adenosine}-\text{S}^{\oplus} \\ \\ \text{CH}_2 \\ \\ \text{CH} \\ \\ \text{H}_2\text{N} \quad \text{COOH} \end{array}$	Protein DNA	glutamate, aspartate A,G

Table 1. Summary of nonenzymatic addition reactions.

link protein carbamylation with some of the pathological consequences of high urea levels. For example, since the oral administration of cyanate was found to lead to cataract formation (5); Harding has postulated that the high incidence of cataracts in the developing world might be attributed to chronic diarrhea and the progressive carbamylation of lens proteins (6).

The nonenzymatic reaction which has been studied the most extensively is the reaction between glucose, a reducing sugar, and protein amino groups. Interest in nonenzymatic glycosylation in vivo began with the observation by Rahbar (7) in 1968 that diabetic patients had elevated amounts of an electrophoretically "fast-moving" hemoglobin. Subsequent work in several laboratories led to the identification of hemoglobin A_{1c} as a modified hemoglobin formed post-translationally by the attachment of glucose to the N-terminal valine of the beta-chain (8-10). The mechanism for this reaction is outlined in Figure 1. A reversible Schiff base adduct is formed between the C-1 aldehyde and the ϵ -amino group of valine. The condensation product, an aldosylamine, then undergoes an Amadori rearrangement to form a 1-deoxyfructosyl adduct. The demonstration by Koenig and colleagues (11) that the amount of hemoglobin A_{1c} reflected the integrated blood glucose over a three to four week period spurred the use of this measurement as a clinical tool in diabetes mellitus (12). Furthermore, the observation that many of the sequelae of this disease occur in tissues that do not require insulin for glucose transport led to the hypothesis that increased protein glycosylation might account for many of the complications in diabetes. A number of proteins have been shown subsequently to be more glycosylated in the diabetic individual. These include albumin (13), myelin (14),

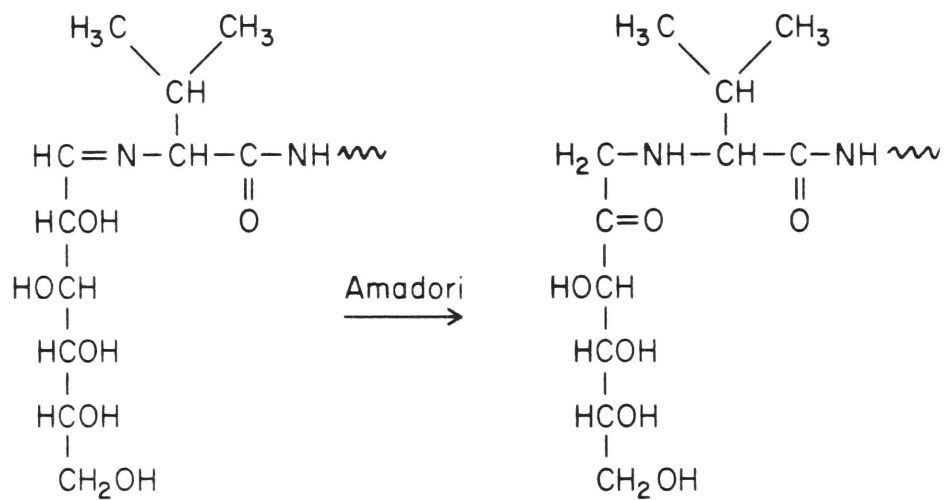


Figure 1. The Amadori rearrangement between glucose and the N-terminal valine of the β -chain of hemoglobin.

tubulin (15), lens crystallins (16), collagen (17), and erythrocyte membrane proteins (18).

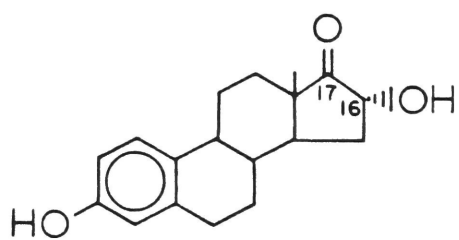
Perhaps the most biologically significant aspects of nonenzymatic glycosylation however, may be attributed to an observation made over 70 years ago by the French chemist Louis Maillard (19). He described a chemical process, known today as nonenzymatic browning or the Maillard reaction, which is initiated by the addition of reducing sugars to amino groups. With time, Amadori and other Schiff base rearrangement products undergo a series of dehydrations, oxidations, and cyclizations to form yellow-brown, fluorescent compounds which contain unsaturated rings and nitrogen heterocycles. When the amino groups of proteins are involved, protein-bound, cross-linking pigments form. Products with the same spectral and fluorescent properties as those of late-stage Maillard products are associated in vivo with long-lived structural proteins, such as collagen and the lens-crystallins (20, 21). In the course of normal aging, long-lived proteins become more fluorescent (22), more crosslinked, and less soluble (23). In the diabetic individual these protein changes are accelerated, as are a number of age-dependant, physiological effects. These include atherosclerosis (24), stiffening of the lungs (25), and the large arteries (26), thickening of the capillary and glomerular basement membranes (27, 28), and periarticular rigidity (29). Acceleration of these age-related complications in the diabetic patients implicates the role of glucose and Maillard products in this form of protein aging.

The third metabolite listed in Table 1 is acetaldehyde. The observation that non-diabetic, chronic alcohol abusers had elevated levels of minor hemoglobins led to the demonstration that acetaldehyde

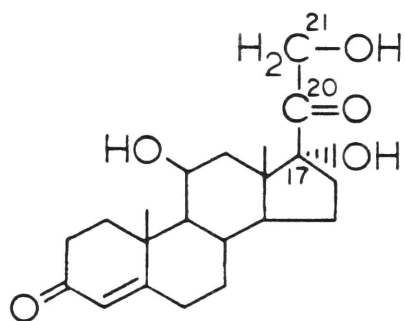
(the oxidation product of ethanol) can form stable addition products with proteins (30). Chemically, these studies proved interesting because tyrosine, a residue which cannot participate in Schiff base formation, was found to be readily modified. Acetaldehyde may be reacting with tyrosine by forming an intermediate, electrophilic immonium cation. These studies led to the hypothesis that protein modification by acetaldehyde may contribute to some of the pathological sequelae of chronic alcoholism, such as peripheral neuropathy and an increased susceptibility to infection, which occur in diabetes as well.

The nonenzymatic reactions of acetyl-CoA and S-adenosyl methionine have been studied by Paik and co-workers (31-32). Although these reactions were readily demonstrable in vitro, their contribution to the numerous acetylation and methylation reactions which occur in vivo is difficult to assess. Of substantial interest is the recent observation by Rydberg and Lindahl (33) that S-adenosyl-methionine can nonenzymatically methylate nucleotide residues. Since the products of these reactions are potentially mutagenic, even a very low frequency of nonenzymatic methylation could have a profound biological effect.

For my thesis studies, I have investigated two novel aspects of nonenzymatic addition reactions. The first aspect (Chapters 2-4) pursues the hypothesis that certain ketolic steroids, that is steroids bearing an unhindered hydroxylcarbonyl function, can form covalent addition products with the primary amino groups of proteins. These steroids include 16 α -hydroxyestrone and the glucocorticoids, exemplified in Figure 2 by cortisol. These steroids were selected for study because elevated levels of these molecules are associated with clinical syndromes. The first,



16α-hydroxyestrone



Cortisol

Figure 2.

16 α -hydroxyestrone, is the product of the hydroxylation of estrone and is an intermediate in the formation of estriol. Both men and women with the autoimmune disease, systemic lupus erythematosus, have been observed to have an increased rate of 16-hydroxylation (34) and an increased urinary level of 16 α -hydroxyestrone (35). The second steroid, cortisol, is a glucocorticoid and the long-term, therapeutic administration of these steroids produces a variety of toxic effects (36). The formation of cataracts for example, is observed in nearly all patients with rheumatoid arthritis who receive 20 mg of prednisone per day for four years (37). It could be reasoned that lens proteins might be susceptible to nonenzymatic modification by glucocorticoids, in analogy to glucose and cyanate which react with the lysine residues in lens crystallins and facilitate the formation of high-molecular weight, refractile aggregates.

The studies to be described were undertaken with the goal of demonstrating that the diverse pathological sequelae of autoimmunity or drug toxicity might be attributed to a common mechanism, i.e. protein modification. In the next chapter, model reactions between albumin and these two steroids are described. Chapter 3 presents in vitro and in vivo studies linking glucocorticoid-lens protein adducts with steroid-induced cataracts. Chapter 4 describes the reaction of 16 α -hydroxyestrone with membrane proteins and the detection of these addition products in patients with systemic lupus erythematosus. Studies on the occurrence of anti-estrogen antibodies are also presented.

The second aspect of nonenzymatic reactions which I have investigated are in vitro studies demonstrating the nonenzymatic modification of DNA by reducing sugars. Evidence for the occurrence of protein-bound Maillard products on long-lived structural proteins led to

the hypothesis that similar products may accumulate on nucleic acids. Since nucleic acids are also long-lived molecules in the resting cell, the accumulation of glucose-derived DNA adducts may lead to abnormalities in DNA function. Many current theories of aging emphasize the concept that the functional decrements characteristic of cellular senescence are due to the progressive accumulation of unrepaired genetic lesions. (121, 122). The long-term modification of DNA by reducing sugars could be a mechanism for the decreased genetic viability and increased tumorigenesis observed in the aged organism. Chapter 5 describes experiments demonstrating that DNA and nucleotides can react with reducing sugars in vitro to form fluorescent Maillard products. In a model prokaryotic system, DNA molecules are inactivated and mutagenized by incubation with a reducing sugar.

2. Nonenzymatic Reactions of 16α -Hydroxyestrone and Cortisol
with Proteins In Vitro.

A. Model studies of the nonenzymatic modification of albumin.

The ability of two ketolic steroids, 16 α -hydroxyestrone and cortisol, to form covalent adducts with human serum albumin was investigated. We hypothesized that the presence of the carbonyl moiety would permit these steroids to form freely reversible Schiff base intermediates with the amino groups of proteins. The adjacent hydroxyl group then would allow a Heyns rearrangement (38) to occur, resulting in a stable ketoamine adduct with the protein. Figure 3 shows this proposed reaction scheme for the estrogen metabolite 16 α -hydroxyestrone. In this case, the Schiff base would form at the C-17 carbonyl and a rearrangement involving the C-16 hydroxyl could occur through the D-ring of the steroid. In the case of the glucocorticoid cortisol (Fig. 4), the reaction would be analogous to the reaction of a ketose with protein amino groups in that the Schiff base and subsequent Heyns rearrangement would occur at the C-17 dihydroxyacetone side chain. The following chapter describes model studies of the reaction between 16 α -[6,7-³H]hydroxyestrone and [1,2-³H]cortisol and human serum albumin.

Methods

Reagents. 16 α -[6,7-³H]Hydroxyestrone (50 Ci/mmol; 1 Ci = 3.7 x 10¹⁰ bequerels) was synthesized by the procedure of S. Ikegawa and J. Fishman (39). This material was stored in toluene/ethanol, 9:1 (v/v) at -80°. [2,4,6,7-³H]Estrone (80 Ci/mmol) and [1,2-³H]cortisol (47 Ci/mmol) were obtained from Amersham and stored at -80°. Radiochemical purity was monitored by HPLC as described below. The steroids were acetylated prior to chromatography by incubating with pyridine containing 10% (v/v) acetic

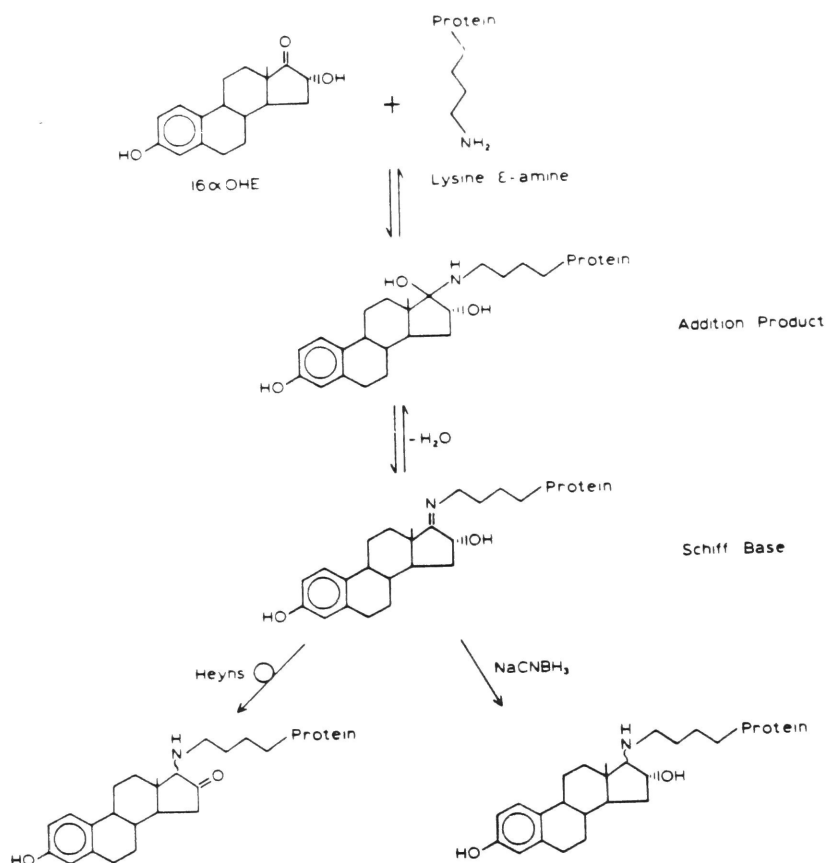


Figure 3. The reaction scheme between 16 α -hydroxyestrone and the ϵ -amino group of lysine residues, showing the adducts formed by the Heyns rearrangement and by reduction with NaCNBH_3 .

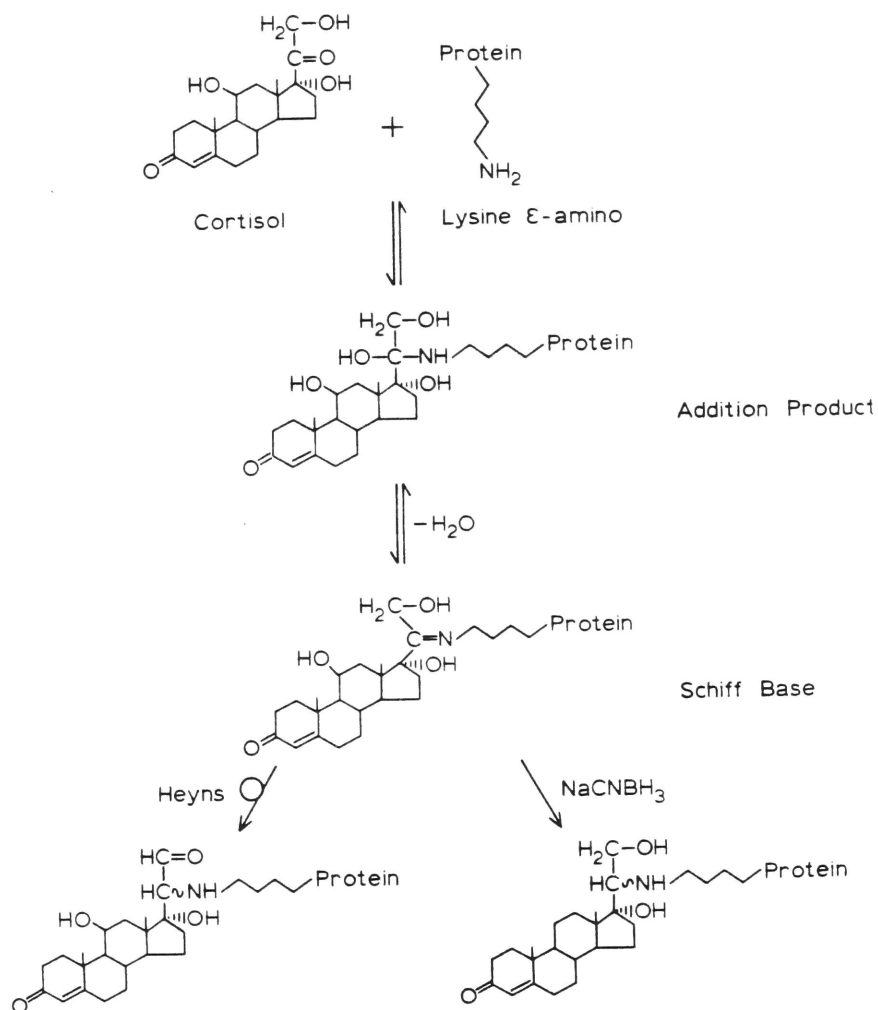


Figure 4. The reaction scheme between cortisol and the ϵ -amino group of lysine residues, showing the adducts formed by the Heyns rearrangement and by reduction with NaCNBH_3 .

anhydride.

Human serum albumin (not defatted) was purchased from Sigma and used without further purification.

Albumin/Steroid Incubations. One hundred and thirty picomoles of either 16α -[^3H]hydroxyestrone, [^3H]estrone, or [^3H]cortisol was placed into microfuge tubes and evaporated under nitrogen. The steroids were dissolved in 60 μl ethanol, followed by the addition of 0.54 ml of 50 mM potassium phosphate buffer, pH 7.4/10% (v/v) ethanol. The final incubation mixture contained 0.13 μM steroid and 40 mg of albumin per ml. For experiments requiring reducing conditions, the mixtures contained in addition 15 μmol of sodium cyanborohydride (NaCNBH_3 ; Sigma)(40). At time zero, the mixtures were transferred from 0° to a 37° water bath. At indicated times, 0.1 ml aliquots were removed, placed inside dialysis tubing (M_r cutoff, 12,000-14,000) and dialyzed for 24 hr against 2 liters of 50 mM potassium phosphate, pH 7.4/10% ethanol. The remaining unreacted steroid was removed by chromatography over a column (1 x 29 cm) of Sephadex G-100 (Pharmacia), which was equilibrated with 50 mM potassium phosphate buffer, pH 7.4/10% ethanol. The flow rate was 3 ml/hr, and the protein eluate was monitored by absorbance at 280 nm with a Uvicord II (LKB Instruments). Fractions (0.5 ml) were collected, added to 6.0 ml of Hydrofluor (National Diagnostics, Sommerville, N.J.), and the amount of radioactivity was determined with a Packard Tricarb scintillation counter.

Albumin Acetylation. A 200 mM solution of acetylsalicylic acid (Malinckrodt) was prepared in 1 ml of 50 mM potassium phosphate (pH 7.4) and the pH was adjusted to 7.4 with 15-20 μl of 45% (w/v) potassium hydroxide. A portion (0.1 ml) of this solution then was added to 0.1 ml

of a human serum albumin solution (100 mg/ml) dissolved in 50 mM potassium phosphate, pH 7.4/10% ethanol and incubated at 37°. At the end of 10 hr this material was dialyzed against 2 liters of 50 mM potassium phosphate, pH 7.4/10% ethanol to remove unreacted acetylsalicylic acid. A control incubation containing no acetylsalicylic acid was also performed. The acetylated and control albumins (2.5 mg each) were then incubated for 42 hr with 18 μ mol of the ^3H -labeled steroid in a volume of 62 μ l and assayed for binding as described above. The experiment that measured reducible bonds contained in addition, 1 μ mol of NaCNBH_3 .

Adduct Characterization. Albumin which had been treated with 16 α - ^3H hydroxyestrone in the presence of NaCNBH_3 for 48 hr was dialyzed against 50 mM potassium phosphate, pH 7.4/10% ethanol to remove free steroid. The protein (4 mg) was subjected to acid hydrolysis by adding 2 ml of 6M HCL in a tube that was sealed and evacuated and incubating the mixture at 110° for 8 hr (41). At the end of this time, the acid was evaporated under nitrogen. The hydrolysate was acetylated for HPLC analysis by dissolving the residue in 1 ml of pyridine (distilled under N_2 and stored over NaOH pellets) and adding 0.1 ml of acetic anhydride. After an incubation at 80° for 30 min, the solvent was evaporated under nitrogen. This material was dissolved in 0.4 ml of 5 mM sodium phosphate, pH 7.4/50% methanol and subjected to HPLC analysis.

A 16 α - ^3H hydroxyestrone-lysine adduct was prepared as a standard. A 50 μ l solution of 50 mM N- α -t-butoxycarbonyl lysine (Vega Biochemicals, Tucson AZ.) dissolved in either 100 mM sodium phosphate, pH 7.4/10% ethanol or in 100 methanol, was incubated with 0.8 μ mol 16 α - ^3H hydroxyestrone and 20 mM NaCNBH_3 for 6 days at 37°. Aliquots (2 or 3 μ l) were removed at intervals. The aqueous solutions were diluted 1:5 and

extracted 3 times with 3 volumes of ether to remove the unreacted steroid. After drying the sample under nitrogen, the α -blocking group was removed by incubation with either 50 μ l of 4 M HCL or 50 μ l of a solution of 50% trifluoroacetic acid in methylene chloride for 20 min at 25 $^{\circ}$ (42). The acid was evaporated under nitrogen, and the products were acetylated by incubating for 30 min at 80 $^{\circ}$ in 0.1 ml of dry distilled pyridine containing 10% acetic anhydride. This solution was then evaporated under nitrogen, and the residue was dissolved in 0.4ml of 5mM sodium phosphate, pH 7.4/50% methanol. The identity of the standard was validated by its predicted chromatographic behavior after removal of the blocking group and by the increased retention time observed when the standard was run in a solvent system containing 1% acetic acid instead of neutral buffer.

HPLC analysis. All HPLC studies were performed on a reverse-phase Waters Associates C₁₈ μ Bondapak column in a Hewlett-Packard 1084B liquid chromatograph interfaced with a Flo-One radioactive flow detector (Radiomatic Instruments and Chemical, Tampa, FL). Sample (30-60 μ l) was injected into a mobile phase of 10 mM sodium phosphate, pH 7.4/50% methanol, which increased linearly to 100% methanol from 0 to 55 min. The flow rate was 1 ml/min. The column eluate was mixed with scintillation fluor (Flo-Scint II); Radiomatic, Tampa, FL) which flowed at a rate of 3 ml/min. The Flo-One microprocessor integrated the amount of radioactivity eluted.

Peptide Mapping. Tryptic peptides were prepared from albumin which had been extensively modified by incubation for 8 days with 3.45 mM steroid supplemented with either 1.26x10⁶cpm of [³H]16 α -hydroxyestrone or 9.00x10⁶ cpm of [³H]cortisol. The reduced incubation contained 20 mM

NaCNBH₃. After extensive dialysis the modified proteins were purified by gel filtration chromatography as described above. The specific activity of incorporation was 6.9×10^3 cpm/mg for 16 α -hydroxyestrone-HSA, 16.9×10^3 cpm/mg for 16 α -hydroxyestrone-HSA (NaCNBH₃ reduced), 36.0×10^3 cpm/mg for cortisol-HSA, and 75.2×10^4 cpm/mg for cortisol-HSA (NaCNBH₃ reduced). The proteins were lyophilized and redissolved in 10 ml of a solution of 6M Gn-HCL and 1 M Tris-HCL, pH 8.8. Disulfide bonds were reduced by adding 25 mg of dithiothreitol and incubating at room temperature for 4 hours under nitrogen. Reduced proteins were then aminoethylated (43) as follows. A 680 mg/ml of N-(β -Iodoethyl)tri-flouroacetamide (Aminoethyl-8, Pierce Chemical Co. Rockford, IL.) was prepared in methanol and 0.56 ml added to the protein solution. This was incubated at 50° for 1 hour, after which another 0.50 ml of Aminoethyl-8 was added and incubation continued for an additional 3 hours. The reaction was terminated by the addition of 0.75 ml of 2 N acetic acid and dialyzed against several changes of 2 liters of 5 mM acetic acid.

After lyophilization, the aminoethylated proteins were redissolved in 2 ml of 0.5 NH₄CO₃ (pH 8.8) and enzymatically hydrolyzed by the addition of 0.27 ml of a 180 U/ml solution (prepared in 0.55 NH₄CO₃) of trypsin (TPCK treated, Worthington, Freehold, NJ). The digestion was allowed to proceed for 2 hours at 37°, after which the solution was heated to 100° for 5 minutes and cooled. The digestion was repeated two more times. The digest was then lyophilized and the peptides resuspended in 2 ml of 0.1% phosphoric acid. Insoluble material was removed by centrifugation. Of the total radioactivity, 82.0% was found to be present within the soluble peptides.

Peptide mapping was performed by reverse-phase HPLC (43) utilizing a

Waters Associates C18 column. Samples were injected into a mobile phase consisting of 0.1% phosphoric acid/2% acetonitrile which increased linearly to 50% acetonitrile from 0 to 40 min. The flow rate was 1 ml/min. Radioactivity analysis was conducted on 0.35 mg of peptides. Six second fractions were collected, mixed with scintillation fluid, and counted in a Beckman LS 7000 liquid scintillation spectrometer.

Results

Figure 5 shows that the incorporation of 16α -[^3H]hydroxyestrone into albumin increases with time in both the presence and the absence of NaCNBH_3 . In the presence of NaCNBH_3 , the rate of incorporation is rapid and plateaus when 80% of the free steroid has reacted. This steroid binds efficiently to albumin, most likely through hydrophobic interactions, and forms reversible Schiff bases with available amino groups. These Schiff bases are chemically reduced by the cyanoborohydride and are trapped as covalent adducts. Under nonreducing conditions, the covalent attachment of 16α -hydroxyestrone proceeds at a slower rate. It is postulated that the presence of a hydroxyl group adjacent to the C-17 carbonyl permits the initial Schiff base to undergo a Heyns rearrangement and form a stable, covalent steroid-protein adduct.

To illustrate this point, albumin was incubated with [^3H]estrone, which has a C-17 carbonyl moiety but lacks the 16α -hydroxyl group. In the presence of NaCNBH_3 , a significant amount of estrone is incorporated into albumin, indicating that the 17-carbonyl of estrone forms a transient Schiff base adduct that can be trapped by reduction. However, no estrone is incorporated in the absence of NaCNBH_3 because the Schiff base cannot undergo a Heyns rearrangement.

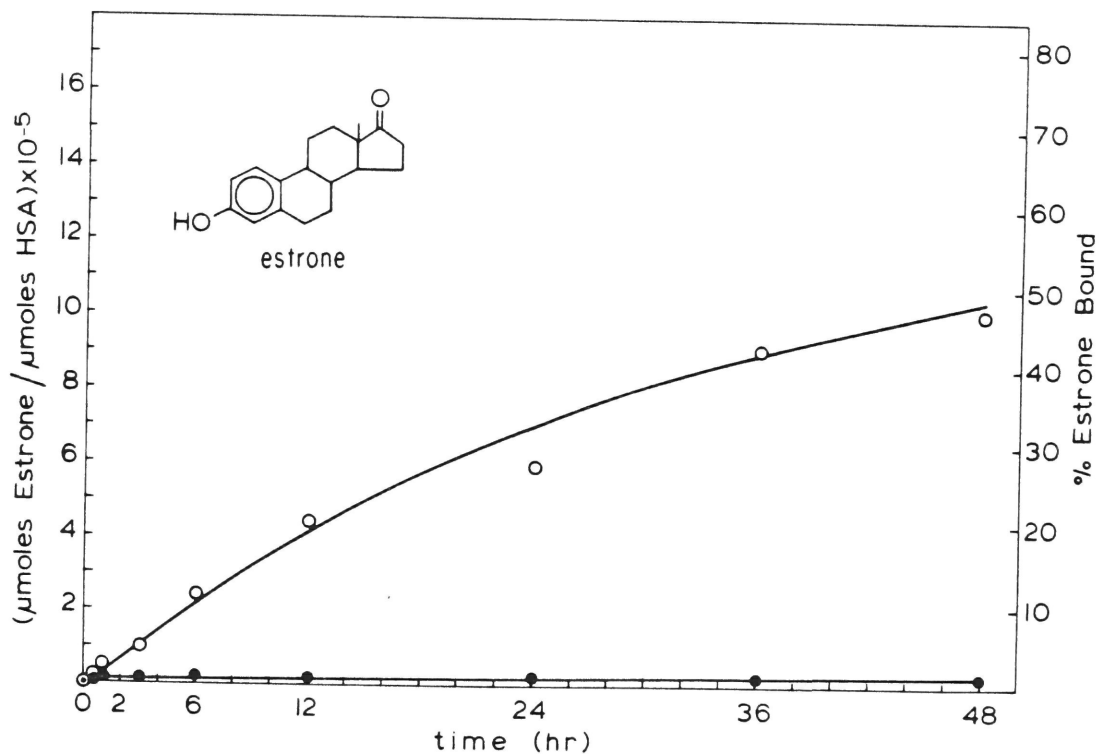
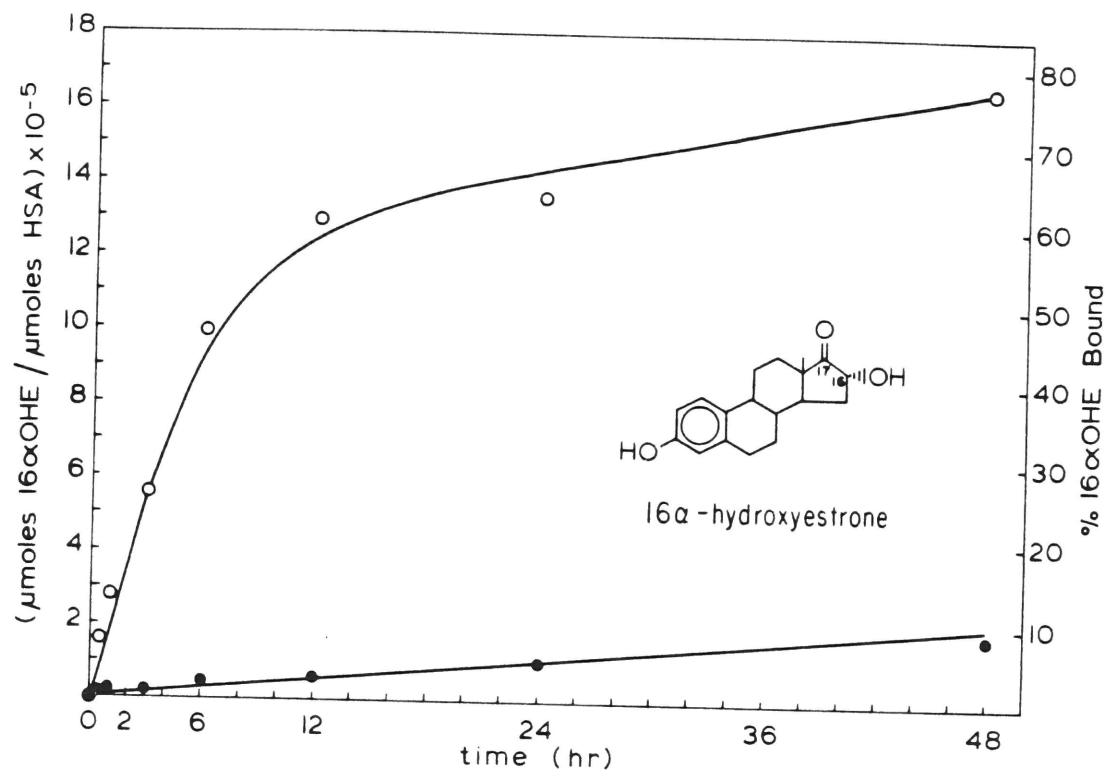


Figure 5. Incorporation of 16α-hydroxyestrone and estrone into albumin, under reducing (o) and nonreducing conditions (●).

The ability of cortisol to form adducts with albumin also was examined. Cortisol possesses two carbonyl groups, one at C-3 and one at C-20. We hypothesized that a Schiff base involving the C-20 carbonyl could readily undergo a Heyns rearrangement with the unhindered C-21 hydroxyl. The formation of cortisol-albumin adducts in both the presence and the absence of NaCNBH_3 is shown in Figure 6. The presence of NaCNBH_3 increases the rate of cortisol incorporation only slightly. It would appear that the Heyns rearrangement is so rapid that the formation of the Schiff base is rate-limiting for this reaction. The great reactivity of the cortisol side chain is well recognized and is exemplified by its spontaneous rearrangement and degradation to acidic products (45).

Previous studies of the nonenzymatic glycosylation of albumin suggested that the Schiff base adduct probably involves the ϵ -amino groups of lysine residues (46). In order to determine whether 16α -hydroxyestrone and cortisol also react at this position, albumin was first acetylated with acetylsalicylic acid, which modifies the lysine located at position 199 of the amino acid sequence of human albumin (47). The acetylated albumin was then incubated with steroid. There was a significant decrease in the formation of steroid adducts with acetylated albumin, thus indicating a significant competition for this reactive lysine residue (Figure 7).

The 16α -hydroxyestrone-albumin adduct, after reduction with NaCNBH_3 , was subjected to acid hydrolysis. The hydrolysate was acetylated and analyzed with HPLC utilizing a reverse-phase column. A prominent peak was observed to elute at 8-9 minutes, which was followed by the elution of several peaks together at 19-23 minutes (Figure 8). The latter peaks correspond to unreacted 16α -hydroxyestrone and products produced by the

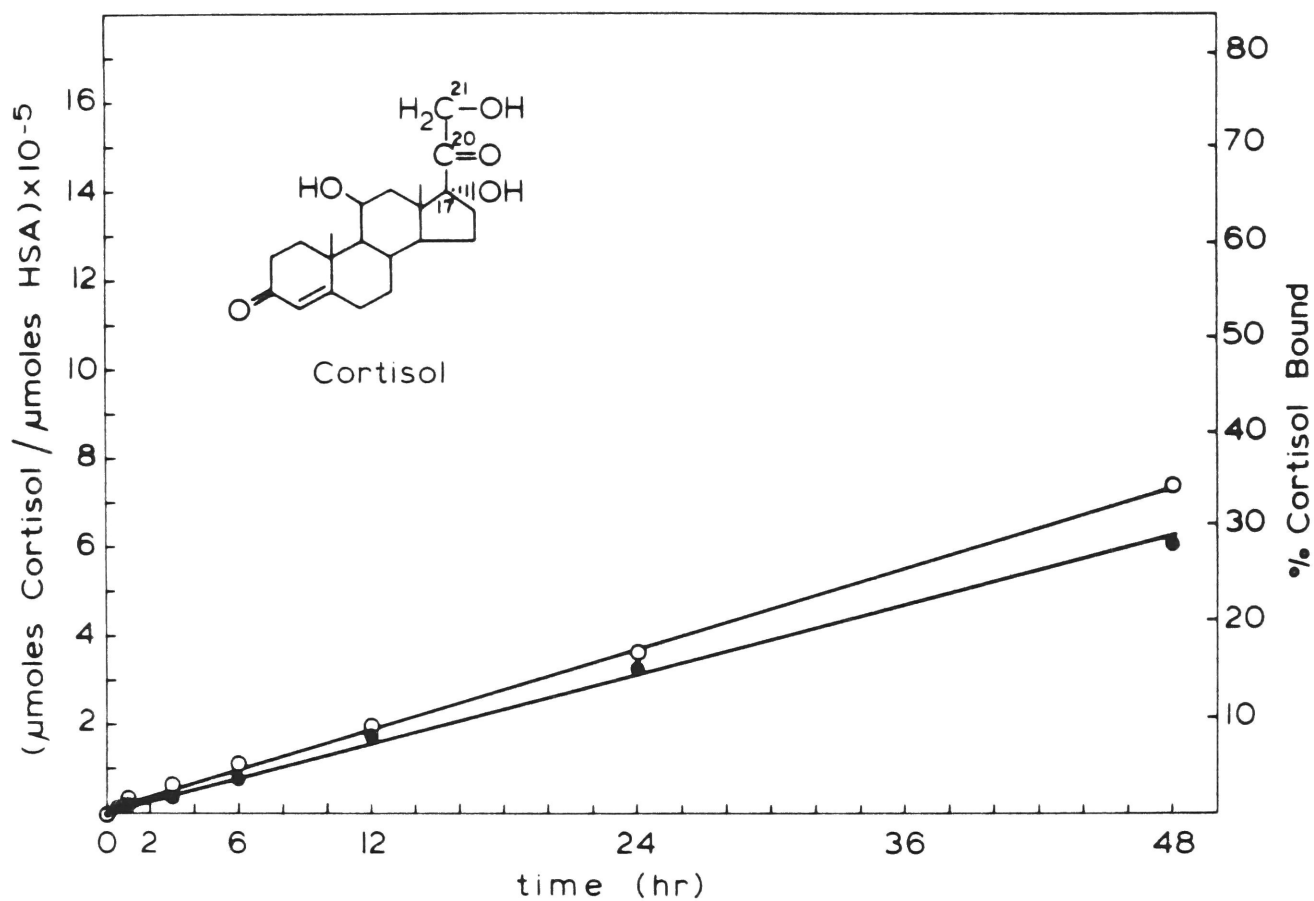


Figure 6. Incorporation of cortisol into albumin, under reducing (o), and nonreducing conditions (●).

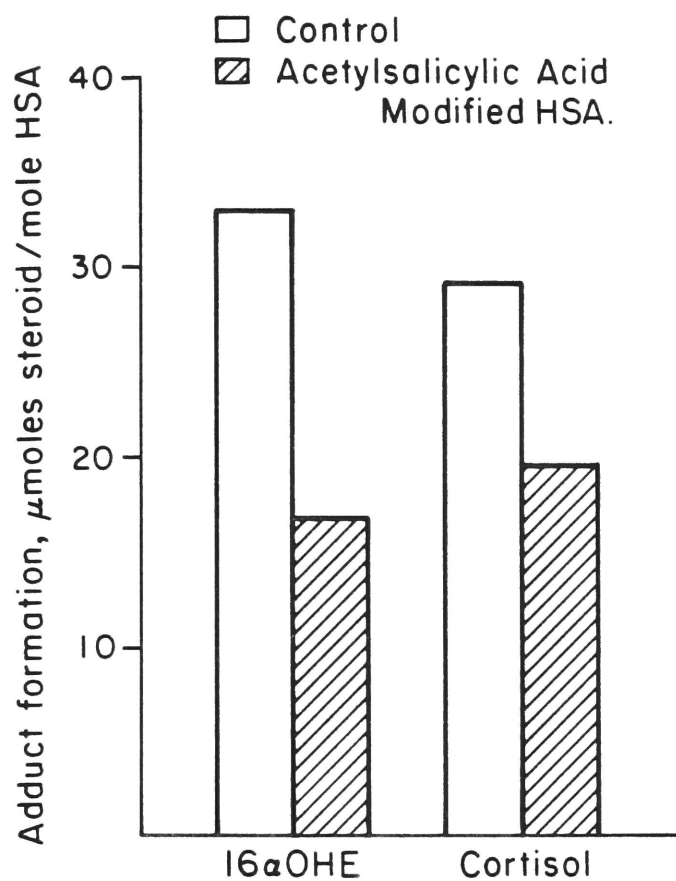


Figure 7. Inhibition of steroid incorporation by acetylation with acetylsalicylic acid.

reduction and acid hydrolysis of this steroid. The first peak was observed to coelute with a synthetic 16 α -hydroxyestrone-lysine adduct standard. These data indicate that 16 α -hydroxyestrone forms an acid-stable adduct with the ϵ -amino group of lysine when incubated with albumin under reducing conditions. An elution profile identical to Figure 8 was obtained after the acid hydrolysis of albumin which had been incubated with 16 α -hydroxyestrone and reduced with NaB[³H]₄ (data not shown).

Chromatography of an acid hydrolysate of 16 α -hydroxyestrone adducts prepared without NaCNBH₃ failed to show a significant peak near the 8-9 minute position. Evidently, the ketoamine Heyns product is destroyed by acid hydrolysis, whereas the α -hydroxyamine product produced by NaCNBH₃ reduction is not.

Figure 9-12 show the reverse-phase HPLC elution pattern of tryptic peptides prepared from steroid modified albumin. Four peptide maps are shown, corresponding to albumin modified by 16 α -hydroxyestrone or cortisol either in the presence or the absence of NaCNBH₃. These preparations were substituted at a molar ratio of steroid to albumin of 2.1:1 for 16 α -hydroxyestrone-HSA, 2:1 for cortisol-HSA, 5.1:1 for 16 α -hydroxyestrone-HSA (NaCNBH₃ reduced) and 3.6:1 for cortisol-HSA (NaCNBH₃ reduced). From these peptide elution patterns it is apparent that both 16 α -hydroxyestrone and cortisol react at discrete sites in the albumin molecule. Two well resolved albumin peptides are labelled by 16 α -hydroxyestrone in the absence of NaCNBH₃. Cortisol appears to label 3 to 5 major peptides, although 3 of these elute very close together in the chromatogram. This poorly resolved triplet may in fact represent peptides which are incompletely digested by trypsin. Digestion may be

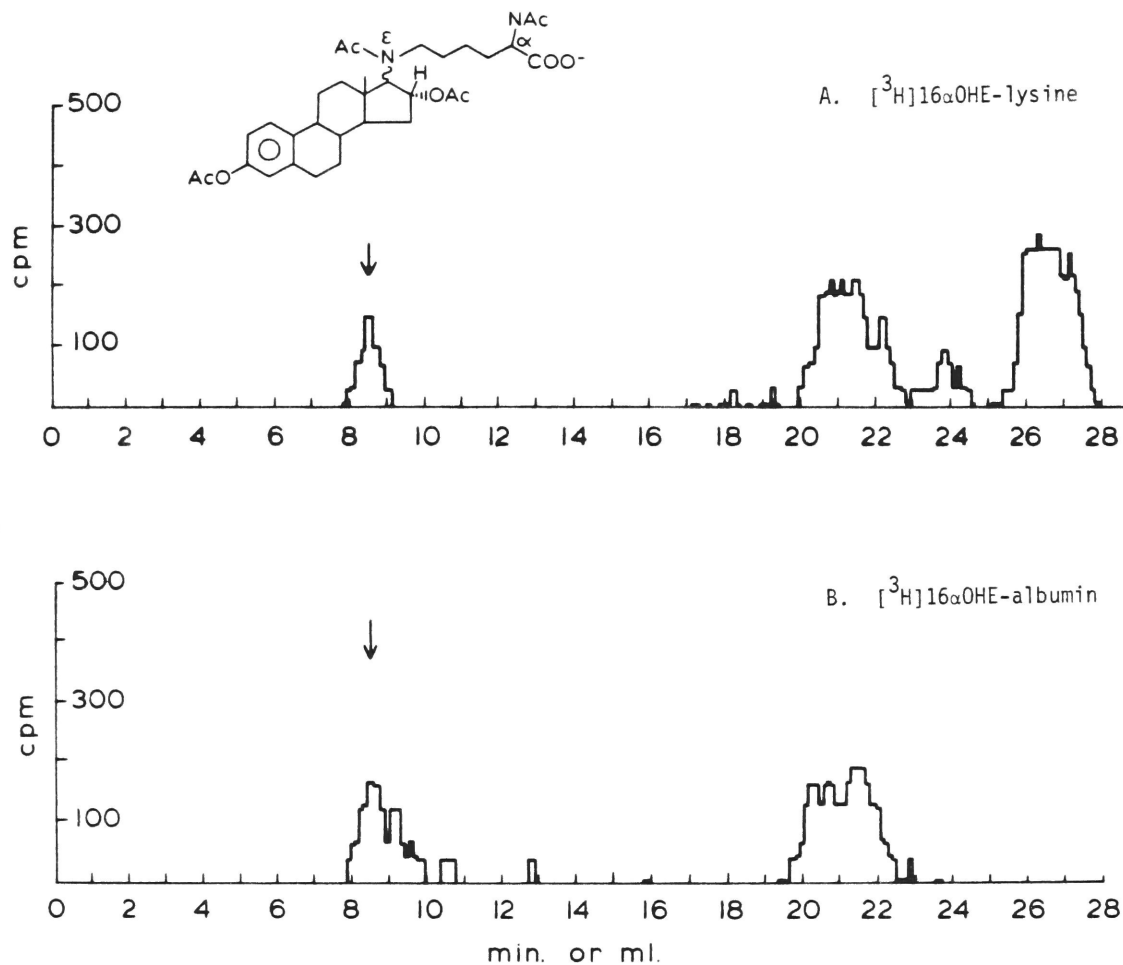


Figure 8. Reverse-phase HPLC elution profiles of a reaction mixture of $[^3\text{H}]16\alpha$ -hydroxyestrone and lysine (A), and an acid hydrolysate of $[^3\text{H}]16\alpha$ -hydroxyestrone modified albumin (B).

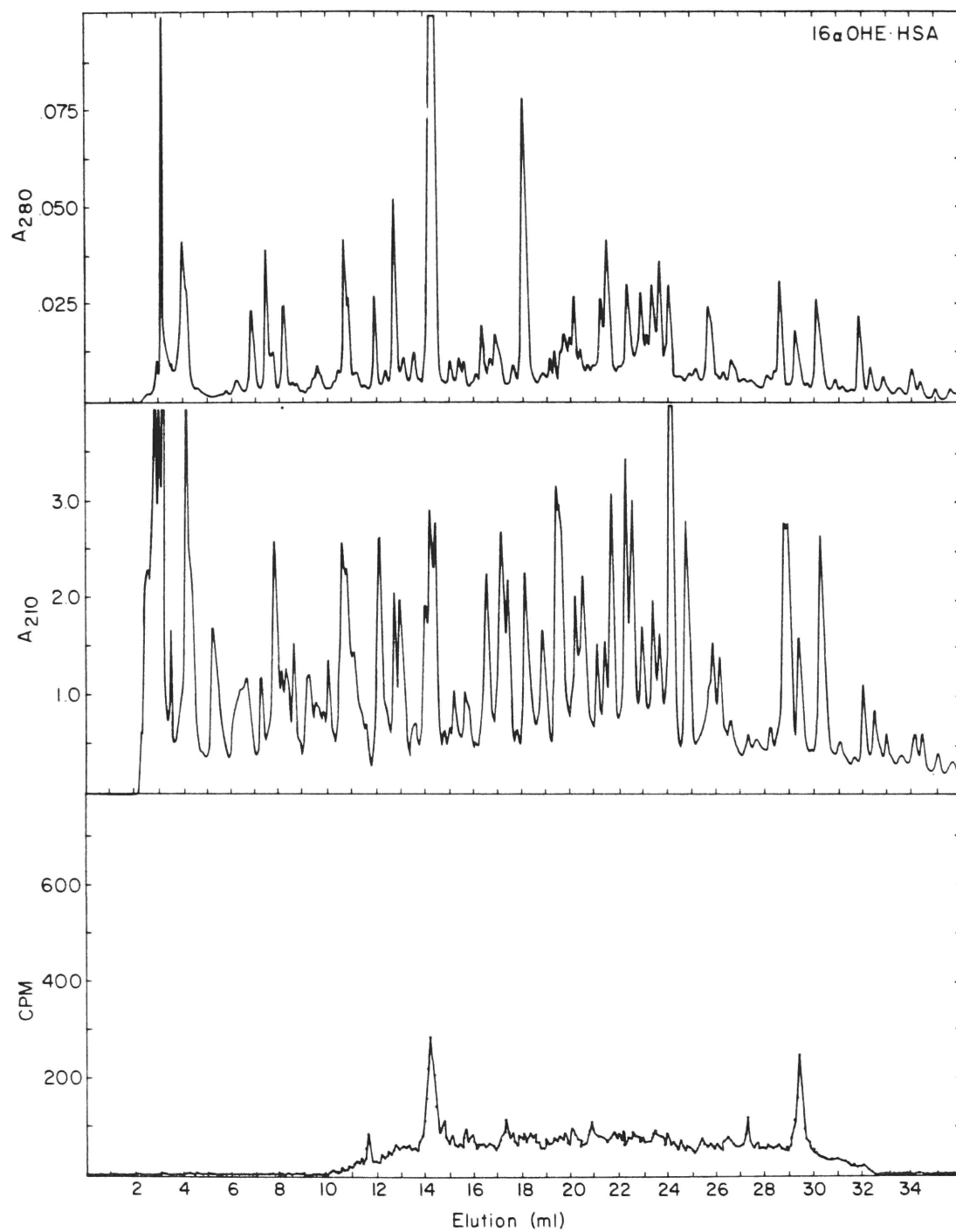


Figure 9. Reverse-phase HPLC tryptic map of 16 α -hydroxyestrone modified albumin.

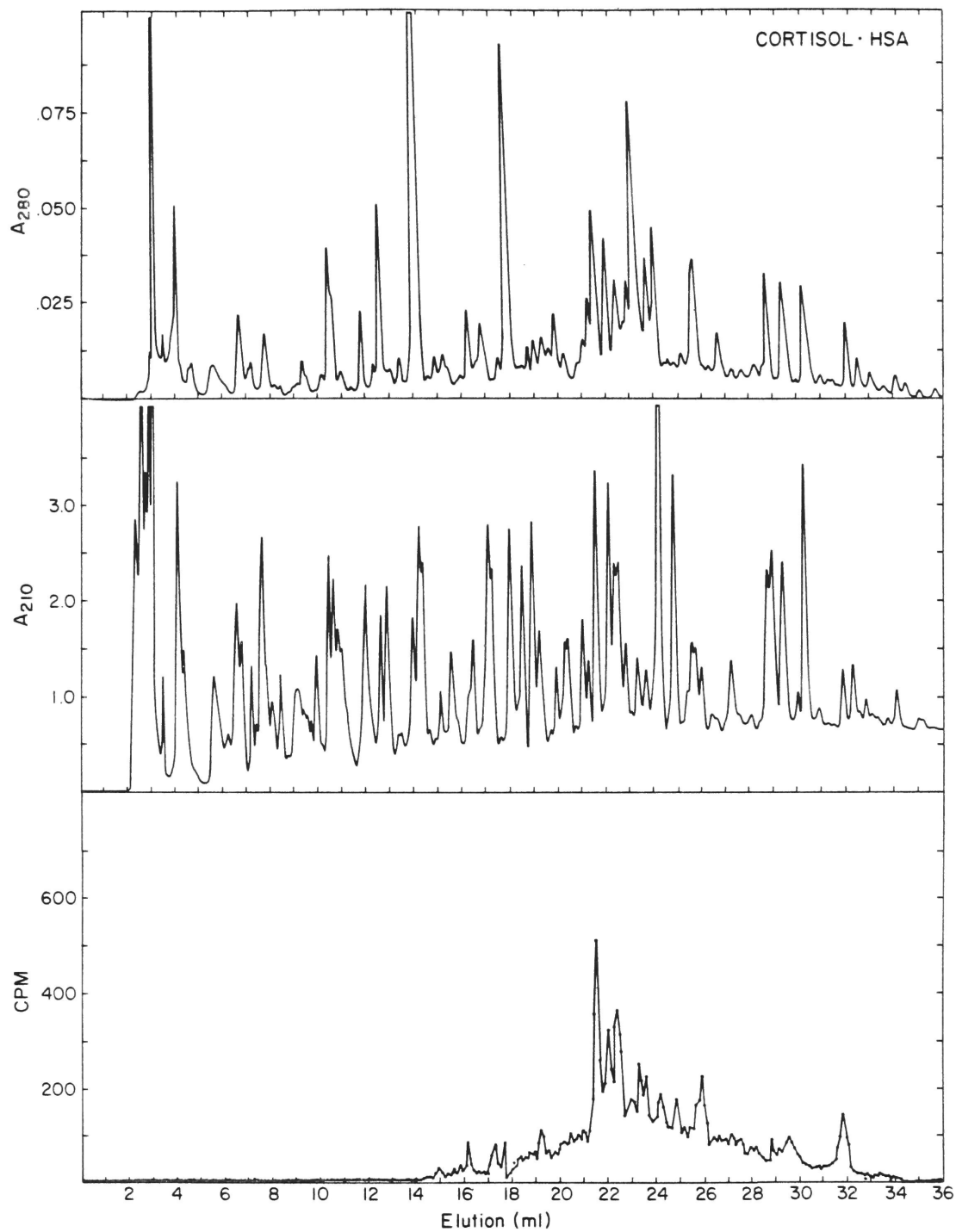


Figure 10. Reverse-phase HPLC tryptic map of cortisol modified albumin.

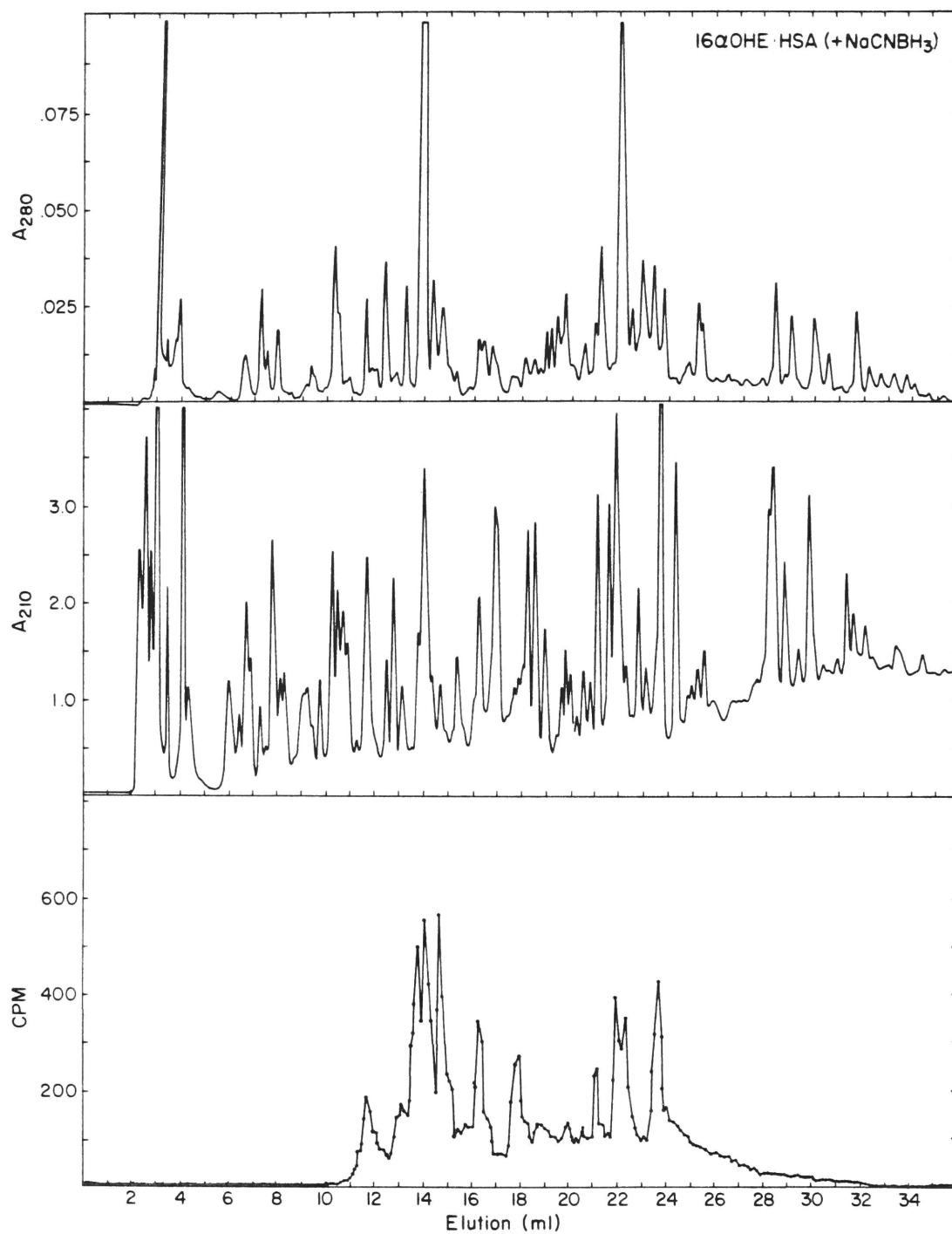


Figure 11. Reverse-phase HPLC tryptic map of albumin modified by 16 α -hydroxyestrone in the presence of NaCNBH₃.

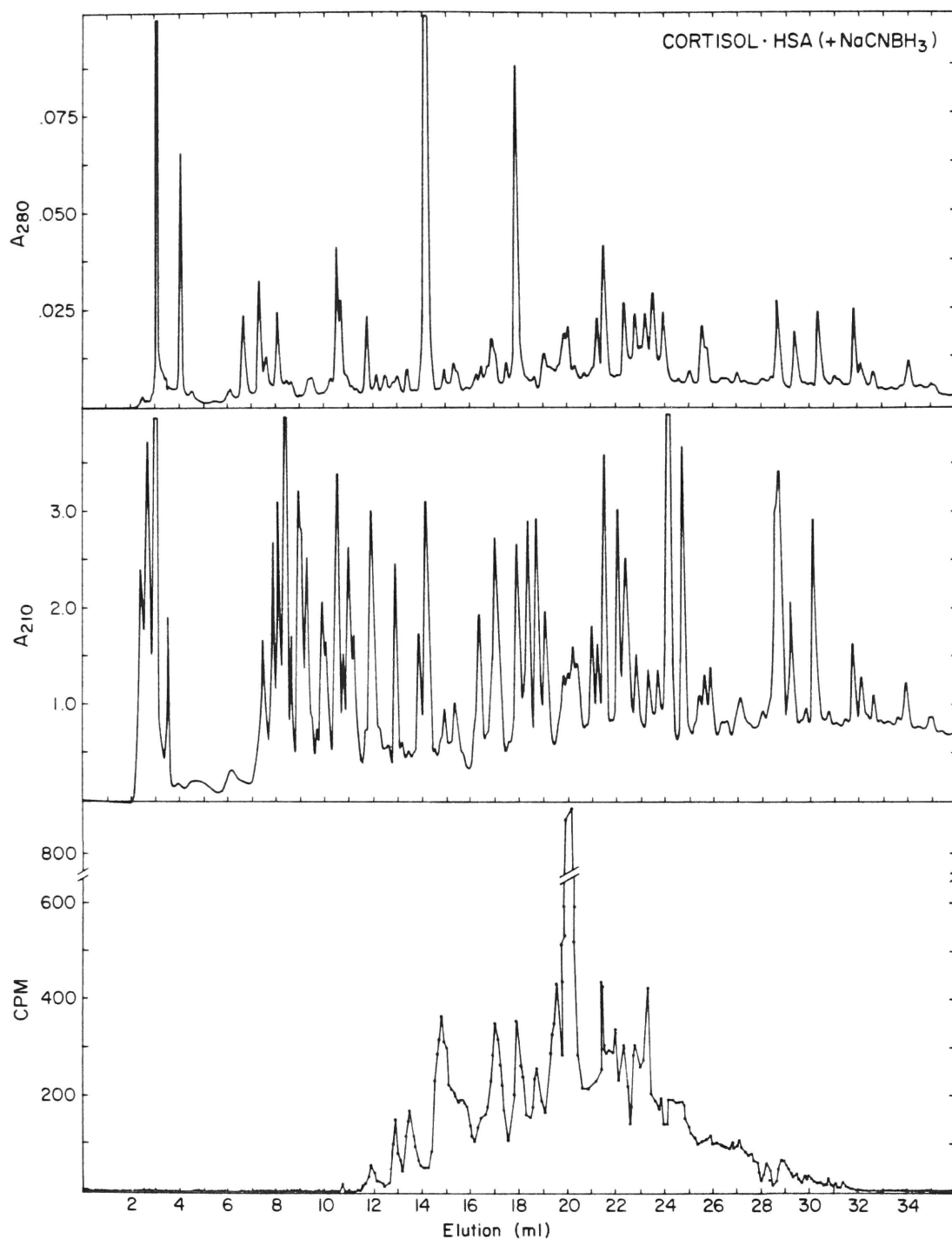


Figure 12. Reverse-phase HPLC tryptic map of albumin modified by cortisol in the presence of NaCNBH_3 .

inhibited by the presence of the cortisol moiety; in analogy to the observation that glycosylated lysine residues are poor substrates for proteolytic enzymes (47).

Inclusion of NaCNBH_3 in the incubation mixtures was found to lead to the modification of additional albumin sites. These lysine residues can readily form Schiff bases with the steroid carbonyl groups, but under normal conditions; i.e. in the absence of NaCNBH_3 , stable rearrangement products do not form. This may be the result of factors in the micro-environment of a particular lysine residue. Important factors might be the presence of an adjacent hydrophobic binding site to orient the steroid, or the ability of nearby amino acids to assist in the proton transfer reaction which produces the Heyns rearrangement.

Although the Schiff base rearrangement products between sugars and amino acids are stable, there is in fact a very slow reversal rate. In order to estimate the reversal rate for the steroid-albumin rearrangement products, albumin was modified with 16α - $[\text{}^3\text{H}]$ hydroxyestrone or $[\text{}^3\text{H}]$ cortisol and dialyzed at 37°C for several months against a buffer containing 50 mM potassium phosphate, pH 7.4 and 10% ethanol.

Figure 13 illustrates the results of these experiments. Two relevant control experiments are also shown. The first shows that $[\text{}^3\text{H}]$ steroids which were reacted with albumin in the presence of NaCNBH_3 are stable and not dialyzable for at least a 70 day period. The second control demonstrates that when these steroids are added to albumin immediately prior to dialysis, greater than 95% of the radioactivity can be recovered in the dialysate within 24 hours. Thus, once the steroid-protein linkage has reversed, the steroid is freely dialyzable and will not reassociate with the albumin via hydrophobic or other interactions. The results of

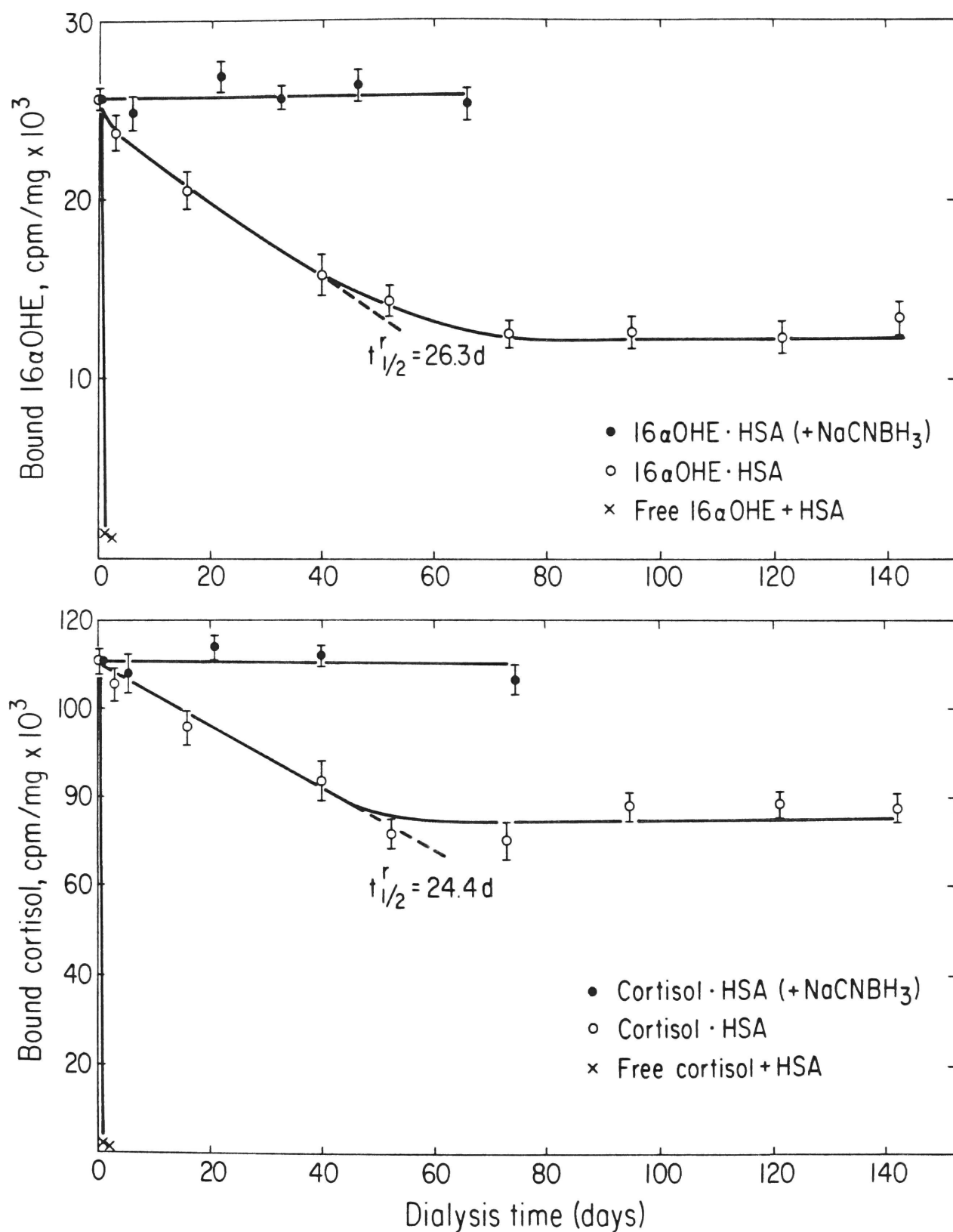
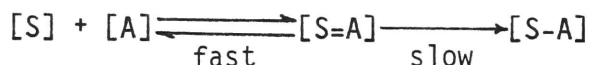


Figure 13. Measurement of the off-rate of steroid-albumin adducts.

these studies indicate that only a portion of the steroid ligands are reversible. In the case of 16 α -hydroxyestrone, this amount represents about 50% of the protein bound radioactivity; and in the case of cortisol, about 30%. These observations may be accounted for by either of two mechanisms. One possibility is that during the long time course of this experiment further reactions - such as an oxidation - may occur, producing a steroid-protein adduct which is chemically irreversible. In the case of the cortisol-lysine Heyns product, this could easily occur by oxidation of the C-21 aldehyde to a carboxylic acid. Another possibility is that some of the reactive albumin sites lead to the formation of Heyns products which are in fact irreversible. For example, these sites may be inaccessible to the water molecule which hydrolyzes the Schiff base and reverses the addition reaction.

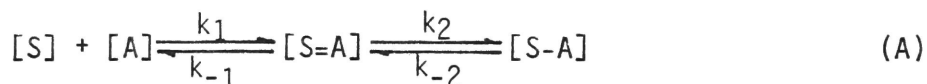
The half life of the rapidly reversing steroid - lysine adduct was calculated from the linear part of the decay slope and found to be 26.3 days for 16 α -hydroxyestrone-HSA and 24.4 days for cortisol-HSA.

It is possible to calculate the rate constants for the formation of the steroid-protein adducts. Reactions involving Schiff bases and their rearrangement products can be written in the following scheme.



where [S] and [A] are the concentration of free steroid and free albumin respectively, [S=A] is the concentration of the Schiff base intermediate, and [S-A] is the concentration of the Schiff base rearrangement product.

In a kinetic form, this becomes:



The rate at which albumin forms the Schiff base linkage (k_1) can be calculated from conditions in which all of the adduct is trapped and the reverse reaction (k_{-1}) cannot occur. These conditions are satisfied in the incubations which contain an excess of NaCNBH_3 . From the linear slope of the incorporation curves (Figs. 5 and 6), k_1 can be readily calculated from:

$$d[S=A] = k_1[S][A]dt \quad (B)$$

Note that k_1 is the overall rate constant for the condensation of the steroid at the multiple sites within albumin and,

$$k_1 = (k_{1a} + k_{1b} + \dots)[S][A]$$

where k_{1a} , k_{1b} , etc. are the rates of condensation for each reactive lysine residue.

$$\text{Therefore,} \quad k_1 = \frac{d[S=A]}{dt} \times \frac{1}{[S][A]} \quad (C)$$

Under the experimental conditions for 16α -hydroxyestrone,
 $[A] = 601.3 \mu\text{M}$, $[S] = 0.13 \mu\text{M}$, $[S=A] = .061 \mu\text{M}$ and $t=6 \text{ hr}$.

Therefore,

$$k_1 = 131 \times 10^{-3} \text{ nM}^{-1} \text{ hr}^{-1} \text{ for } 16\alpha\text{-hydroxyestrone.}$$

Similarly for cortisol,

where $[S=A] = .045 \mu\text{M}$ at 48 hr.

$$k_1 = 12 \times 10^{-3} \text{ nM}^{-1} \text{ hr}^{-1} \text{ for cortisol.}$$

The overall rate (k_{obs}) for the formation of a stable rearrangement product can be calculated from reactions performed in the absence of NaCNBH_3 . Under the reaction conditions described for Figure 4, albumin amino groups are present in large excess and equation (A) can be represented by the pseudo first-order expression:



In the case of the reaction between 16α -hydroxyestrone and albumin $k_{\text{obs}}[\text{A}] = k_2$ since $k_2 \ll k_1$ (Fig. 4) and $k_2 \gg k_{-2}$ (Fig. 13). k_2 can be calculated from the amount of incorporation at 48 hours, where the rate is still linear.

Therefore: $k_2 = 0.0021 \text{ hr}^{-1}$ for 16α -hydroxyestrone.

It is interesting to compare these values to those calculated for the Amadori reaction between glucose and hemoglobin (48). In this case, the rate of Schiff base formation was found to be: $0.3 \times 10^{-3} \text{ mM}^{-1} \text{ hr}^{-1}$, and the rate of the Amadori rearrangement is $.0055 \text{ hr}^{-1}$. Therefore, the rate of Schiff base formation for 16α -hydroxestrone with albumin is 2300x slower than for glucose with hemoglobin; and the rate of the Heyns rearrangement is about 2.6x slower.

These rate values probably represent overestimates since the in vivo rate of reaction may be slower than that measured in vitro. For the Amadori product between albumin and glucose for example, the in vivo rate is slower by about 60% and may be due to competition between glucose and other ligands (49).

B. Structural analysis of steroid-lysine adducts.

Structural studies of steroid-adducts were performed on model compounds prepared by the incubation of either 16α -hydroxyestrone (16α OHE) or cortisol with lysine derivatives blocked in the N- α -position. The following section describes the synthesis of these model steroid-lysine addition products and the elucidation of their structure.

Methods

Synthesis of N α -acetyl, N ϵ -acetyl, N ϵ -(3,16 α -diacetyl-1,3,5(10)-estratrien-17-yl)-lysine. A 1 ml solution containing 450 mM N- α -t-Boc-L-lysine (Vega Chemicals, Tuscon, Az.), 34 mM 16α -hydroxyestrone (Steraloids, Wilton, N.H.), and 86 mM NaCNBH₃ (Sigma Chemical Co.) was prepared in methanol/pyridine (3:1 v/v). A parallel incubation was supplemented with 1 μ Ci of 16α -[6,7-³H]hydroxyestrone. These were incubated at 37 $^{\circ}$. At daily intervals, 25 μ l aliquots were withdrawn and the reaction products analyzed by reverse-phase HPLC as described on page 17. At the end of 8 days the entire mixture (0.8 ml) was removed, the solvent evaporated under a stream of N₂ and the t-Boc group removed by adding 1 ml of a 50% mixture of trifluoroacetic acid and methylene chloride (42). This was incubated at room temperature for 20 min. The solvent then was evaporated and the products acetylated by adding 0.5 ml of pyridine and 0.05 ml of acetic acid anhydride and incubating at 80 $^{\circ}$ for 30 minutes. The solvent was then evaporated under N₂ and the residue redissolved in 1 ml methanol. Insoluble material was removed by centrifugation and the supernatant stored at -20 $^{\circ}$ prior to purification. 0.1 ml aliquots were purified by reverse phase HPLC (p. 17) and the peak

fractions containing 16 α -hydroxestrone-lysine pooled. 0.57 mg of product was isolated. This corresponds to a yield of 5.2%, based on the amount of 16 α OHE starting material.

Synthesis of N α -(9-fluorenylmethyloxycarbonyl), N ϵ -(20-cyano-3-oxo-11,17 α ,21-trihydroxy-4-pregnen-20-yl)-lysine. A 2 ml solution containing 100 mM cortisol (Steraloids), 54 mM N- α -Fmoc-L-lysine (Bachem, Inc. Torrance, CA) and 54 mM NaCNBH₃ was prepared in a buffer containing 25 mM potassium phosphate pH 7.4, 25% methanol and 25% pyridine. A second solution was prepared which was supplemented with 1 μ Ci of [1,2-³H]-cortisol. These solutions were incubated at 37 $^{\circ}$ for 6 days. On days 2 and 4 an additional 54 mmol of NaCNBH₃ was added to inhibit the formation of brown products. At the end of six days, 1.6 ml of each incubation was removed and diluted with 1.6 ml of methanol. The reaction mixtures were analyzed by reverse phase HPLC utilizing a Waters Associates C-18 column. Products were monitored by absorbance at 300 nm. The solvent system consisted of a gradient of 5 mM sodium phosphate, pH 7.4 and methanol which increased linearly from 40% to 80% methanol from 0 to 25 minutes. Two additional products (peaks I and II, Fig. 15) were identified and isolated by injecting, repetitively, 200 μ l of the incubation mixture. 1.73 mg of peak II was isolated, corresponding to a yield of 1.5% (based on cortisol starting material).

Analysis. Absorption spectra were recorded on a Hewlett Packard 8450A spectrophotometer. For mass-spectroscopy, 50 to 100 μ g of each sample was desalted by reverse-phase HPLC utilizing a C-18 column and gradient elution with water-methanol. Fission fragment ionization mass spectra were obtained on the Rockefeller University custom built time-of-flight mass spectrometer (51). Chemical ionization experiments

were made with a VG 70-250 (Altringham, U.K.) double focusing magnetic sector instrument and the desorption chemical ionization probe for sample introduction.

Proton NMR spectra were recorded in methanol- $^2\text{H}_4$, $\text{Me}_2\text{SO}-^2\text{H}_6$, and $\text{Me}_2\text{SO}-^2\text{H}_6/\text{chloroform}-^2\text{H}_3$ (2/1, v/v). A Nicolet NTC-300 (wide-bore) spectrometer was operated at 300 MHz in the Fourier transform mode. Chemical shifts are in ppm downfield from tetramethylsilane.

Results

The incubation of $16\alpha\text{OHE}$ with N- α -t-BOC-lysine and NaCNBH_3 was found to lead to the production of a single polar product which eluted very early in a reverse-phase HPLC system. On the basis of UV absorbance and the incorporation of $[^3\text{H}]16\alpha\text{OHE}$, this product was suspected to contain the major structural elements of $16\alpha\text{OHE}$. Treatment of the product with standard de-blocking conditions decreased its HPLC retention time, suggesting the incorporation of N- α -t-Boc lysine. Furthermore, the de-blocked product co-chromatographed with both the radioactive acid-hydrolysis product of $[^3\text{H}]16\alpha\text{OHE}$ modified albumin which had been reduced with NaCNBH_3 , and with $16\alpha\text{OHE}$ modified albumin which had been reduced with $\text{NaB}[^3\text{H}]_4$ (data not shown).

Approximately 0.5 mg of the $16\alpha\text{OHE}$ addition product was isolated by repetitive purification on HPLC. Figure 14 shows the proton NMR spectrum of the acetylated $16\alpha\text{OHE}$ addition product recorded in methanol- $^2\text{H}_4$. A portion of a spectrum recorded in $\text{Me}_2\text{SO}-^2\text{H}_6$ is shown in order to more clearly demonstrate the resonances of the C-16H, the C-17H, and the amide N-H of the lysine side chain. The application of shielding constant calculations to reference spectra of $16\alpha\text{OHE}$ -diacetate and estrone acetate

indicated that in the proposed structure in Fig. 14, the 16-CH proton should appear in the 4.0 to 4.5 ppm region. We assign the peaks at 4.4 and 4.05 ppm (methanol- $^2\text{H}_4$) as the 16-CH resonances of the 17- β and 17- α side chain epimers, respectively (these peaks appear at 4.35 and 3.9 ppm in the $\text{Me}_2\text{SO}-^2\text{H}_6$ spectrum). By comparison to reference spectra of N-acetyl lysine, the larger peak between them (4.3 ppm in methanol- $^2\text{H}_4$, 4.15 ppm in $\text{Me}_2\text{SO}-^2\text{H}_6$) is the lysyl α -CH. The two NMR peaks in the 5.5 ppm region (methanol- $^2\text{H}_4$) are assigned as amide rotamers and are more clearly defined in the non-exchanging solvent $\text{Me}_2\text{SO}-^2\text{H}_6$. The peaks assigned to 17-CH appear at 3.4 and 3.5 ppm in $\text{Me}_2\text{SO}-^2\text{H}_6$ for the α - and β -side chain epimers, respectively; these peaks both appear as partly resolved doublets, as would be expected for the 17-CH, coupled only to 16-CH. The 16-CH peaks are broader, reflecting more complex coupling to both 17-CH and 15-CH $_2$. These data are consistent with the ϵ -lysyl amide linkage being at C-17 of the estrogen nucleus and not at an alternative site, such as C-16. The overall spectrum is consistent with the assignment of this product as the tetra-acetyl derivative of N^ϵ -(3,16 α -dihydroxy-1,3,5(10)-estratrien-17-yl)lysine. The presence of two stereoisomers at the C-17 position indicates that NaCNBH_3 reduces the initial Schiff base to produce both α - and β -17-lysyl epimers.

Initial attempts to synthesize a cortisol-lysine adduct were hampered by the tendency of these reactions to undergo nonenzymatic browning and yield a complex mixture of fluorescent products. Supplementing these incubations with a large excess of NaCNBH_3 inhibited the formation of brown products. The use of the N- α -Fmoc-lysine derivative (52) enabled us to monitor reaction products by absorbance at 300 nm. Cortisol absorbs weakly at this wavelength and Fmoc containing products are readily

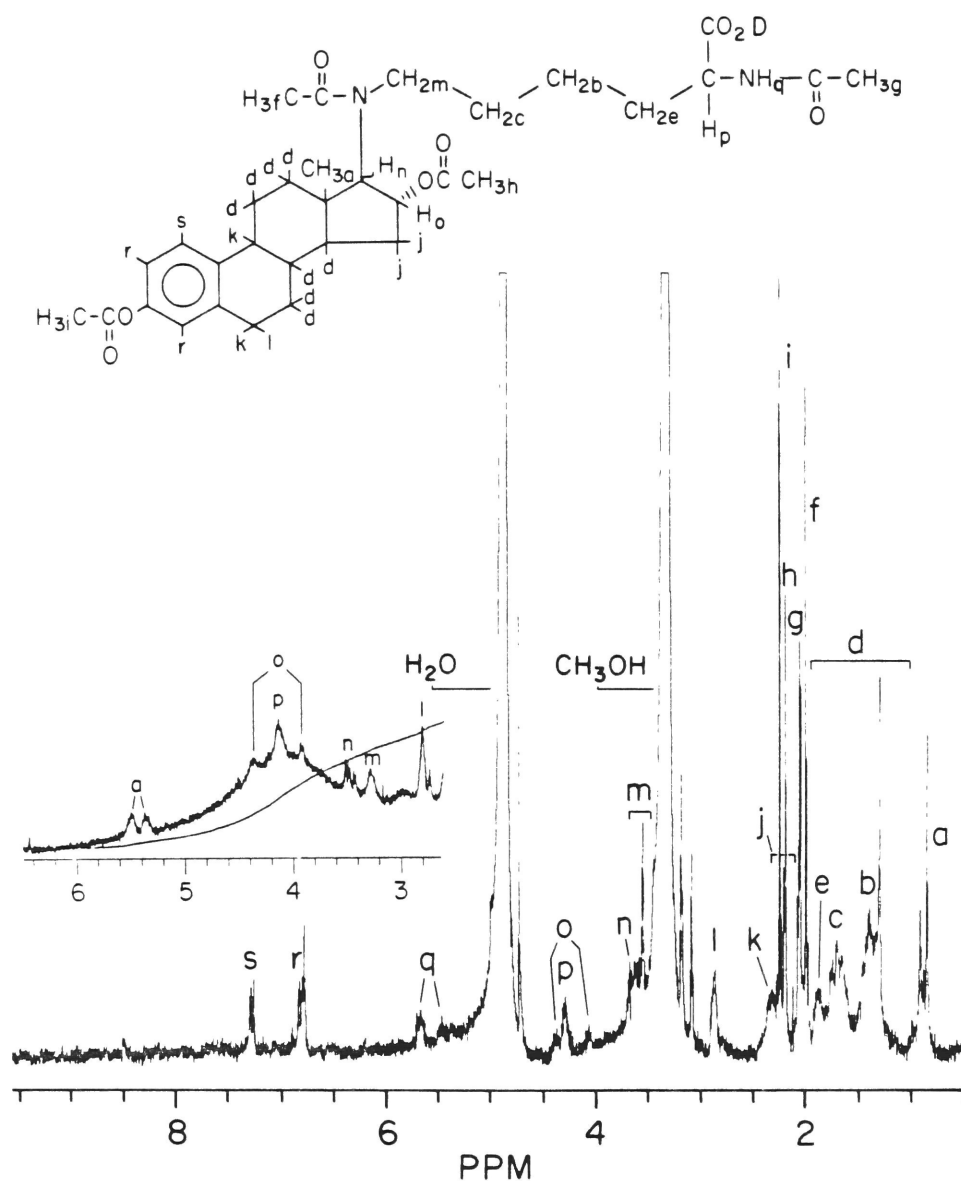


Figure 14. Proton nuclear magnetic resonance spectra of the isolated 16 α -hydroxyestrone-lysine conjugate. Full spectrum is recorded in methanol-²H₄. Inset is in Me₂SO-²H₆.

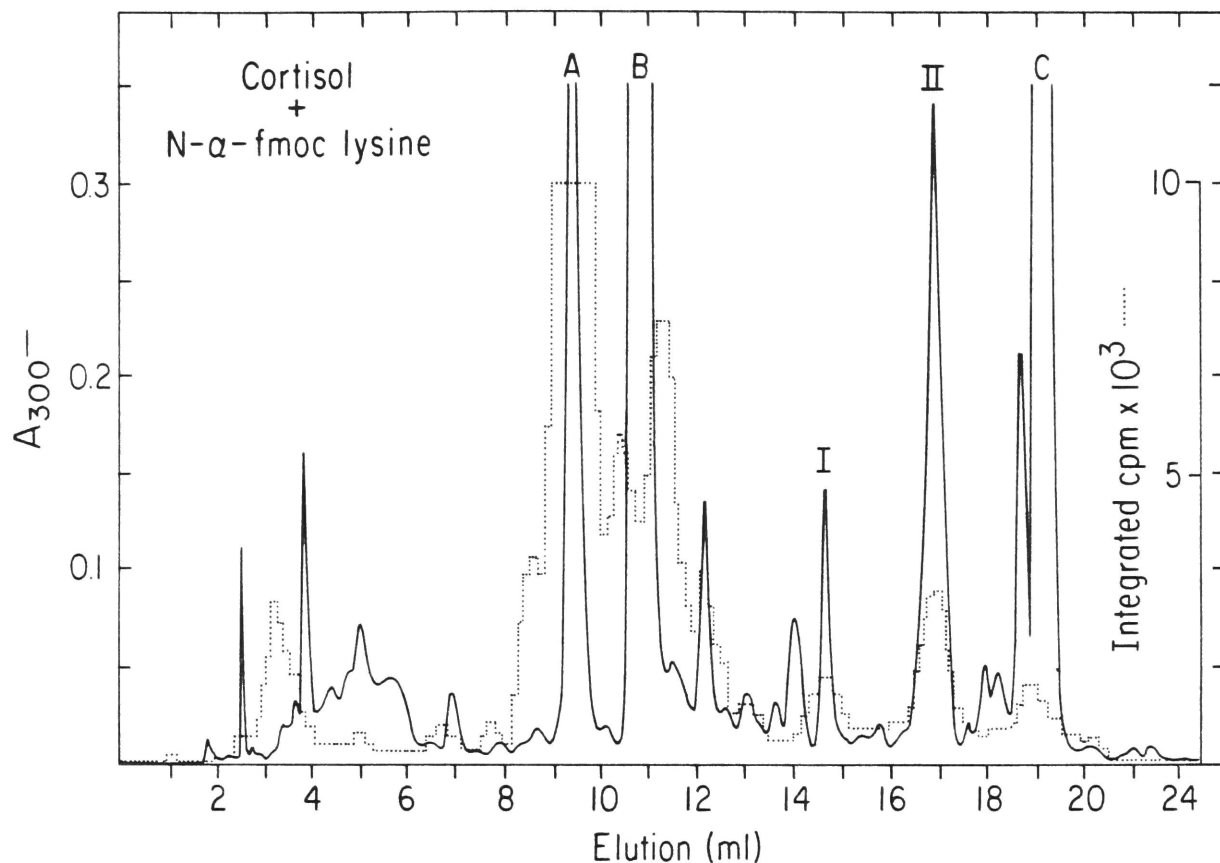


Figure 15. Reverse-phase HPLC elution profile of a 6 day incubation mixture of N- α -Fmoc-lysine, cortisol, [³H]cortisol, and NaCNBH₃. A = cortisol, B = N- α -Fmoc-lysine, C = free Fmoc. Peaks I and II designate the major cortisol-N- α -Fmoc-lysine addition products.

identified. Figure 15 shows the HPLC elution profile of an incubation mixture which had been supplemented with [^3H]cortisol. The peaks designated A,B, and C correspond to cortisol, N- α -Fmoc-lysine, and fmoc respectively. From the ratio of the tritium content and the extinction at 300 nm, it is apparent that there are two cortisol-lysine conjugates (designated peaks I and II). Analysis of absorbance spectra also confirmed that peaks I and II contained equimolar amounts of cortisol and N- α -Fmoc-lysine (data not shown).

Figure 16 shows the proton NMR spectrum of the peak II product. Resonances were assigned by comparison to reference spectra of cortisol and N- α -Fmoc-lysine recorded under analogous conditions. It is apparent that the peak II conjugate contains an intact cortisol and N- α -Fmoc-lysine residue linked in the cortisol C-17 side-chain. The absence of a C-20 proton and the similarity of the C-21 proton resonances between cortisol and the lysine adduct was unexpected (Fig. 17), and suggested that the structure might be an unreduced Schiff base at the C-20 position. A possible alternative structure, that of lysine linked at the C-21 position, was considered unlikely since the resulting α -amino ketone would produce chemical shifts for the C-21 protons that are upfield from the observed positions.

The positive fission fragment ionization spectrum of this product contained a main ion of m/z 761.4 ± 1.0 . This mass was taken to comprise the $\text{M}+\text{Na}^+$ ion for the cortisol-lysine product. Because of the relatively large error limits of this mass determination, a new synthesis of the Peak II cortisol-lysine product was undertaken. Additional NaCNBH_3 was utilized in an attempt to increase yield and prevent side reactions. Spectroscopic analysis of this new preparation demonstrated that it was reduced

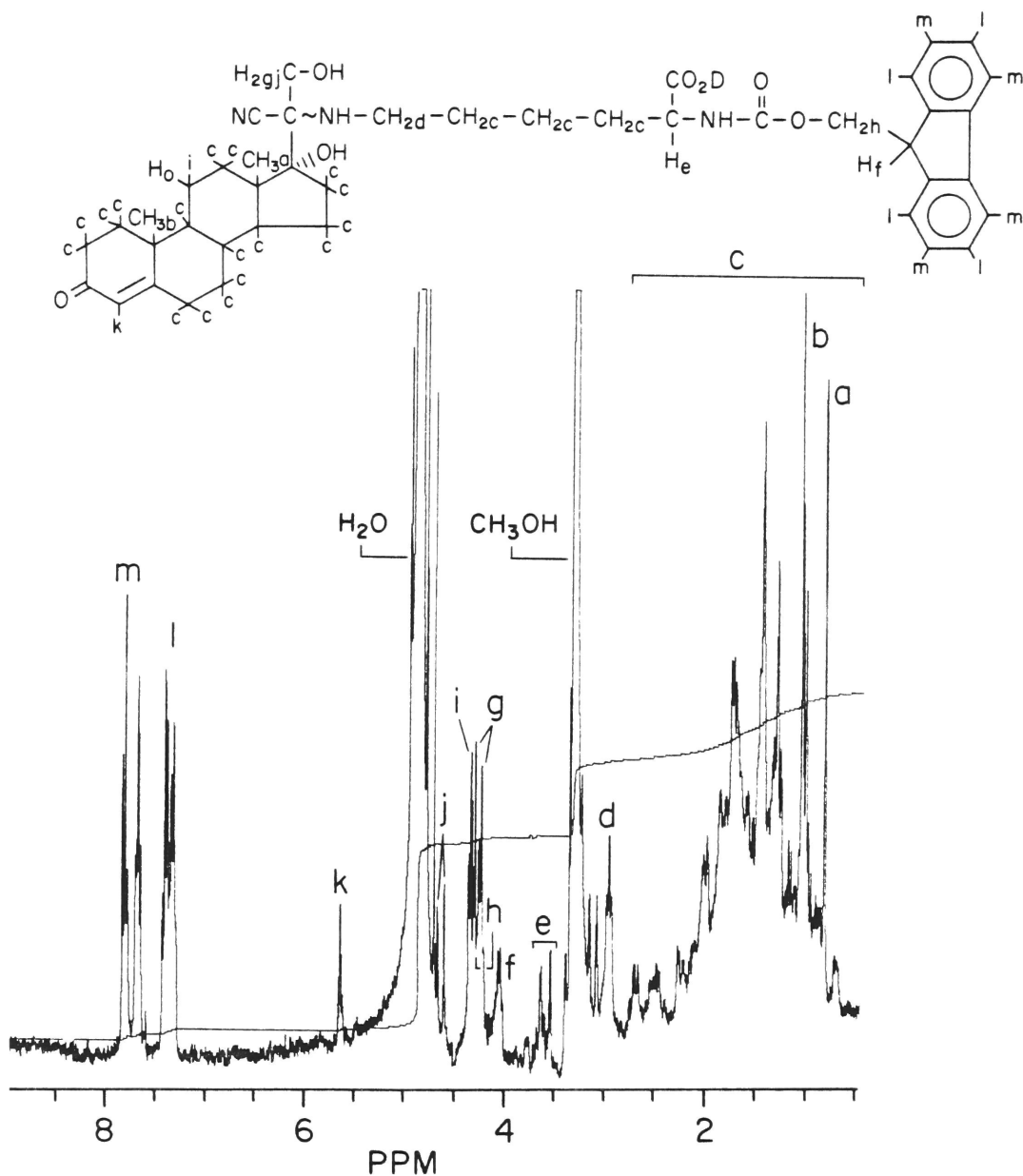


Figure 16. Proton nuclear magnetic resonance spectrum recorded in methanol- $^2\text{H}_4$ of the Peak II cortisol-lysine adduct.

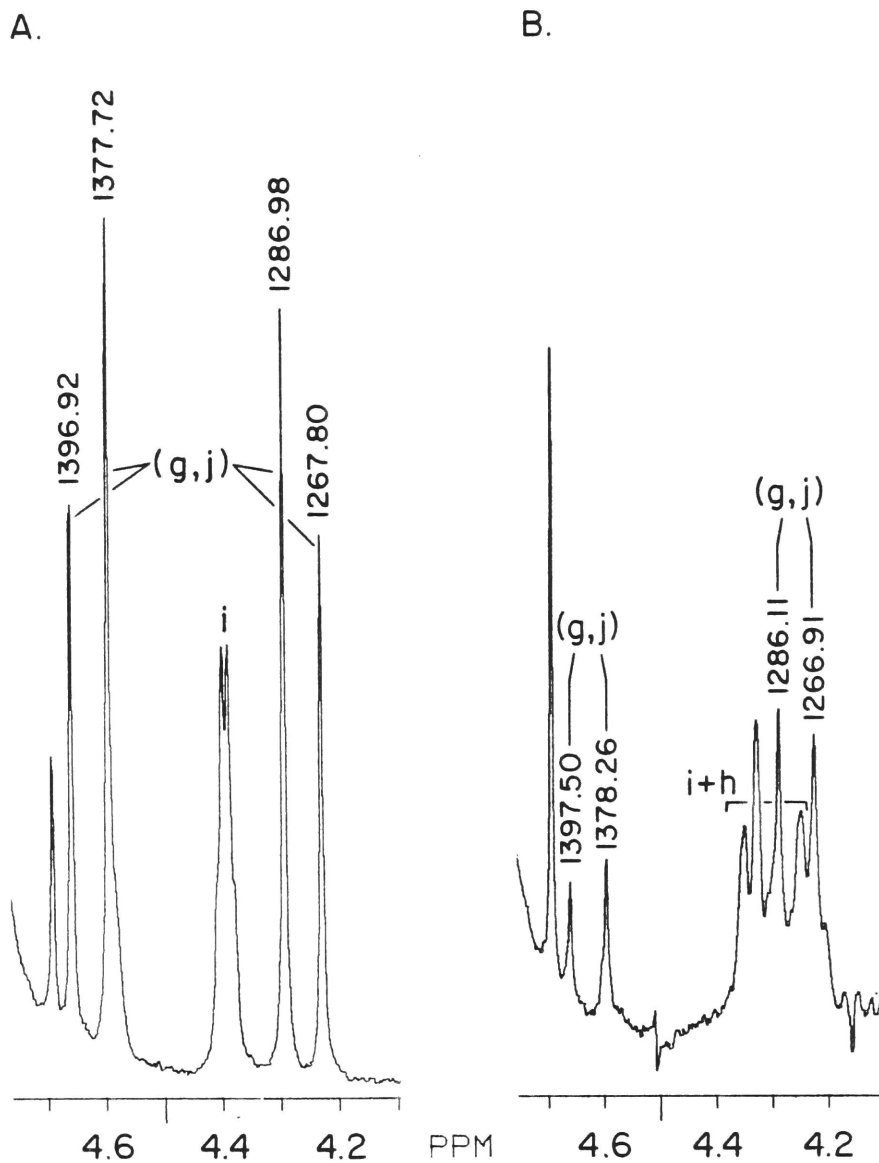


Figure 17. Expanded spectrum (methanol- $^2\text{H}_4$) illustrating the similar chemical shifts of the C-21 protons in cortisol (A) and the Peak II conjugate (B).

at the position of the C-3 carbonyl. A fission fragment ionization spectrum of higher quality was obtained for this cortisol-lysine product, and demonstrated an ion with m/z 764.2 ± 0.2 ($M+Na$)⁺. In addition, the negative fission fragment spectrum showed an ion of m/z 740.0 ± 0.5 which comprises the ($M-H$)⁻ ion. These results constitute strong evidence that the molecular weight of the first preparation is 739 and that of the second preparation is 741. Each of these values is greater, by 25 mass units, than a reduced cortisol-lysine Schiff base. On the basis of these data and mechanistic considerations, we conclude that adventitious cyanide, generated from the breakdown of cyanoborohydride, had added to the Schiff base intermediate to form a 20-cyano amine structure.

Figure 18 summarizes the reaction mechanism which led to this isolated product. A Schiff forms at the C-20 carbonyl. Steric constraints within the cortisol side chain most likely prevent cyanoborohydride from reducing the Schiff base. Cyanide is liberated by the breakdown of cyanobobrohydride, and being a small nucleophile, readily adds to to the C-20 position. It is conceivable that the Schiff base is partially stabilized by hydrogen bonding between the ϵ -nitrogen and the adjacent C-17 and C-21 hydroxyl groups.

NMR analysis of th Peak I cortisol-lysine product indicated that this conjugate also was linked in the cortisol side chain (data not shown). Additional structural studies were hampered by the fact that this compound underwent further reaction to form brown products. By analogy to known reactions between reducing sugars and amino acids (53), it seems likely tha peak I is an unreduced product and that it is either a stable Schiff base or the Heyns rearrangement product. Both of these structures would be expected to continue to react, forming brown, complex rearrangement

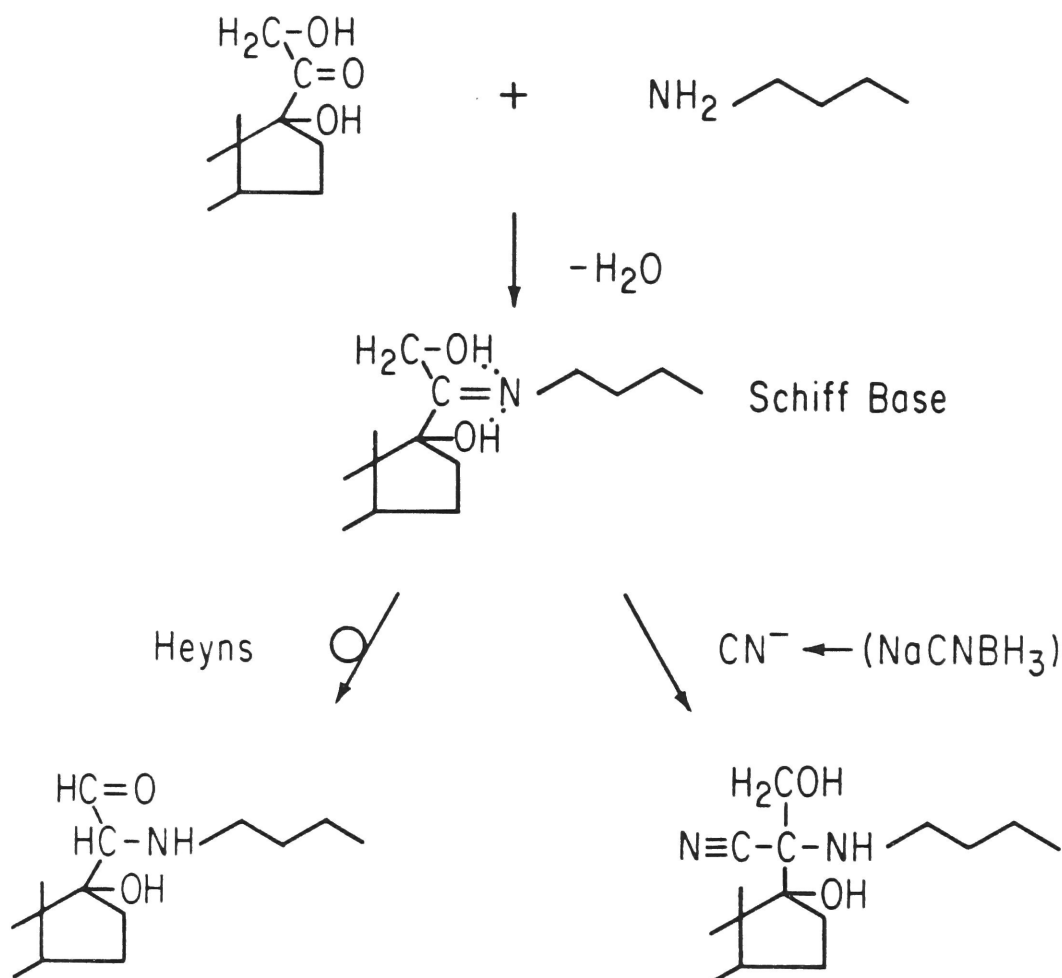


Figure 18. Proposed reaction scheme leading to the Peak II product (right), and the mechanism of the Heyns rearrangement.

products.

In conclusion, two model steroid-lysine conjugates have been synthesized, isolated, and their structures determined. Figure 19 shows space-filling models of the reduced 16 α -hydroxyestrone-lysine adduct and the cortisol-lysine Heyns product. The product formed from the reaction of 16 α OHE and lysine was found to have a structure predicted for a reduced Schiff base between these two molecules. A stable, cortisol lysine addition product was similarly synthesized and isolated. This compound was found to have an incorporated cyano moiety, produced by the nucleophilic addition of a cyanide ion which is generated during the course of the incubation. The reaction between cortisol and primary amino groups differs from other Schiff base rearrangement reactions, such as those involving 16 α -hydroxyestrone or reducing sugars, in that the Schiff base intermediate is not reducible with cyanoborohydride. This would account for the observation that the rate of cortisol incorporation into albumin is not accelerated by the presence of cyanoborohydride.

An interesting hypothesis emerged from these studies. Previous work has demonstrated that steroids which have glucocorticoid activity invariably contain a ketol side-chain at the C-17 position of the steroid nucleus. It is possible that the side chain enables these steroids to interact with the glucocorticoid receptor by forming a partially stabilized Schiff base at the C-20 carbonyl.

Figure 19. Space-filling models of the reduced 16 α -hydroxyestrone-lysine adduct (upper panel) and the cortisol-lysine Heyns product (lower panel).



3. Nonenzymatic Modification of Lens Proteins by
Glucocorticoids in Steroid-Induced Cataracts.

A. Introduction.

The biological effects of glucocorticoids are diverse and widespread. They influence protein, fat, and carbohydrate metabolism and electrolyte and fluid balance. In the human, the physiology of virtually every organ system is influenced by these hormones (54).

Therapeutically, glucocorticoids are administered either in physiological doses to correct adrenal insufficiency or in pharmacological doses to treat inflammatory conditions, collagen diseases, and certain other disorders. For many chronic diseases, they are often the last course for the alleviation of severe inflammation. It is also primarily in these cases that the adverse affects of glucocorticoids become apparent. Common manifestations include cataract formation, an immunosuppression leading to an increased susceptibility to infection, osteoporosis, a proximal myopathy, and behavioral disturbances (36). Perhaps one of the hallmarks of glucocorticoid toxicity is the development of bilateral cataracts. In 1960, Black et al. reported a high incidence of posterior subcapsular cataracts in patients receiving moderately high doses of corticosteroids (55). Since then, this phenomenon has been well-documented (56). For example, cataracts are observed in nearly all patients who receive 20 mg of prednisone per day for four years for the treatment of rheumatoid arthritis (37). The development of steroid-induced cataracts correlates well with the dosage and duration of therapy and generally halts after the withdrawal of steroids (57).

A cataract is a partial to complete opacification of the lens of the eye. The opacity may be located on the surface of the lens or within the crystalline material of the lens. The location of the lens relative to other structures in the eye is shown in Figure 20. A cross-section of the

lens, taken through the equator is displayed in Figure 21. A fibrous capsule surrounds the lens. The substance of the lens consists of highly differentiated lens fiber cells which primarily contain the specialized proteins called crystallins. These cells have the highest protein content of any tissue in the body, approximately 35% by weight (58). The cells originate near the equator, grow and elongate into lens fibers, and migrate toward the center of the lens. The lens cells depend entirely on the surrounding media, i.e. the aqueous and vitreous humors, for their nutrient supply, oxygen, as well as for the removal of waste products.

The types of opacities induced by glucocorticoids are characteristic because in initial stages, they always occur in the posterior subcapsular region. Other chemical agents also lead to posterior subcapsular opacities. Grant's compendium: "Toxicology of the eye" lists the following as inducing these types of changes: bis-(phenylisopropyl)-piperazine, busulfan, corticosteroids, dinitrophenol, dinitro-o-cresol, tripaparanol, and cyanate (59). Hyperglycemia, in addition, leads to cataracts of posterior subcapsular morphology. Figure 21 shows that although epithelial cells cover the anterior surface of the lens there are no epithelial cells present on the posterior side. The absence of an epithelium is believed to make this region sensitive to opacification since many agents might otherwise be removed or detoxified by the epithelial cells.

A number of cataractogenic agents, specifically glucose, cyanate, and busulfan have been shown to react with the amino groups of proteins to form covalent-addition products. Glucose and cyanate in particular, have been shown to modify the lysine residues of lens proteins, inducing

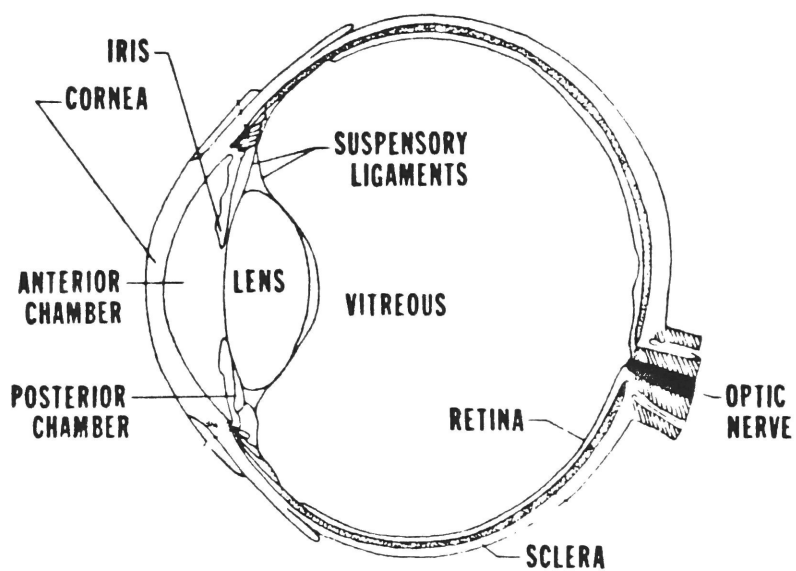


Figure 20. A schematic cross-section of the eye, showing the relationship of the major structures.

LENS IN CROSS-SECTION

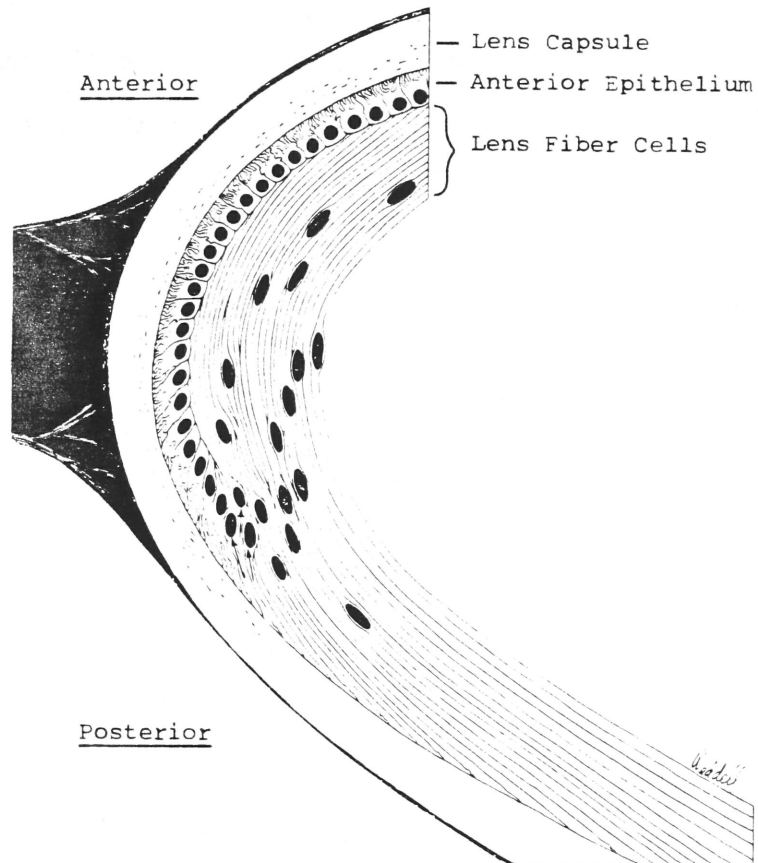


Figure 21. Schematic cross-section of the lens taken through the equator.

sulphydryl oxidation, and the formation of light-scattering aggregates (16, 60). On the basis of these observations, it seemed reasonable to pursue the hypothesis that glucocorticoid modification of lens proteins might be involved in the induction of lens opacification. The following chapter describes the development of two experimental models for the steroid-induced cataract, the detection of the glucocorticoid adducts in human steroid-induced cataracts, and studies of the effect of glucocorticoids on lens crystallins in vitro.

B. Glucocorticoid-lens protein adducts in two experimental models of the steroid-induced cataract.

Investigations into the pathogenesis of the steroid cataract have been hampered by the lack of suitable experimental models for the induction of these lesions. In vivo, both the rat and the chick appear to be resistant to the cataractogenic effect of glucocorticoids (61, 62) although a potentiating effect for the experimental dinitrophenol and galactosemic cataract does occur (63, 64). There have been two reports of long-term glucocorticoid therapy producing lens changes in the rabbit. In one study (65), the effects were cortical and anterior subcapsular while in another (66), posterior subcapsular changes were described, but only in rabbits which had simultaneously developed diabetes. Recently, Nishigori et al.(67) have described the induction of perinuclear opacities by glucocorticoids in the developing chick embryo. Chick lenses cultured in the presence of glucocorticoids show opacification (68), but these experiments utilized very high concentrations (1 mM) of prednisolone.

The following section describes two models for the steroid-induced cataract. The first is an in vitro model utilizing the rat lens cultured with low concentrations of prednisolone. The second is an in vivo model developed in the rabbit. In this animal model, specific posterior subcapsular opacities were induced by injecting glucocorticoids directly into the vitreous chamber of the eye.

Methods

Reagents and Animals. Steroids were purchased from Steraloids (Wilton, N.H.). [1,2,6,7-³H]Cortisol (80 Curies/mmol, 1Ci = 3.7×10^{10}

becquerels), [1,2,4,6,7-³H]dexamethasone (77 Curies/mmol), and [2,4,6,7,-³H]prednisolone (57 Curies/mmol) were obtained from Amersham (Arlington Heights, IL). Radiochemical purity was assessed by chromatography on reverse phase HPLC as described earlier (p.17). Male Sprague-Dawley rats (70-90 g) and young (4-6 wk) female New Zealand White rabbits were obtained from Taconic Farms (Germantown, NY). Outbred rabbits of the same sex and age were obtained from a local vendor.

Lens Culture. Rat lenses were excised and washed at room temperature with Medium 199 (Gibco, Grand Island, NY) supplemented with 5% heat inactivated fetal bovine serum, 100 U/ml penicillin G, and 25 µg/ml amphotericin B. Lenses were cultured on wire grids (69) in 1 x 3.5 cm tissue culture wells in Medium 199 containing 5% heat-inactivated fetal calf serum and 100 U/ml penicillin G. Each well contained 2-3 lenses. Incubation was at 37° in a humidified incubator containing an atmosphere of 5% CO₂ and balance air.

Glucocorticoids were dissolved in culture media containing 10% ethanol and 3 µl added to each well, yielding a final concentration of 0.01% ethanol and either 5 to 50 nM glucocorticoid or 35 µCi/ml [³H]prednisolone (a final concentration of 5 nM). Control medium used in the steroid-free incubations contained 0.01% ethanol as well. Culture medium was replaced on alternate days. Reverse phase HPLC analysis of the culture media after 48 hr of incubation demonstrated that less than 4% of the added [³H]prednisolone had undergone conversion to other compounds.

Fractionation of lens proteins. Lens proteins were fractionated with modifications of the procedure described by Kramps, et al. (70). Three rat lenses or a single rabbit lens was placed in an ice-cold glass tissue

homogenizer which contained 1-2 ml of 0.1 M Tris-HCl buffer, pH 7.4 and 0.02% NaN₃. The lenses were homogenized under a nitrogen stream with 8 strokes of a ground glass pestle. The mixture was centrifuged at 20,000 x g for 30 min at 4°, and the supernatant solution (water-soluble fraction) stored at 4° under nitrogen. The sediment was resuspended in 10 ml of 0.1 M Tris-HCl buffer (pH 7.4), centrifuged again, and the supernatant removed. The washed sediment was then resuspended in 1.5 ml of 0.1 M Tris-HCl buffer (pH 7.4), containing 7 M urea and 0.02% NaN₃. This material was vortexed at intervals and allowed to stand for 1 hr at 4°. After centrifugation as above, the supernatant solution (urea-soluble fraction) was stored under N₂ at 4°. The urea-insoluble fraction was resuspended in 10 ml of the same buffer and washed one additional time. The amount of total protein in the soluble fractions was determined by the method of Bradford (71), utilizing dye reagent supplied by Bio-Rad Laboratories and lyophilized bovine serum albumin as a standard. Urea-insoluble protein was measured after solubilization in SDS and dialysis as described below.

Water-soluble and urea-soluble fractions were dialyzed overnight (MW cutoff 3,500) at room temperature against a buffer containing 0.1M Tris-HCl, pH 6.8, 10 mM 2-mercaptoethanol and 0.1% SDS. The urea-insoluble fraction was first dissolved in a minimum volume of 10 mM Tris-HCl buffer (pH 6.8) containing 10 mM 2-mercaptoethanol, 1 mM EDTA, and 4% SDS before dialysis

Immunoprecipitation of Cultured Rat Lens Proteins. Dialyzed protein fractions were obtained from lenses cultured in the presence of [³H]prenisolone and digested by incubating a 50 µl aliquot with 50 µl of 1 mg/ml proteinase K at 37° (described below). The reaction was terminated

after 1 hour by adding 100 μ l of 0.1 M Tris-HCl buffer, pH 7.4 containing 7 M urea and 0.02% NaN₃. Two-hundred microliters of antiserum (1:3000, described on p.68) was then added to each sample. The blank samples contained normal rabbit serum at the same dilution. Samples were incubated at 4° for 10 hr. Bound material was separated by adding 0.8 ml of a saturated ammonium sulphate solution and collecting the precipitate by centrifugation for 10 minutes in an Eppendorf microfuge (Model 5414). Aliquots from the soluble fraction were removed and analyzed for radioactivity by liquid scintillation counting.

Noncovalently bound material was removed from human lens protein fractions by dialysis against a buffer containing SDS (described below), or by extraction with ethyl ether (3X). The efficiency of extraction was estimated by adding a standard amount of [³H]prednisolone (3,000 cpm) to the protein fraction and counting the extractable tritium. Recoveries of greater than 95% of the added tritium were observed consistently. A portion of the lens protein fraction was subjected to radioimmunoassay while another portion was assayed after digestion with proteinase K. Identical values were obtained with the dialyzed and the ether-extracted protein samples.

SDS-polyacrylamide Gel Electrophoresis. Dialyzed lens protein fractions were analyzed for radioactivity and total protein and then diluted 1:1 in a sample buffer containing 0.1 M Tris-HCl buffer pH 6.8, 40% glycerol, 40 mM DTT, 2 mM EDTA, 1% SDS, and 0.02% bromophenol blue. The mixtures were heated at 100° for 3 min. Twenty-five μ g of protein containing approximately 5,000 cpm of tritium was loaded on polyacrylamide slab gels containing 13% acrylamide and 0.35% bisacrylamide and electrophoresed in the presence of 0.1% SDS utilizing the buffer system

described by Laemmli (72). The protein standard mixture (low molecular weight) was obtained from Bio-Rad Laboratories. Gels were stained for protein with Coomassie Brilliant Blue R-250 and then fluorographed (73) in order to localize tritium incorporation. Exposures were performed at -80° with Kodak X-OMAT AR5 X-ray film.

Induction of Cataracts in the Rabbit. Rabbits were housed singly, rested for one week after arrival, and fed water and standard rabbit chow ad libitum. Steroids were administered intravitreally by the following procedure. The pupils of the eyes to be injected were first dilated with 1-2 drops of Mydriacyl (Alcon Laboratories). The rabbits were then anesthetized by ether inhalation and 1 drop of local anesthetic (Alcaine, Alcon Labs.) applied to the cornea. The eye was gently proptosed with blunt forceps and a 27 gauge needle inserted into the cornea in order to drain the aqueous humor. This needle was then removed and 0.2 ml of steroid preparation (1 mM or 0.1 mM in sterile saline) injected into the vitreous chamber by inserting a needle (27 gauge) through the posterior aspect of the sclera. For radioisotope studies, each eye received 0.2 ml of a sterile solution containing 100 μ Ci of [3 H]cortisol, [3 H]dexamethasone, or [3]prednisolone. During this procedure, the penetrating needle could be visualized through the lens and the dilated pupil. Rabbits were then returned to their cages and their eyes periodically examined with an ophthalmoscope.

At intervals after injection, the animals were sacrificed by injection with 2.5 ml of T-61 euthanasia solution (Hoechst). The eyes were then excised and the posterior chamber exposed by perpendicular incisions in the posterior sclera. The vitreous was then carefully removed and lens changes examined and photographed with a Zeiss binocular dissecting

microscope. Lenses were processed for electron microscopy or frozen at -80° prior to protein fractionation.

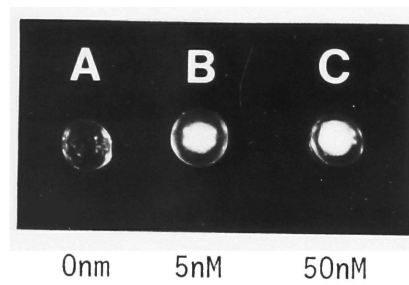
Electron Microscopy. Electron microscopy studies were performed on a control lens and a representative cataractous lens removed 4 days after the administration of prednisolone (200 nmoles). Lenses were fixed at 4° in triple aldehyde fixative (25% formalin, 1% acrolein, 1% glutaraldehyde, 0.1M cacodylate buffer pH 7.4, and 5 mM CaCl_2). Sections were stained with saturated uranyl-acetate (50% ethanol) and lead citrate (74) and analyzed with a JEOL 100B electron microscope at an accelerating voltage of 80 kV.

Results

The interaction of lenses with glucocorticoids was studied initially in an in vitro system utilizing whole, explanted rat lenses cultured with varying amounts of glucocorticoids. In the presence of 5 to 50 nM prednisolone, an opacification developed in the lenses in a time and concentration dependent manner. In contrast, no morphological changes could be detected under similar incubation with 5 to 50 nM of pregnen-tetrol-one (4-pregnen-11 β ,17 α ,20 α ,21 α -tetrol-one), a non-ketolic analog in which the C-20 carbonyl is reduced to a hydroxyl group (Fig. 30) Figure 22 shows the appearance of three representative lenses after 11 days of culture. Lens A shows no opacification and is typical of lenses incubated without prednisolone or with pregnen-tetrol-one. Lenses B and C were incubated with 5 and 50 nM prednisolone respectively, and show a centrally located opacification which increases with prednisolone concentration. This appearance differs from the posterior subcapsular changes which occur in the human in vivo. In an in vitro situation, the normal anatomic

Figure 22. Three representative rat lenses after eleven days of culture. Lenses incubated with the non-ketolic analog pregnen-tetrol-one had the appearance of lens A. The small light reflections visible in lens A are artifacts of photography.

Effect of Prednisolone on Cultured Rat Lens



orientation of the lens is lost and the development of opacification may not exactly parallel the in vivo changes.

Single lenses which were incubated with 50 nM prednisolone were removed at intervals and the total proteins extracted into water-soluble, urea-soluble, and urea-insoluble fractions. As a function of time in culture, there is a decrease in the amount of water-soluble proteins and a commensurate increase in the amounts of urea-soluble and urea-insoluble proteins (Fig. 23). Control lenses incubated without prednisolone did not show any change. This result is consistent with previous studies showing that there is a decrease in water-soluble proteins with cataract formation in vitro and in vivo (75, 70).

The possibility that glucocorticoids might form covalent adducts with proteins in the intact rat lens was analyzed by culturing lenses in the presence of 5 nM [^3H]prednisolone. These lenses were then fractionated into water-soluble and urea-soluble fractions. After dialysis against a buffer containing 0.1% SDS, these fractions were analyzed for protein content and incorporated radioactivity. Figure 24 shows that there is a time dependent increase in protein associated prednisolone in the water-soluble and urea-soluble lens fractions. By 11 days, the urea-soluble proteins contain somewhat more incorporated tritium than the proteins in the water-soluble fraction. Figure 24 also shows that in each fraction, virtually all the incorporated radioactivity is precipitable with antiserum specific for proteins modified prednisolone (described on p.68). Therefore, the occurrence of tritiated proteins must be due to covalent modification by [^3H]prednisolone, and not to [^3H]prednisolone metabolism with subsequent incorporation of the tritiated intermediates into newly synthesized macromolecules.

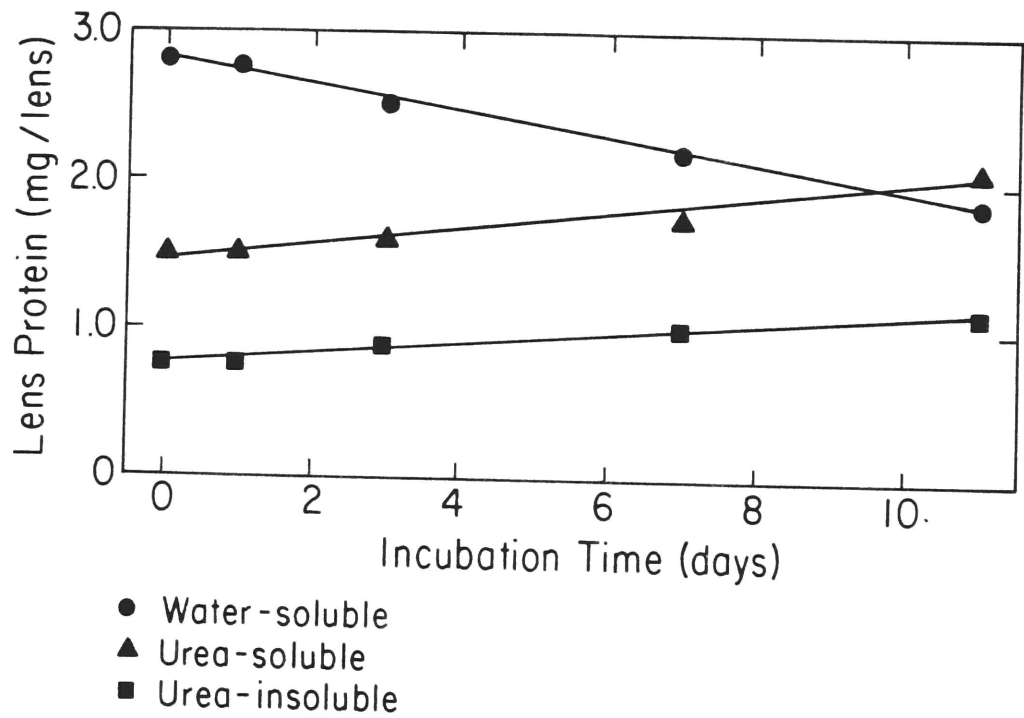


Figure 23. Single rat lenses incubated with 50 nM prednisolone were removed at the indicated times and the total protein fractionated into water-soluble, urea-soluble, and urea-insoluble protein. Lenses incubated without prednisolone did not show any change.

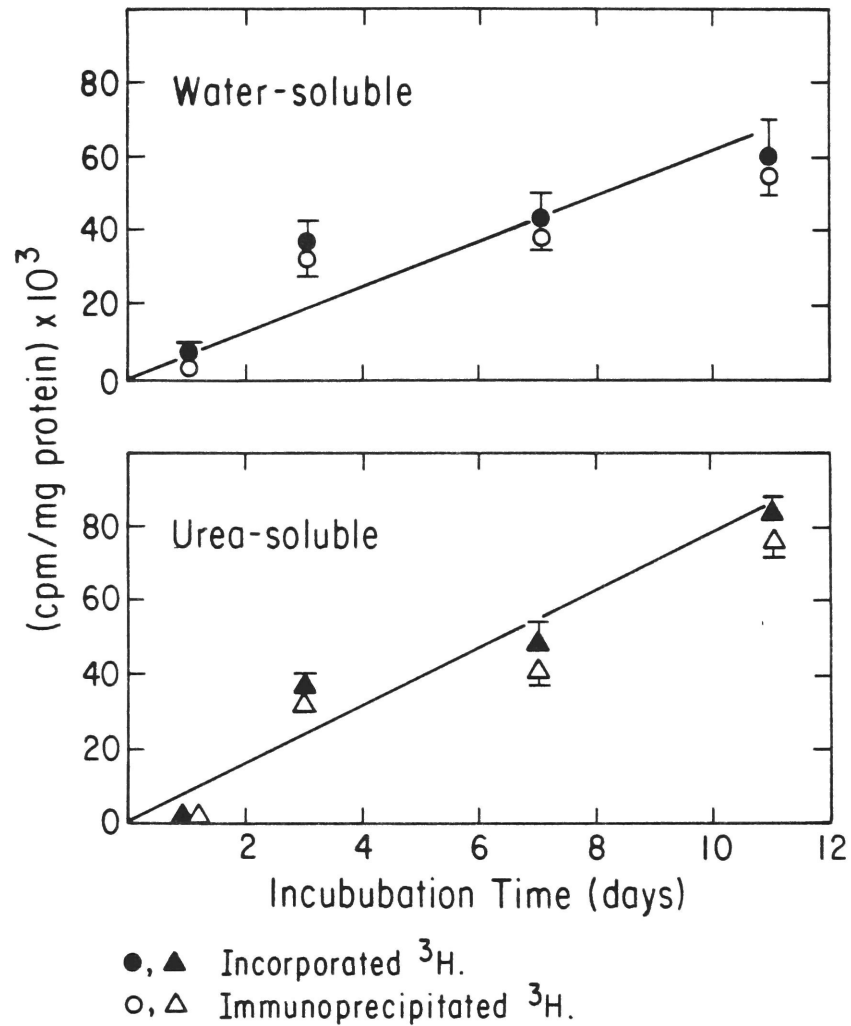


Figure 24. Lenses incubated with 5 nM [³H]prednisolone were removed at the indicated times and the total proteins fractionated into water-soluble and urea-soluble protein. Each point shows the mean \pm 1 S.E.M. for duplicate determinations.

The water-soluble, urea-soluble, and urea-insoluble extracts were then electrophoresed under denaturing conditions in the presence of 0.1% SDS. Figure 25 shows the Coomassie blue staining pattern of each lens protein fraction and the fluorographs of these fractions as a function of time the lens was in culture. Negligible changes were observed in the Coomassie blue staining of the water-soluble and the urea-soluble protein fractions during incubation. Protein bound [^3H]prednisolone can be seen to be present in both water-soluble and urea-soluble proteins. The labeled urea-soluble proteins contain a slightly higher specific radioactivity than the labeled water-soluble proteins, and the incorporation of tritium into both protein fractions increases with incubation time. These results confirm the observations shown in Fig. 24. In both the water-soluble and the urea-soluble fractions, the two predominant species of [^3H]prednisolone labeled proteins have an approximate molecular weight of 18,000 and 22,000. By comparison with a previous description of rat lens proteins (76), it would appear that the 18,000 MW protein can represent either α or γ crystallins and the 22,000 MW protein is a subunit of β crystallins. A small amount of [^3H]prednisolone incorporation is also detectable in a much higher molecular weight protein. The identity of this protein is unknown.

Since the addition reaction between ketolic steroids and proteins is slow, we reasoned that the introduction of a high, local concentration of glucocorticoid might lead to sufficient protein modification so as to produce experimental lesions in vivo. This idea was tested by injecting glucocorticoids directly into the vitreous chamber of rabbit eyes. In initial studies, young (4-6 week old) rabbits were administered 200 nmoles

Figure 25. SDS polyacrylamide gel electrophoresis and fluorography of lens protein fractions after incubation of rat lenses with 5 nM [^3H]prednisolone. MW: molecular weight, WS: water-soluble fraction, US: urea-soluble fraction, UI: urea-insoluble fraction. CB: Coomassie Blue staining of each lens protein fraction. 0, 7, 11 = gel fluorographs after 0, 7, and 11 days of culture.

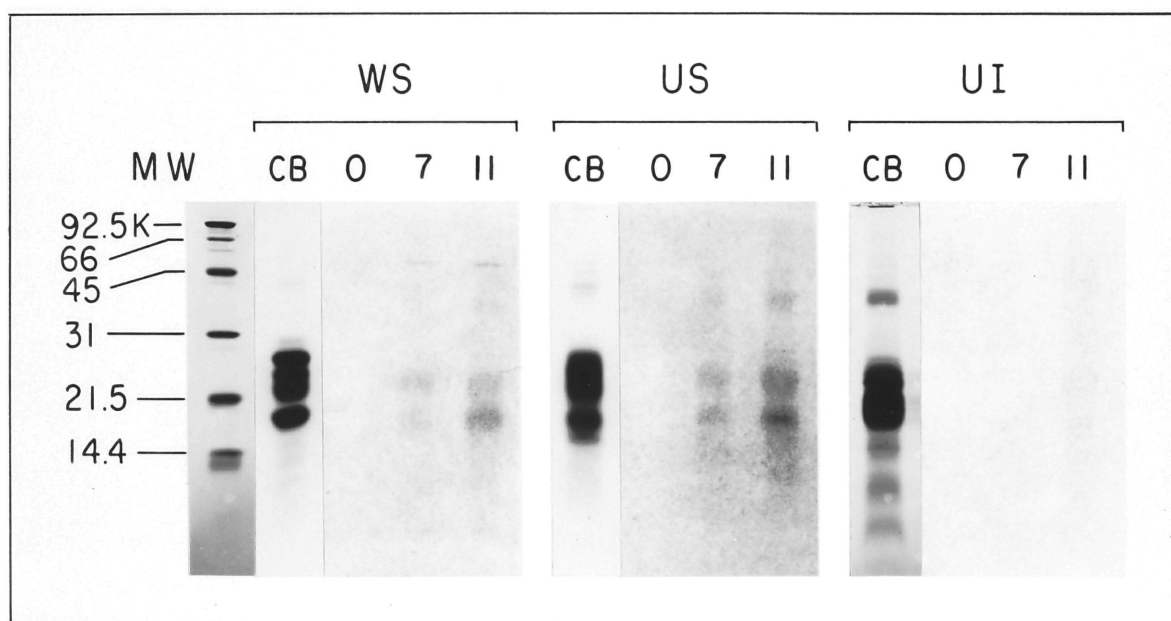


Figure 26. Posterior views of two lenses photographed through a binocular dissecting microscope. A: control lens. B: lens removed eight days after the intravitreal injection of 200 nmoles of prednisolone. The central reflection in both lenses is an artifact of photography.

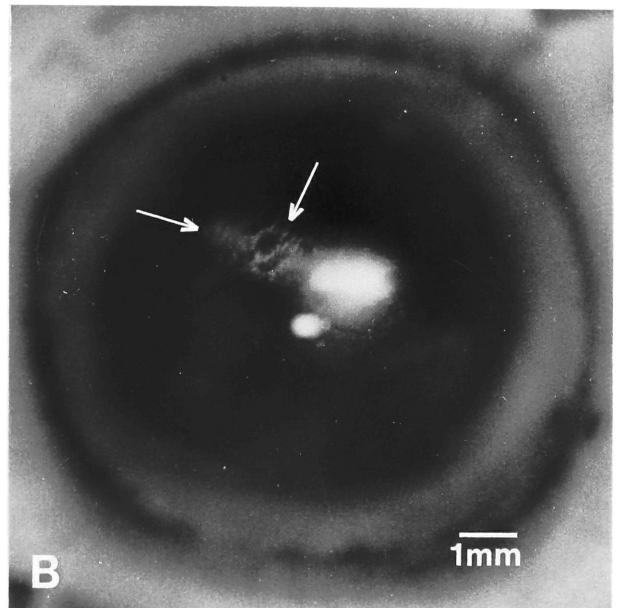
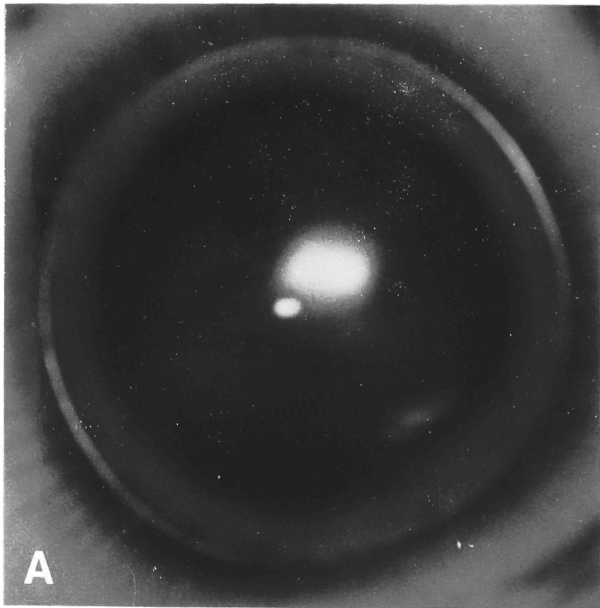
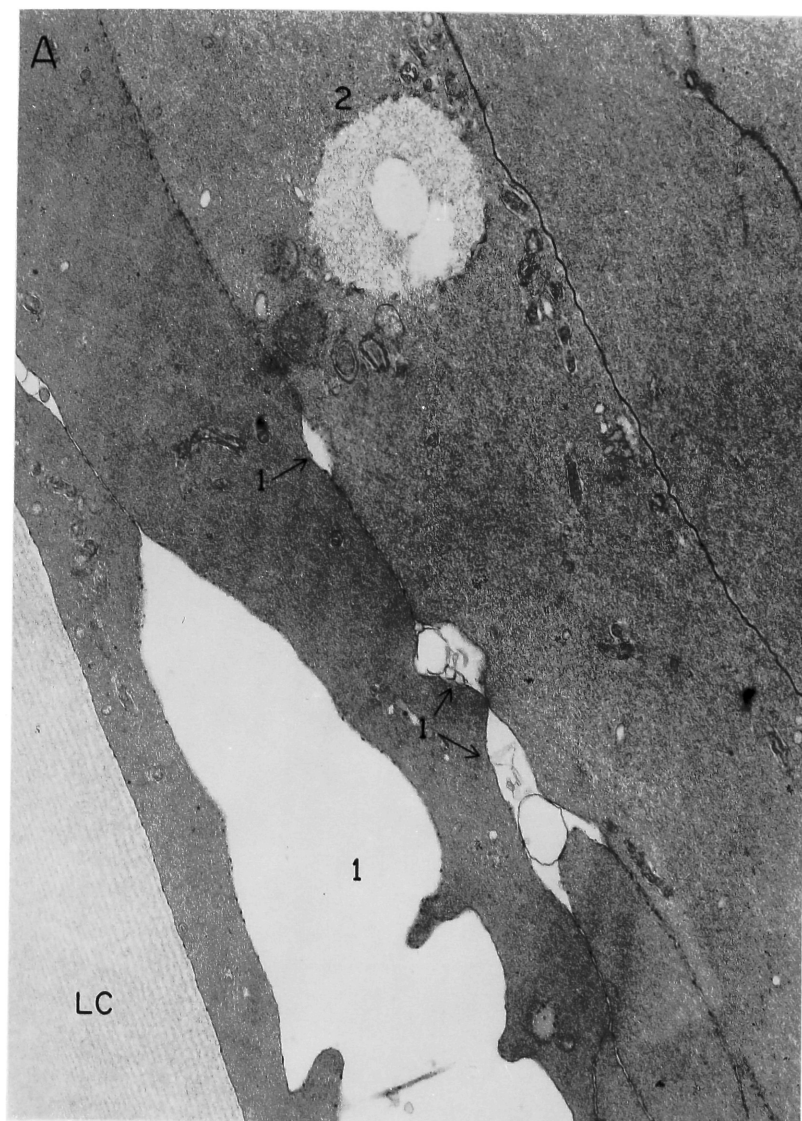


Figure 27 and 28. Representative electron micrograph sections of the posterior region of a lens removed four days after injection with 200 nmoles of prednisolone. Sections were stained with uranyl acetate and lead citrate. LC: lens capsule, 1: area of fiber cell separation. 2: circular lesions within lens fiber cells. Final magnification = 25,000x.



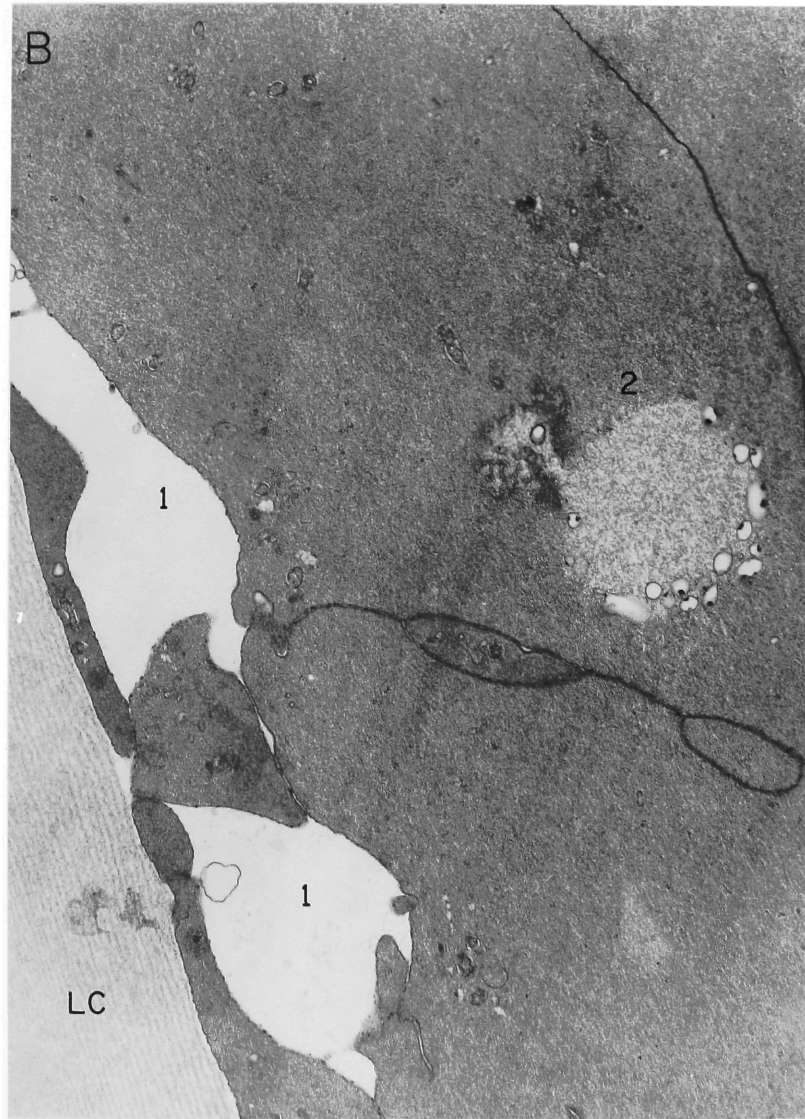
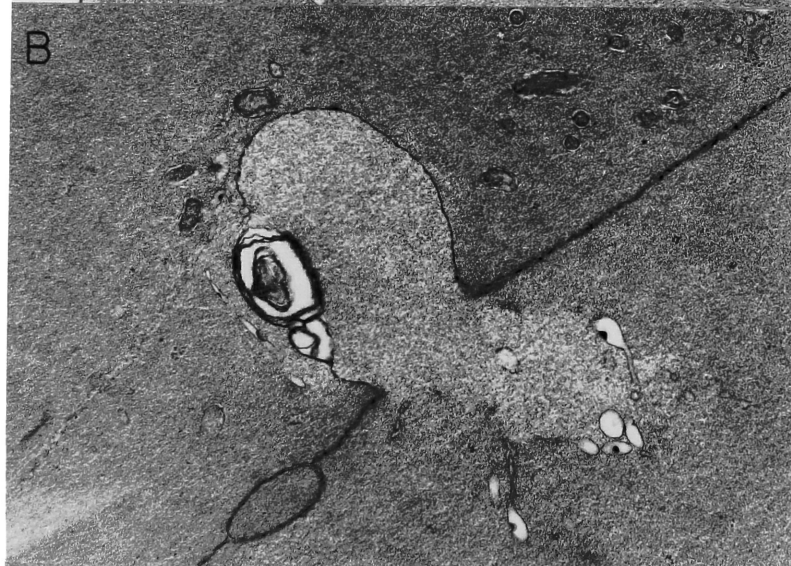
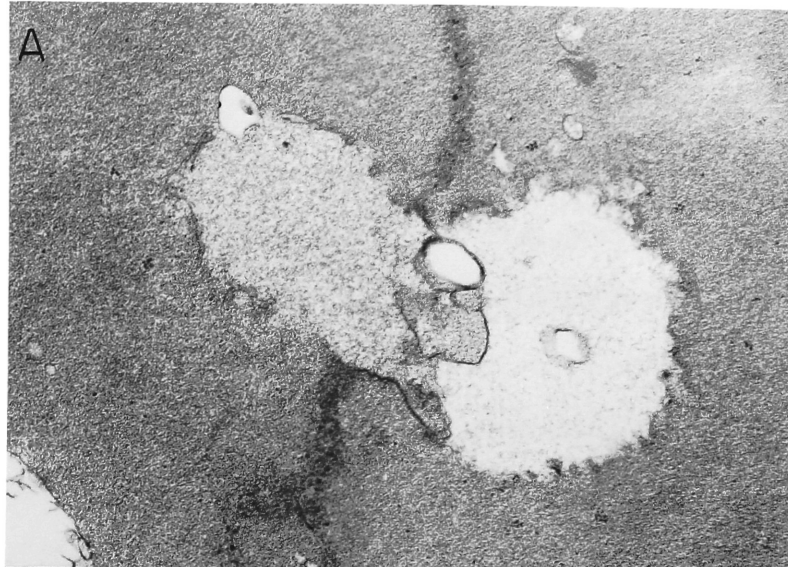


Figure 29. Enlarged views showing circular regions of lower electron density within the lens fiber cells. Final magnification: A = 33,333x; B = 41,666x.



of prednisolone by injection into the vitreous chamber of one eye. Contralateral eyes received an equivalent volume (0.2 ml) of saline. Rabbits were examined daily by direct illumination with an ophthalmoscope and all portions of the eye evaluated. Lens changes in the prednisolone treated eyes were first noted 48 to 72 hours after injection and appeared as punctate, refractile deposits near the posterior pole. Figure 26 shows posterior views of two representative lenses removed eight days after injection. An opacification is apparent and consists of white, granular lesions located immediately below the posterior capsule.

Figures 27-29 shows a series of electron micrographs obtained from the posterior subcapsular region of a prednisolone treated lens 4 days after injection. Two classes of lesions are present in these sections. The first is a disruption of the normal architecture in the subcapsular lens fiber cells ("1", Figs. 27,28). Fiber cells adjacent to the capsule have separated along their membranes to produce large, vacuolated regions. Figure 27, in particular, displays an intermembranous region in which this separation appears to be in the process of developing. The second type of damage ("2", Figs. 27, 28) consists of circular lesions within the lens fiber cells. These changes appear as areas of lower electron density within the matrix of the crystallins. Figures 29A and 29B show higher magnification views in which two of these lesions appear to penetrate the fiber cell membranes. Also present are associated vacuoles, electron-dense granules, and a multi-vesiculated structure. The origin of these intracellular changes is unclear, but they presumably represent a disruption of normal lens crystallins.

Table 2 summarizes data for the induction of lens changes by 5 different steroids. A total of 6 out of 6 prednisolone injected eyes

showed posterior subcapsular opacifications. This number also includes 3 outbred rabbits which were studied. Opacification was induced only by glucocorticoids having a reactive C-20,21 hydroxylcarbonyl function; e.g. cortisol, dexamethasone, and prednisolone (Fig. 30). Lens changes were not produced by glucocorticoid analogs which either lack the C-21 hydroxyl (17 α -hydroxyprogesterone) or in which the C-20 carbonyl is reduced to a hydroxyl (pregnen-tetrol-one). Cortisol and dexamethasone also produced opacities at a ten-fold lower dose (20 nmoles) in two rabbits which were tested.

The occurrence of glucocorticoid-lens protein adducts was examined in three rabbits injected with 100 μ Ci of either cortisol, dexamethasone, and prednisolone. After eight days, the lenses were removed, homogenized, and extracted into water-soluble, urea-soluble, and urea-insoluble fractions. After extensive dialysis against SDS to remove noncovalently bound material, the amount of protein-bound tritium was measured. Figure 31 shows the distribution of radioactivity in these [3 H]glucocorticoid treated lenses. In the case of each steroid, the greatest proportion of incorporated radioactivity appears in the urea-soluble protein fraction. Injection with [3 H]dexamethasone also results in the incorporation of radioactivity in water-soluble as well as urea-soluble protein. The reason for this difference between dexamethasone and the other glucocorticoids is unclear. Perhaps dexamethasone is unique in reacting with water-soluble proteins to a significant degree. Alternatively, if the labeled urea-soluble protein is aggregated water-soluble crystallins, these dexamethasone modified proteins may represent aggregates which have not yet become insoluble.

Steroid	Dose (nmoles)	Eyes Injected	Posterior Subcapsular Changes
Cortisol	200	2	2
	20	1	1
Dexamethasone	200	2	2
	20	1	1
Prednisolone	200	6	6
	20	1	0
17 α -OHP ¹	200	3	0
	20	1	0
Pregnen- tetrol-one ²	200	4	0

¹17 α -hydroxyprogesterone.

²24-pregnen-11 β ,17 α ,20 α ,21-tetrol-3-one.

Table 2. Summary of experimental rabbits injected with different steroids.

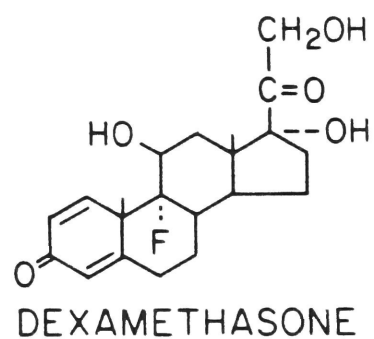
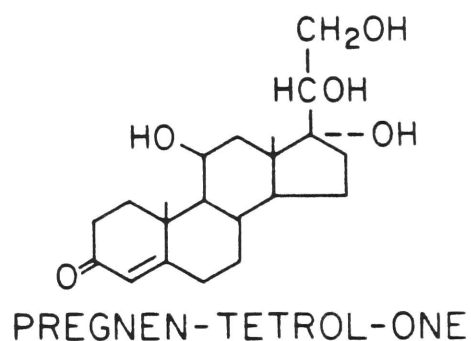
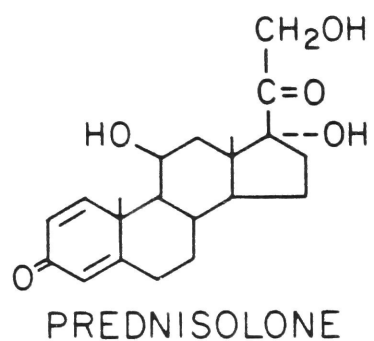
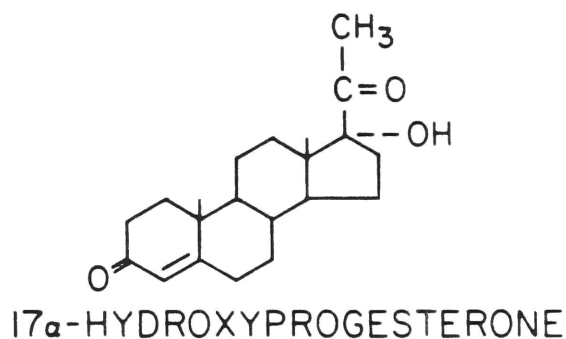
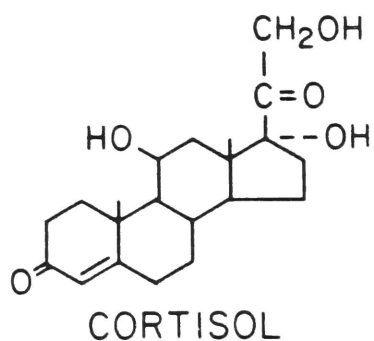


Figure 30. Structures of steroids assayed for cataractogenic activity. Pregnen-tetrol-one is 4-pregnen-11 β ,17 α ,20 α ,21-tetrol-3-one.

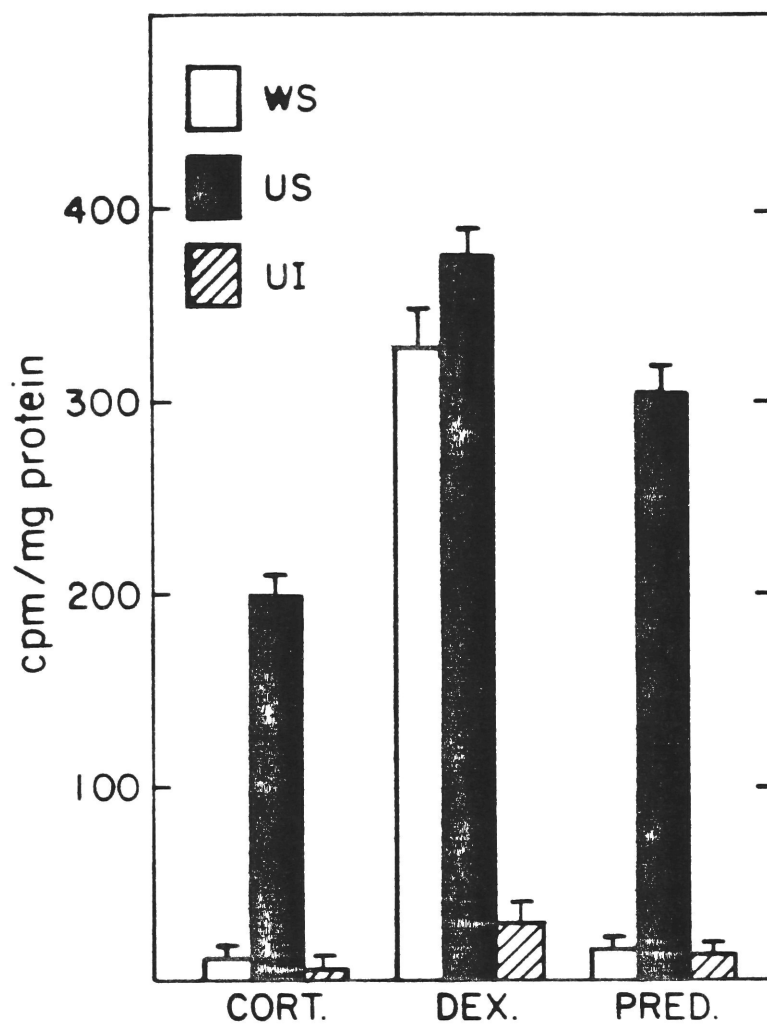


Figure 31. Distribution of protein-bound radioactivity in lens protein fractions 8 days after the intravitreal injection of 100 μ Ci of [3 H]cortisol, [3 H]dexamthasone, or [3 H]prednisolone. WS: water-soluble fraction, US: urea-soluble fraction, UI: urea-insoluble fraction.

C. Detection of prednisolone-lens protein adducts formed in vivo.

In order to detect the occurrence of lens proteins modified by prednisolone in vivo, a radioimmunoassay was developed for the measurement of prednisolone-albumin addition products. Rabbit antisera was raised against albumin which had been nonenzymatically modified with prednisolone and used in a competition assay with [^3H]prednisolone. The following section describes the details of this assay and the detection of prednisolone adducts in human steroid-induced cataracts.

Methods

Antiserum production and radioimmunoassay procedure. Synthetic prednisolone-protein adducts were prepared as follows. Fifty microliters of a 12.6 mg/ml solution of prednisolone was added to a 0.45 ml solution of HSA in 50 mM potassium phosphate buffer, pH 7.4, containing 10% ethanol. Ten microliters of a 1M solution of NaCNBH_3 was then added and the solution incubated for 6 days at 37° . In order to calculate the degree of prednisolone incorporation, a second mixture was prepared which contained, in addition, 4×10^5 cpm of [^3H]prednisolone. Unbound material was separated by extensive dialysis against a buffer of 50 mM potassium phosphate pH 7.4 containing 10% ethanol, and gel filtration over a column of Sephadex G-100, equilibrated with the same buffer. A molar ratio of prednisolone to HSA of 3.1:1 was obtained.

After lyophilization, the prednisolone conjugated albumin was dissolved in sterile saline and the concentration adjusted to 2 mg/ml. One ml was emulsified with 1 ml of complete Freund's adjuvant (Miles Laboratories, Elkhart, IN) and injected into each of two New Zealand White

rabbits. Each rabbit received four intradermal injections over the back (200 μ g each) and one injection in each hindquarter (100 μ g). This procedure was repeated at weekly intervals for 6 weeks. The rabbits were then rested for two weeks, following which a booster injection of 1 mg in Freund's incomplete adjuvant was administered. The animals were bled (50 ml) on the tenth day after this injection. Sera were prepared, cleared of debris by centrifugation at 500 x g and stored at -80° . Similar antibody titres to prednisolone-albumin were observed in the two rabbits which were immunized. Radioimmunoassays were performed in 1.5 ml microfuge tubes (Brinkman Instruments, NY) which contained the test antigen in 100 μ l of a buffer composed of 50 mM Tris-HCl (pH 8.0), 100 mM NaCl 0.1% gelatin and 0.02% NaN_3 . Diluted antiserum (200 μ l) which contained 5,000 cpm of $[^3\text{H}]$ prednisolone was then added, the samples incubated at 4° for 12-16 hr, and antibody bound material separated from free material by precipitation with 50% saturated ammonium sulphate. The optimal dilution of antisera which bound 50% of the 94 fmole of $[^3\text{H}]$ prednisolone present in the assay was found to be 1:3,000. The K_A for the binding of prednisolone was calculated by Scatchard analysis to be $8.7 \times 10^8 \text{ M}^{-1}$. Antiserum specificity was tested by assaying various steroids, synthetic prednisolone-protein adducts, and enzymatically digested adducts for cross-reactivity. The molar amount required to reduce the binding of $[^3\text{H}]$ prednisolone by 50% compared to unlabeled prednisolone was calculated from the standard curve. Sensitivity was defined as the value of 2 standard deviations of the mean of duplicate blank values (77).

Enzymatic Hydrolysis. Water-soluble proteins were digested by adding 5 mg of protein to an equal volume of digestion buffer (0.1 M Tris-HCl, pH 8.8 containing 1 mM EDTA and 1 mM DTT) and adding 1 mg/ml proteinase K

(78). The urea-soluble fraction was digested by first adding a two-fold volume excess of 0.1 M Tris-HCl buffer, pH 8.8. This dilution reduces the urea concentration to a degree which does not inhibit protease activity. The urea-insoluble fractions were solubilized by resuspending in 1 ml of the digestion buffer containing proteinase K. All digestions were carried out at 37°. The extent of digestion was estimated by the generation of ninhydrin positive material (79). Proteolysis was judged to be complete at 1 hr. The reactions were terminated by adding 1 ml of 0.1 M Tris-HCl containing 7 M urea. Aliquots of digested samples were then subjected to radioimmunoassay and immunoprecipitation as described above.

Human Lens Specimens. Normal human lenses were obtained from the Eye-Bank for Sight Restoration, Inc. (New York, NY). The assayed samples (n = 9) were from individuals aged 41 to 69 years. The diabetic (n = 5), traumatic (n = 1), and senile (n = 11) cataractous lenses were from the New York Hospital, Department of Surgical Pathology. The diabetic cataracts were from patients of ages 59 to 62 years. Three of these lenses were from individuals with Type 1 diabetes mellitus who also suffered from complications of retinopathy and nephropathy. The senile cataracts were obtained from patients of ages 59 to 87 years. No additional conditions known to contribute to cataract formation were present in these cases.

The glucocorticoid-induced cataractous lenses (n = 7) were the generous gifts of Drs. A.J. Bron, J.J. Harding, and V. Monnier. Relevant clinical information is summarized below.

Lens A: A 67 year old male patient with rheumatoid arthritis and chronic bronchitis who received 5 - 7.5 mg of prednisolone per day for at least 10 years. In addition, this patient received 7.5 mg of cortisone

per day for 1 year. Other likely steroid-induced complications included glaucoma and peptic ulcer.

Lens B: A 72 year old female patient with sarcoidosis who received an ophthalmic preparation of 0.125 prednisolone phosphate two times per day for about 8 years. This patient also suffered from glaucoma.

Lens C: A 73 year old female with rheumatoid arthritis who received a minimum of 5 mg of prednisolone per day for 5 years. During this time the patient also received alternate day therapy of 0.6 mg of betamethasone.

Lens D: A 61 year old male with bronchial asthma who received a minimum of 10 mg of prednisolone per day for more than 10 years.

Lens E: A 74 year old male with bronchial asthma. He received a minimum of 5 mg of prednisolone for 5 years.

Lens F: A 56 year old female patient with rheumatoid arthritis and anterior uveitis. This person received an ophthalmic preparation of 0.125 prednisolone phosphate 4 times per day for 2 years. In addition, dexamethasone ointment was given 3 times per day for 2 years.

Lens G: A 75 year old female patient with bronchial asthma, glaucoma, and diabetes mellitus (Type II). This patient received 2.5 mg of prednisolone for 5 years.

In all cases, nuclear sclerosis and posterior subcapsular changes were evident by ophthalmological examination. Lenses A-C were lyophilized and stored at 4°. Lenses D-G were received whole and stored at -20°.

Results

The incubation of prednisolone with albumin in the presence of NaCNBH_3 produced an addition product which contained approximately 3 moles of prednisolone per mole of albumin. Polyclonal antisera was produced to

this synthetic adduct and utilized in a competitive radioimmunoassay with [^3H]prednisolone. Figure 32 shows a series of antigen inhibition curves. The antisera was found to have a higher affinity for free prednisolone than for the immunogen: prednisolone modified albumin. Enzymatic digestion of prednisolone-albumin by protease K increased the reactivity of the prednisolone protein adduct forty-fold, most likely by destroying a large portion of the interfering tertiary structure of the protein. A similar increase in reactivity was observed after enzymatic digestion of lens proteins modified by incubation with prednisolone in vitro. The minimum detectable amount of antigen at the 95% confidence limit was calculated to be 0.10 pmoles for prednisolone and 0.33 pmoles for prednisolone-peptides.

Table 3 summarizes cross-reactivity studies with several steroids and prednisolone-protein derivatives. Other glucocorticoids react with the antisera to a varying degree, although the 9-fluorocompounds, dexamethasone and betamethasone, react poorly (0.1%). A possible complication in the use of the prednisolone-albumin immunogen is that NaCNBH_3 may reduce additional Schiff base adducts which form at the C-3 position of prednisolone. This site would not normally be expected to form stable adducts with proteins under aqueous conditions. The antisera however, appears to be primarily recognizing a D-ring conjugated hapten since glucocorticoids with structural changes in ring D (i.e. prednisolone hemisuccinate, 4-androsten-11 β -ol-3,17-dione) cross-react to a much greater degree than those with large changes in ring A (i.e. tetrahydrocortisol).

This radioimmunoassay was then used to analyze enzymatic digests of human lens proteins for the presence of prednisolone adducts. Normal and

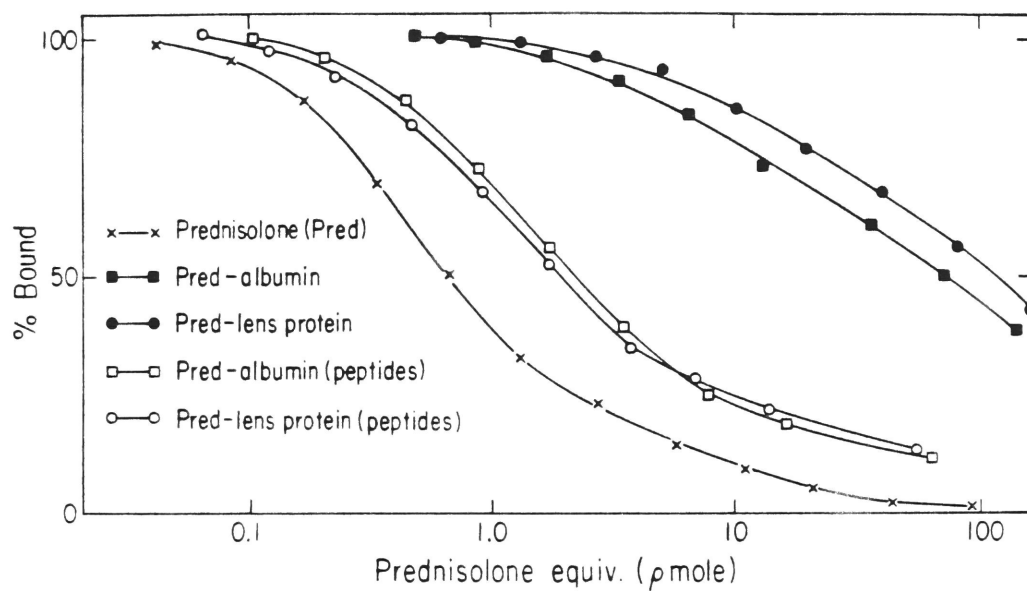


Figure 32. The relative percentage bound versus log dose curves for anti-prednisolone-albumin antiserum with five antigens utilizing [3 H]prednisolone as the competitor. Each point represents the mean of duplicate determinations.

COMPOUND	% CROSS REACTIVITY AT 50% BINDING
Prednisolone	100
Prednisolone-hemisuccinate	41
Cortisol	32
Prednisone	18
4-Androsten-11 β -ol-3,17-dione	1.5
Dexamethasone	0.1
Betamethasone	0.1
Tetrahydrocortisol	0.04
Prednisolone-albumin	1.0
Prednisolone-lens protein	0.7
Prednisolone-albumin (peptides)	40
Prednisolone-lens protein (peptides)	41
Dehydroepiandrosterone	<0.001
Etiocholanolone	<0.001
17 β -Estradiol	<0.001

Table 3. Percent cross-reaction relative to prednisolone of anti-prednisolone-albumin antiserum with selected compounds.

cataractous human lenses were homogenized and the proteins fractionated into water-soluble, urea-soluble, and urea-insoluble portions. Low molecular weight material was removed by extraction with ethyl ether or extensive dialysis against 0.1% SDS. The water and urea-soluble lens protein fractions were then analyzed by competitive radioimmunoassay with [^3H]prednisolone. These fractions and the urea-insoluble fraction also were analyzed after digestion with proteinase K. Antibody binding activity was found in the urea-soluble fraction of 5 out of the 7 steroid-induced cataracts assayed. The binding activity in these lenses was found to increase with enzymatic digestion in an identical manner as the lens proteins modified with prednisolone in vitro. None of the normal, other cataractous lenses, or other lens protein fractions displayed antibody binding activity at an assay sensitivity of 0.33 pmole of prednisolone equivalents.

Table 4 expresses this activity in terms of prednisolone equivalents which were calculated from a standard curve of water-soluble lens proteins which had been modified by prednisolone in vitro and digested with proteinase K. These values may not reflect prednisolone adducts alone since several of the lenses were obtained from patients who were receiving other steroids such as cortisone (lens A), betamethasone (lens C), and dexamethasone (lens F). The degree to which these steroid-protein adducts contribute to the antibody binding activity cannot be determined, but is presumably low since these glucocorticoids were administered in much lower amounts and, in the case of dexamethasone and betamethasone, the antiserum reacts poorly with these steroids.

The cataractous lenses analyzed were from patients who had received relatively low doses of corticosteroids (10 mg/day) for a varying number

Lens Type		N	Urea-Soluble Protein
Normal:		9	N.D.
Cataractous:	Traumatic	1	"
	Diabetic	5	"
	Senile	11	"
	Steroid	7: A	45.3*
		B	67.3
		C	5.3
		D	7.3
		E	6.2
		F	N.D.
		G	N.D.

*pmoles prednisolone equiv./mg protein.

N.D.: less than 0.33 pmoles prednisolone equiv.

Table 4. Glucocorticoid-lens protein adducts in human lenses.

of years. The highest measured levels of prednisolone-protein adducts were from two patients (A and B) who had been treated with prednisolone for more than eight years. Of interest is that patient B had received only an ophthalmic preparation of prednisolone, demonstrating that topical as well as systemic routes of administration can lead to adduct formation in vivo. The two steroid-induced cataractous lenses (F and G) that did not display antibody binding activity were obtained from patients who had received the lowest cumulative dose of glucocorticoids of the lenses assayed. Presumably, these lenses had levels of steroid adducts which were below the sensitivity of the radioimmunoassay.

The differences observed in the amount of steroid-protein adducts present in the human cataractous lenses is most likely the result of exposure to different cumulative doses of prednisolone. Other factors may play an important role in the onset of lens opacification. These include inherent biochemical differences in the susceptibility of the lens to protein modification. The simultaneous accumulation of other covalent adduct products, perhaps as a result of nonenzymatic glycosylation or carbamylation, may influence the course of lens opacification. The report of a more rapid onset of unilateral cataract in the eye of diabetic patients which had been treated with glucocorticoids points to the possible additive effect of agents that can covalently modify proteins (80).

Table 5 summarizes data for prednisolone-lens protein measurements in the rat, the rabbit, and the human. Of note is that these cataracts are associated with approximately equivalent amounts of prednisolone adducts. It would appear that when the lens has accumulated this much modification, a visible opacification occurs. In all three examples,

	Protein Fraction		
	<u>Water-Soluble</u>	<u>Urea-Soluble</u>	<u>Urea-Insoluble</u>
Rat	1-10*	1-10	N.D.
Rabbit	N.D.	5.3	N.D.
Human	N.D.	5.3-67.3	N.D.

*pmoles prednisolone equiv./mg protein

N.D.: less than 0.33 pmoles prednisolone equiv.

Table 5. Summary of prednisolone-lens protein adducts measured by radioimmunoassay.

prednisolone addition products were detected in urea-soluble protein. The rat cataract, which differs in being nuclear in localization, also shows glucocorticoid adducts in water-soluble protein.

There are two possible explanations for why prednisolone tends to be associated with urea-soluble protein. One is that prednisolone may concentrate in the hydrophobic environment of the membrane and react preferentially with proximal membrane proteins. The second possibility is that soluble crystallins, once modified by prednisolone, may undergo structural alterations which lead to precipitation and association with membranes. This second possibility is consistent with the decrease in water-soluble protein which is seen with the in vitro rat cataract (Fig. 23) and with the membrane associated changes observed in the electron micrographs of rabbit cataracts (Figs. 27-29).

The unusual finding from these studies is that visible opacifications appear to be associated with very low levels of protein modification. One mechanism for the role of protein modification in the steroid-induced cataract is that prednisolone may attach to the lens crystallins and induce sufficient conformational changes so as to lead to disulfide cross-linking and the formation of high molecular weight aggregates. Evidence for this mechanism has been presented for both the hyperglycemic and the cyanate induced cataract (16, 60). Alternatively, because the level of modification is so low, it is possible that the modification and inactivation of an enzyme or membrane ionophore is the event which initiates lens opacification. ATP metabolism in the lens for example, has been shown to be affected by the administration of cortisol *in vitro* (81, 82).

D. Studies of the effect of prednisolone modification on lens crystallins in vitro.

In order to gain insight into the role of glucocorticoid modification in cataractogenesis, the effect of incubating prednisolone with lens proteins in vitro was studied. The following studies demonstrate that the modification of lens crystallins by prednisolone imparts on the proteins an increased susceptibility to sulphhydryl oxidation, leading to the development of high-molecular weight aggregates which scatter light.

Methods

Preparation of Lens Protein. Normal human lenses were decapsulated and homogenized on ice in a glass tissue homogenizer containing nitrogen saturated phosphate buffered saline (PBS, 1.5 ml per lens). The mixture was centrifuged at 20,000×g for 30 min. at 4°, and the supernatant solution (water-soluble fraction) recovered. After dialysis (MW cutoff 3500) against PBS, the protein solution was filtered through a sterile Millipore membrane (0.45 µm cutoff).

Acetylated lens protein was prepared by incubating a solution of lens protein (10 mg/ml) with 15 mM acetylsalicylic acid for 18 hr at 37°. Unreacted acetylsalicylic acid was removed by dialysis against PBS. The degree of acetylation was estimated by a parallel incubation which contained, [¹⁴C]acetylsalicylic acid (26.2 mCi/mmol, Amersham). A specific activity of 10 mole/mg lens protein was obtained.

Urea-soluble protein was obtained from a human steroid-induced cataract as described on p. 54. This lens corresponds to lens "A" in Table 4.

Lens Protein Incubations. A 20 mM solution of prednisolone was prepared in methanol and 10 μ l added to 1 ml of sterile lens protein solution (10 mg/ml). The final concentration of prednisolone was 200 μ M and methanol was 1%. Control incubations contained 1% methanol as well. Radioactive incubations contained 200 μ M prednisolone supplemented with 2 μ Ci/ml of [2,4,5,7- 3 H]prednisolone (61 Ci/mmol). All incubations were in sealed tubes, under nitrogen, in the dark, and at 37°. Alternatively, some incubations were performed in sterilized, polystyrene cuvettes (Fisher Scientific, Pittsburgh, PA). The development of opalescence in the incubated solutions was measured by the optical density at 550 nm (16). At intervals, sterile aliquots were removed and the [3 H]prednisolone incorporation measured by precipitation with 10 trichloroacetic acid (83).

Gel Filtration Chromatography. The formation of high-molecular weight aggregates in the lens protein incubations was analyzed by chromatography over a column (1.5 cm x 48 cm) of BioGel A-5m (BioRad Laboratories) equilibrated with 50 mM potassium phosphate buffer (pH 7.4) containing 0.02% NaN₃. The column was calibrated with the following molecular weight standards: blue dextran, thyroglobulin (bovine), gamma globulin (bovine), ovalbumin (chicken), myoglobin (equine), vitamin B₁₂, and [3 H]prednisolone. Samples (5 mg) were chromatographed at room temperature at a flow rate of 3.4 ml/hr. Lens proteins incubated with [3 H]prednisolone were dialyzed against PBS prior to chromatography. Fractions (0.85 ml) were collected and analyzed for protein by the method of Bradford (71). Aliquots (100 μ l) also were counted by liquid scintillation in order to detect tritium incorporation.

Lens proteins were reduced by treatment with 50 mM dithiothreitol

(DTT, Sigma) for 1 hr. For gel filtration analysis of these samples, the resin was equilibrated with a buffer containing 50 mM potassium phosphate (pH 7.4), 0.02% NaN₃, and 30 mM DTT.

Urea-soluble protein was analyzed by the same chromatographic system with the exception that the column buffer contained in addition, 10% ethanol. The presence of prednisolone-protein adducts was analyzed by radioimmunoassay after enzymatic hydrolysis with proteinase K as described on p. 68. Five adjacent fractions were pooled for these determinations.

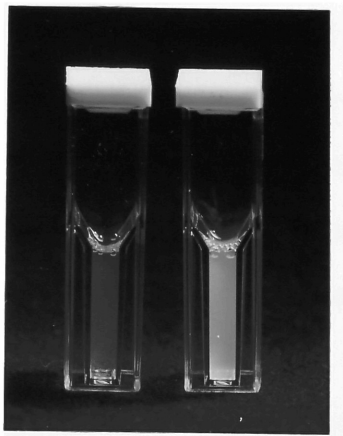
Peptide Mapping. Tryptic peptides were prepared from high-molecular weight complexes isolated by gel-filtration chromatography of lens proteins incubated with [³H]prednisolone. Fractions 29-32 were pooled, yielding 0.51 mg of protein with a specific activity of 2.8×10^5 cpm/mg. The proteins were then reduced, aminoethylated, hydrolyzed with trypsin, and analyzed by reverse-phase HPLC as described on p. 17.

Results

The incubation of prednisolone with human water-soluble lens proteins leads to the gradual development of opalescence of the solutions. In initial experiments, the rate of opalescence was found to vary with both prednisolone and lens protein concentration. Figure 33 shows two representative solutions photographed after 50 days of incubation. Lens protein incubated with 200 μ M prednisolone (right) display marked light scattering, while a control solution containing lens protein alone (left) shows only a modest opalescence.

Figure 34 shows the time-course for this phenomenon, measured by the absorbance at 550 nm.. Incubation with prednisolone (200 μ M) leads to a gradual increase in optical density which becomes significantly more rapid

Figure 33. The appearance of two lens protein solutions after 50 days of incubation at 37⁰. The solution on the right contained 200 μ M prednisolone.



after 30 days. A control solution shows only a moderate, linear increase in optical density. A prednisolone incubation supplemented with 2 μ Ci of [3 H]prednisolone demonstrates that covalent prednisolone-lens protein adducts form at a linear rate (Fig. 34B). Presumably, after 30 days of prednisolone incorporation, aggregates which are large enough to scatter light begin to form and result in a more rapid increase in optical density.

The interpretation that the nonenzymatic addition of prednisolone to lens proteins is involved in the development of light scattering was confirmed by pretreating proteins with acetylsalicylic acid. This reagent, under mild conditions, acetylates the free amino groups of proteins, particularly the ϵ -amino groups of lysine residues. As shown in Fig. 34, lens proteins in which the primary amino groups have been chemically blocked fail to incorporate prednisolone and do not become opalescent.

Calculations from the prednisolone incorporation curve show that at 35 days, 650 pmoles of prednisolone is incorporated per mg of lens protein. Assuming a crystallins subunit molecular weight of 20,000 daltons, this calculates to be an average molar modification of 1 prednisolone molecule per 77 crystallins subunits. Although this is 10 to 50 times the amount observed in either the experimental or the human steroid cataract, the level of modification is still extremely low. This would suggest that the conformation of lens proteins must be extremely sensitive to the presence of the prednisolone ligand.

Several studies have emphasized the role of sulphydryl oxidation in the aggregation of lens proteins in vitro and in vivo (16, 70, 84-87). We examined the effect of adding the reducing agents dithiotreitol (DTT) or

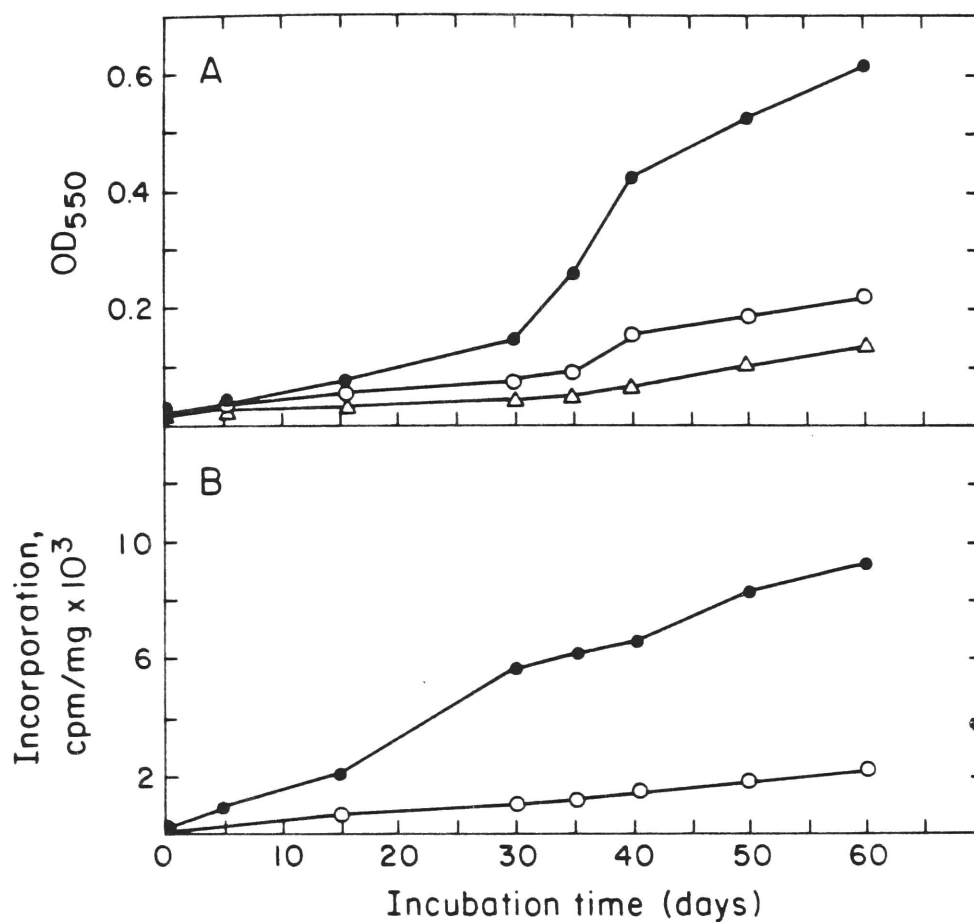


Figure 34. The time course for the development of opalescence (A) and prednisolone incorporation (B). (●): lens proteins incubated with prednisolone, (○): acetylated lens proteins incubated with prednisolone, (Δ): control lens proteins incubated alone.

glutathione (GSH) on the light scattering elements present in the lens protein incubations. As shown in Figure 35, the addition of DTT was found to rapidly reverse much of the opalescence which had developed during incubation. Glutathione had only a minimal effect. This is probably due to the inability of the glutathione tripeptide to penetrate extensively aggregated proteins and reduce the disulfide crosslinks. A similar discrepancy in the reducing action of GSH and DTT has been observed for glucose induced lens protein aggregation in vitro and in vivo (16, 87).

The lens protein aggregates were further investigated by gel filtration chromatography on Bio-gel A-5. Figure 36 shows the elution profile of water-soluble lens proteins after 1 and 50 days of incubation. The day 1 pattern shows 5 major protein peaks, which correspond to the major crystallins proteins present in the human lens (γ , β_1 , β_h , α , α HMW, (88)). It is apparent that after 50 days of incubation, there is an increase in the amount of high molecular weight proteins present. In comparison to the control incubation, the prednisolone incubated solution shows a greater than two-fold increase in the amount of high-molecular weight proteins present. The aggregates elute in the column void volume, indicating that they are composed of molecules having a globular molecular weight of at least 5×10^6 daltons. These aggregates are near if not already at the size estimated by Benedek to be sufficient to scatter light in the lens (50×10^6 daltons, (89)).

Figure 36 also shows that there is a significant decrease in the amount of β_h and β_1 crystallins present in the prednisolone incubation. Therefore, the high-molecular weight species appear to be complexes of β_h , β_1 , and presumably some α crystallins.

Figure 37 shows the gel filtration pattern of lens protein incubations

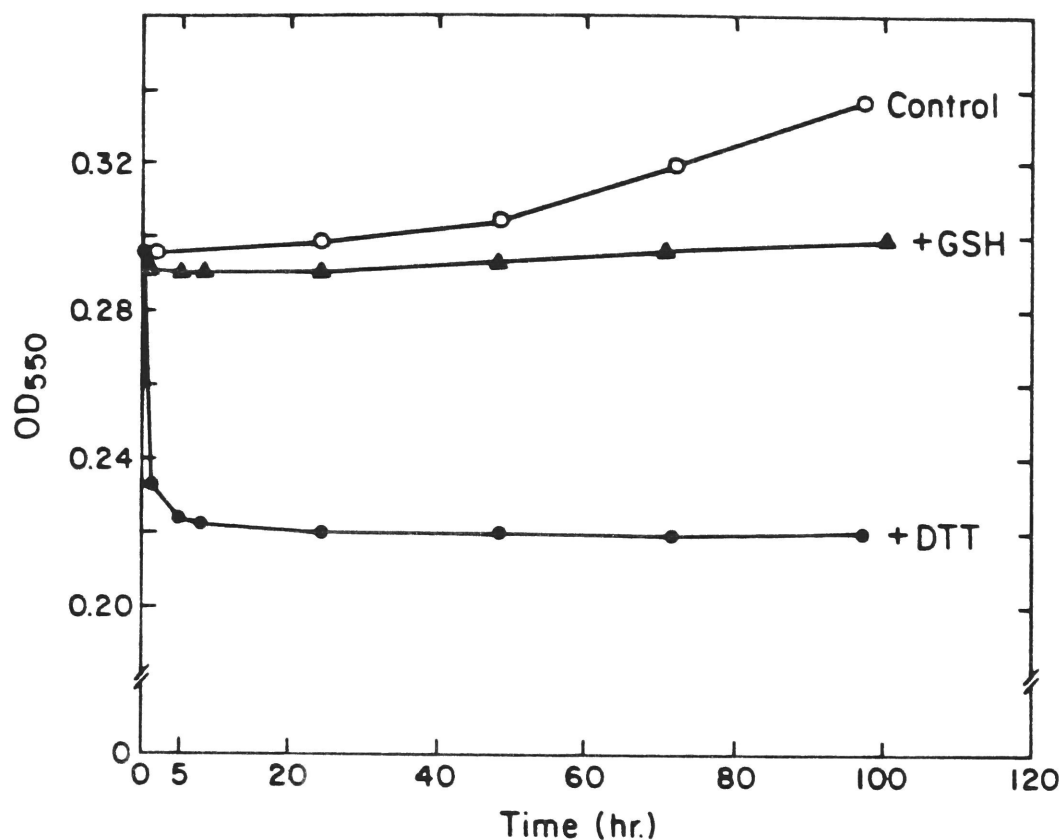


Figure 35. The effect of the reducing agents GSH and DTT on the prednisolone-induced opalescence. The final concentration of each reducing agent was 1 mM.

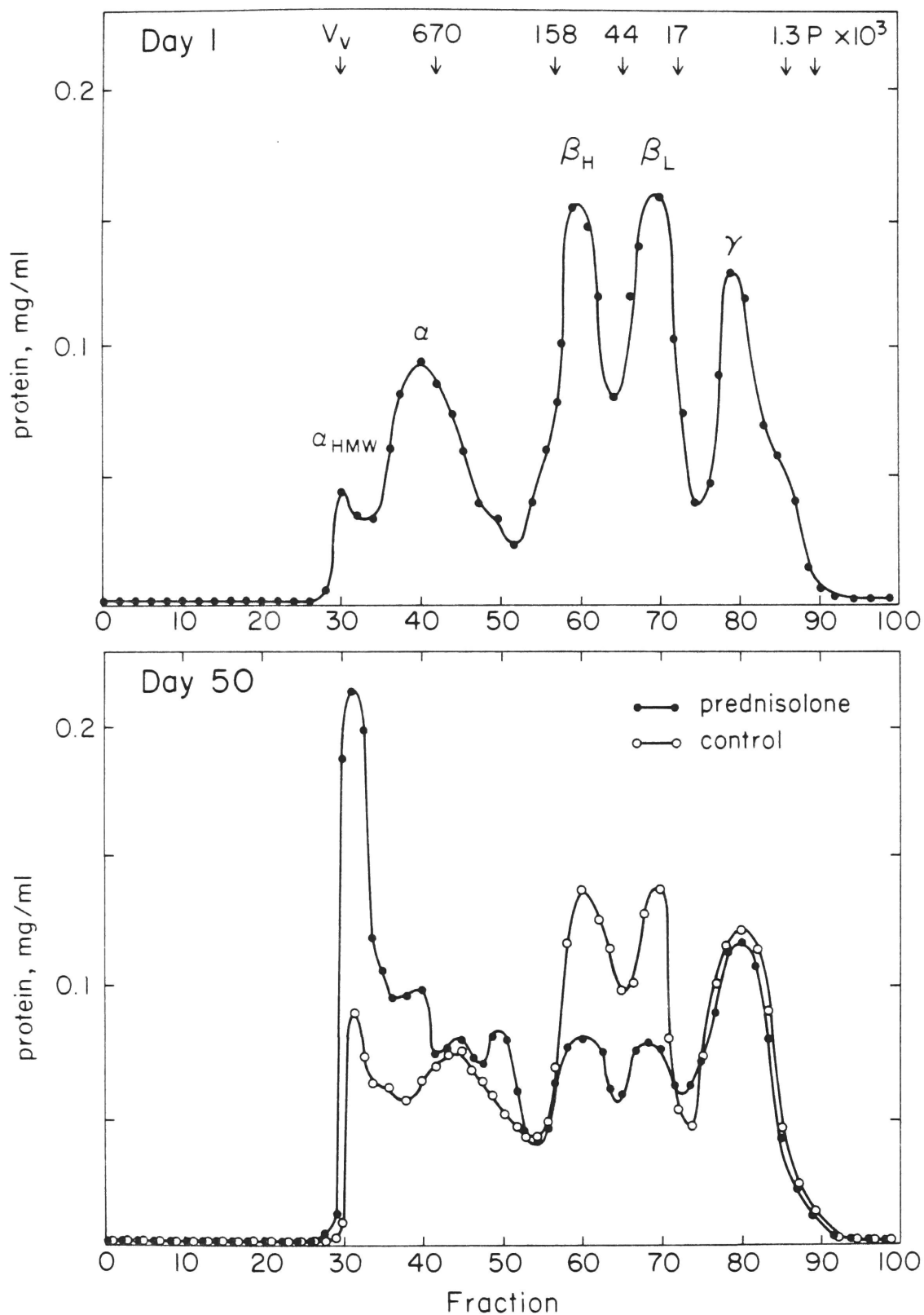


Figure 36. Gel filtration chromatography of water-soluble lens proteins. Top: after 1 day of incubation. Bottom: after 50 days. The numbers denote M_r standards, V_V = void volume, P = prednisolone.

which had been supplemented with [^3H]prednisolone. The majority (55%) of the incorporated radioactivity is associated with the rapidly eluting high-molecular weight complexes. The addition of DTT (Fig. 37, bottom) decreases the amount of high molecular weight protein present by about 50%. With DTT treatment, there is an increase in the amount of protein eluting in the region of α , β_h , and β_l crystallins and there is a corresponding redistribution of protein incorporated [^3H]prednisolone.

Figure 38 shows the reverse-phase HPLC elution of tryptic peptides prepared from the high-molecular weight aggregates isolated by Biogel-A5 chromatography. The majority of the incorporated prednisolone is present in 4-6 peptides, with small amounts present in other peptide peaks. It would appear that although the high-molecular weight complexes are composed of several crystallins types, there is only a small number of lysines within these proteins which can react with prednisolone.

Having determined that the majority of prednisolone which is incorporated in vitro is present in high-molecular weight complexes, we decided to analyze protein from a human steroid-induced cataract which previously was shown to contain prednisolone-protein adducts. Figure 39 shows the gel-filtration profile of urea-soluble protein derived from lens A (Table 3) chromatographed on Biogel A-5 in the presence of 10% ethanol. Fractions were pooled and analyzed for prednisolone adducts by radioimmunoassay. Almost all of the protein bound prednisolone is associated with high-molecular weight proteins which elute early in the chromatogram. Preincubation of these proteins with dithiothreitol decreases the amount of this high molecular weight material and increases the amount of protein present in the α and β regions of the chromatogram. In addition, about half of the prednisolone protein adducts redistribute

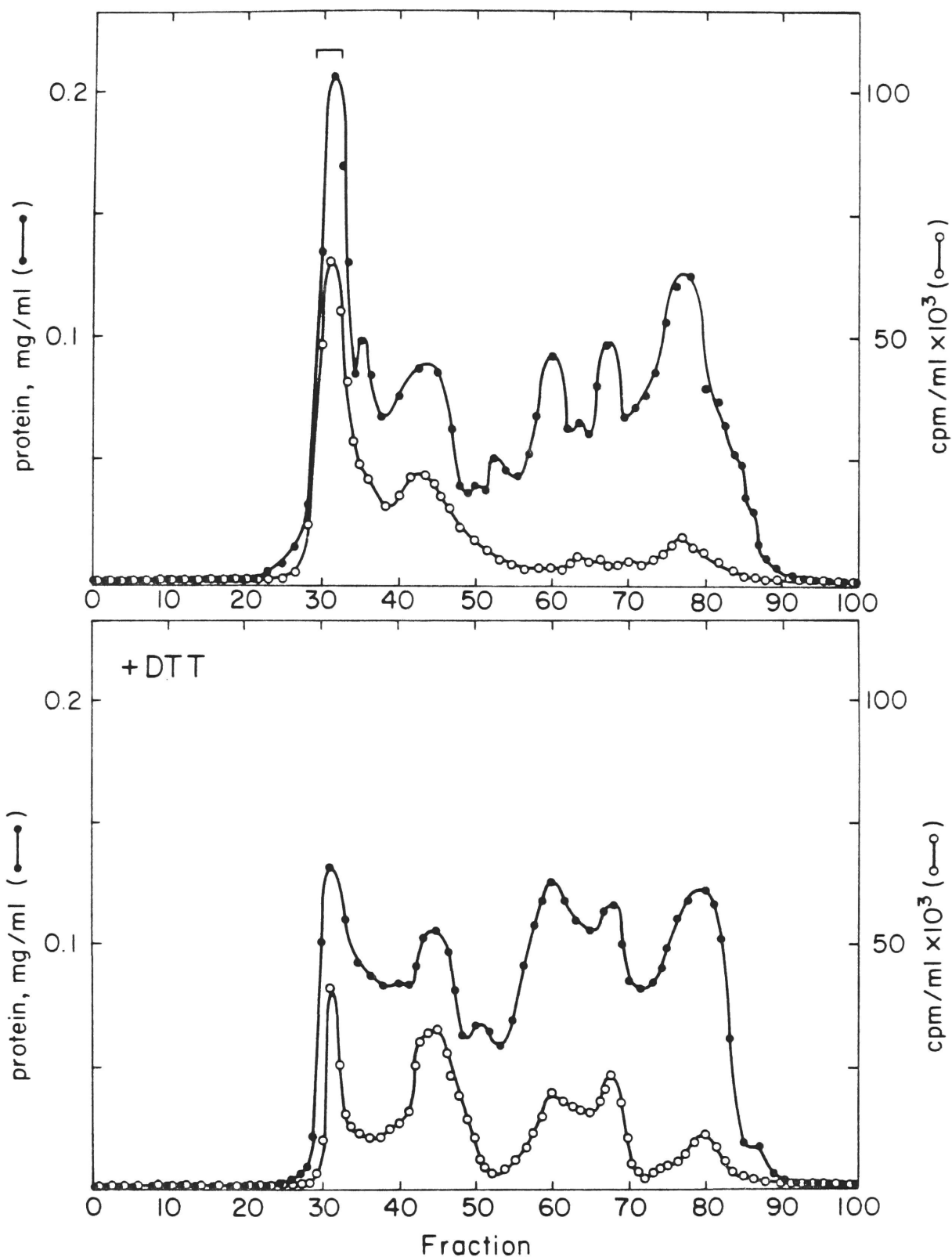


Figure 37. Gel filtration of lens proteins incubated with [3 H]prednisolone. Top: lens proteins at Day 50; the horizontal bar shows fractions pooled for peptide mapping. Bottom: Day 50 proteins reduced with DTT.

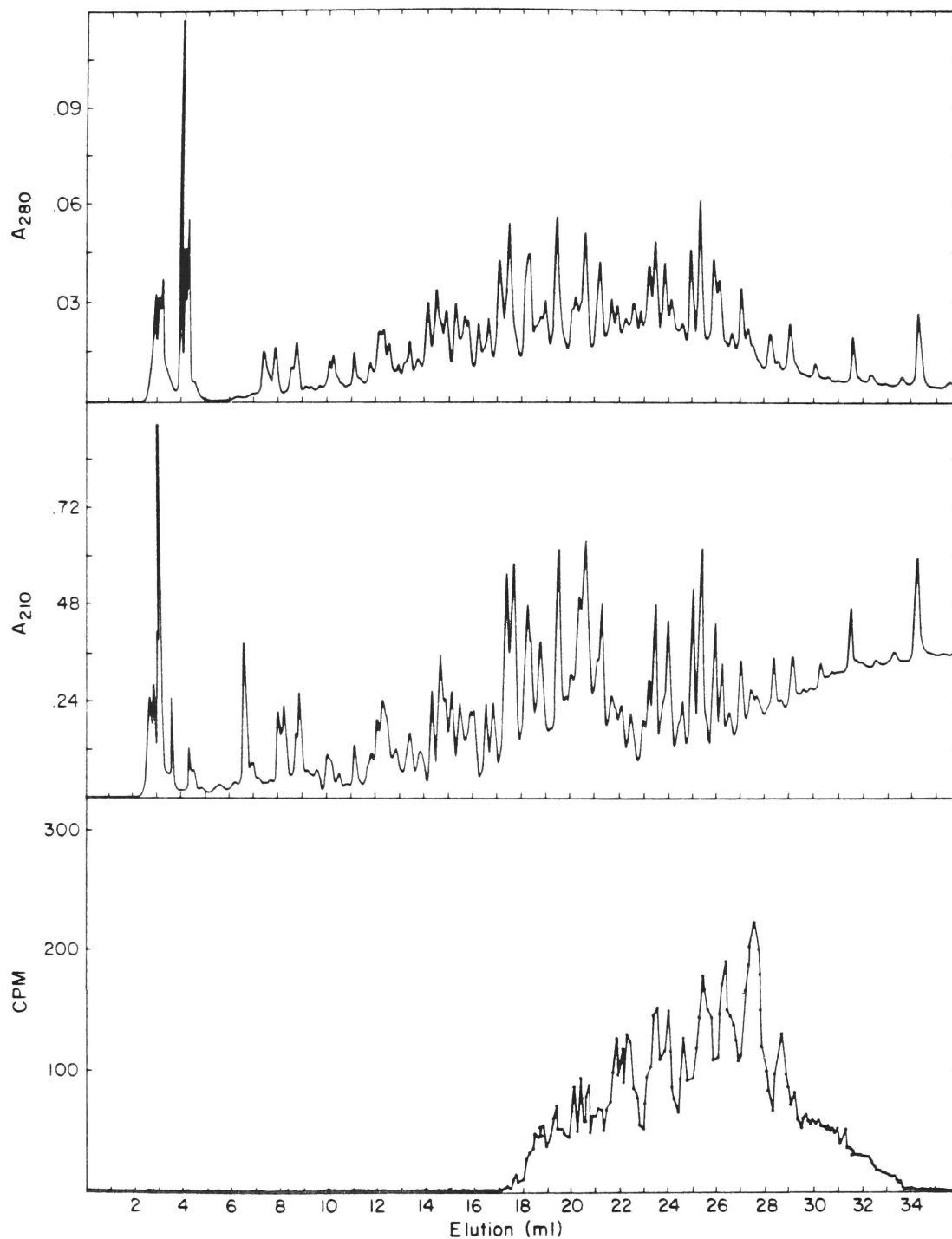


Figure 38. Reverse-phase HPLC tryptic map of the high-molecular weight complexes isolated from lens proteins incubated with [3 H]prednisolone.

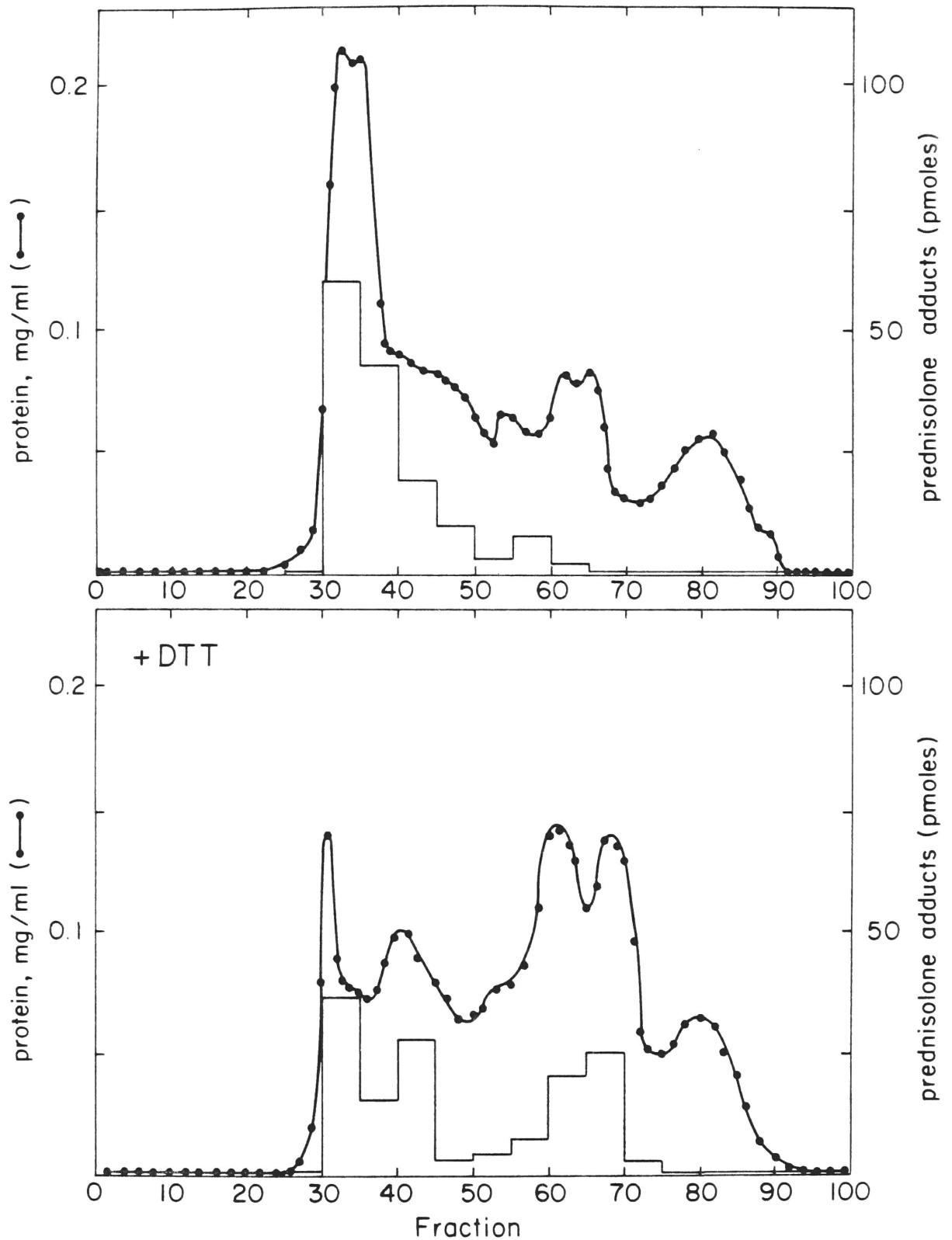


Figure 39. Gel filtration analysis of the urea-soluble protein obtained from a human steroid-induced cataract. Bottom: after reduction with DTT.

to these lower molecular weight regions. These results indicate that in vivo, prednisolone modified lens proteins are also present as disulphide-linked complexes which are reducible with DTT.

E. Conclusion.

The experiments which have been described provide a biochemical basis for how glucocorticoids can react with lens crystallins to cause cataract formation (Figure 40). Prednisolone enters the lens fiber cells and reacts with the amino groups of the lens crystallins. This presumably induces a conformational change and the unmasking of protein sulphhydryl groups. Disulphide bond exchange then occurs and protein aggregates form, leading eventually to complexes which refract light. These aggregates would readily become membrane associated and soluble only in urea.

In the native state, the crystallins proteins exist as hydrophobically associated polymers of molecular weights between 200,000 and 1,000,000 daltons (88). Disulphide exchange therefore, would only need to involve about 50 molecules in order to generate aggregates of the size estimated by Benedik (50×10^6 daltons) to be sufficient to scatter light (89).

The acetylation experiments indicate that lens proteins can be protected in vitro from the damaging effects of glucocorticoid modification. It is surprising that acetylation of lens proteins does not induce aggregation, even when the proteins are modified to a 1000 fold higher specific activity than they are with prednisolone. Evidently, the presence of acetyl moieties does not alter significantly the conformation of lens crystallins. Prednisolone may also be attaching to specific sites in the crystallins proteins where its presence readily disrupts protein conformation and induces disulphide exchange.

The lens may serve as a useful model for the biochemical effects of glucocorticoid modification. It would be interesting to investigate other toxic manifestations of glucocorticoids and examine whether the

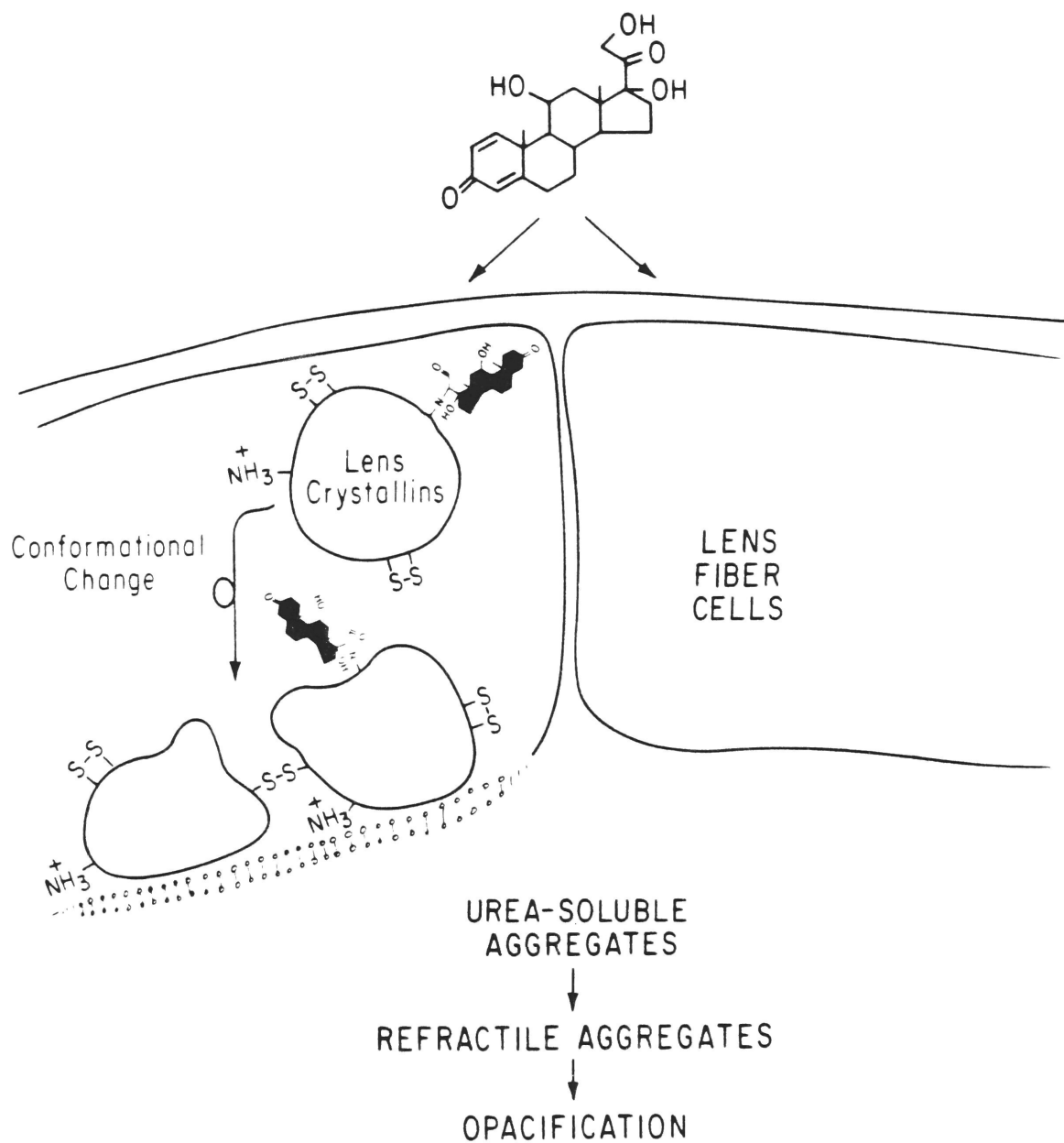


Figure 40. Proposed mechanism for the steroid-cataract.

modification of critical proteins is also involved. In addition, the protective effect of acetylation suggests a novel therapeutic use of aspirin, or other acetylating agents in protecting biologically critical proteins from the damaging effects of protein modification.

4. Nonenzymatic Modification of Proteins by 16α -Hydroxysterone
in Systemic Lupus Erythematosus.

A. Introduction.

Systemic Lupus Erythematosus or SLE, is considered to be the prototype of a multisystem, autoimmune disease (90). Although SLE is often thought to be a rare disease, recent statistics have shown it to be as prevalent as 1 in 8000. The clinical manifestations of this disease are diverse, and include fever, an erythematous rash, polyarthralgia and arthritis, polyserositis, anemia, thrombocytopenia, and renal, neurologic, and cardiac abnormalities (Table 6). In most cases, these symptoms pursue an irregular, but relentless course in which episodes of active disease are interspersed with periods of complete or near-complete remission. The patients who eventually succumb usually do so because of vascular lesions affecting the kidneys, central nervous system, or other vital organs; many die of the complications of secondary infections (92).

Although the etiology of SLE is not known, much of the pathology can be attributed to immune complex deposition resulting from the production of autoantibodies, or antibodies to self-antigens. Antibodies have been identified against an array of nuclear and cytoplasmic components which are neither organ, nor species specific. In particular, the presence of high titres of anti-DNA antibodies can be diagnostic of SLE. Other immunological abnormalities have been found to occur in SLE, including defects in T-cell function, and deficiencies in certain components of the complement system.

An unusual feature of SLE is its strong predilection for women. Several observations have implicated the role of female hormones in either the onset or the severity of the disease (93). For example, SLE

Clinical manifestations during the course of systemic lupus erythematosus

<i>Manifestation</i>	<i>Cumulative percentage of patients</i>
Arthritis and arthralgias	92
Fever	84
Skin eruptions	72
Lymphadenopathy	59
Renal involvement	53
Anorexia, nausea, vomiting	53
Myalgia	48
Pleuritis	45
Central nervous system abnormalities	26

SOURCE: *After Dubois.*

Laboratory abnormalities in systemic lupus erythematosus

<i>Abnormality</i>	<i>Percent of patients</i>
HEMATOLOGIC	
Anemia (Hb < 11 g/100 ml)	72
Leukopenia (WBC < 4500/mm ³)	61
Thrombocytopenia (platelets < 100,000/mm ³)	15
Positive direct Coombs test	14
Circulating anticoagulants	Rare
IMMUNOLOGIC	
Positive tests for ANA	99
Positive LE cell tests	60-80
Hypocomplementemia	75
Increased γ globulin (> 1.5 g/100 ml)	60-77
Positive tests for rheumatoid factors	20
Biologic false positive tests for syphilis	15

Table 6. Clinical and laboratory abnormalities present in SLE (91).

is most common during the reproductive years and the vast majority of patients who present with the disease during this time are female (almost 13:1, female:male). There is in addition, a high incidence of SLE among males with Klinefelter's syndrome (the XXY genotype). A number of reports have described the adverse effects of estrogens on the course of the disease. Among these are birth control agents (94,95), the menstrual period (96), and the possible adverse effect of pregnancy both during (97) and after parturition (98).

A number of investigations have examined the pattern of sex steroid metabolism in patients with SLE. Although both male and female patients with SLE had normal levels of estrogen (99), Lahita and co-workers analyzed in more detail, estrogen metabolism in these patients. Figure 41 shows the pathway of estrogen metabolism in the human. In initial studies, the injection of 6,7- $[^3\text{H}]$ estradiol was found to lead to increased amounts of urinary 16 α -hydroxyestrone; in preference to metabolites of 2-hydroxylation (35). Subsequent work showed an overall increase in the rate of 16-hydroxylation in both male and female patients with SLE (34); and with the development of a specific radioimmunoassay, an increase in the plasma level of the metabolite 16 α -hydroxyestrone (100, 101).

Having determined that 16 α -hydroxyestrone (16 α OHE) can react non-enzymatically with protein amino groups, we considered two mechanisms by which this protein modification activity might contribute to either the etiology or the pathology of SLE. Pregnancy and phasic estrogen secretion during the menstrual cycle increases the level of 16-hydroxylated metabolites and may exacerbate the disease by increasing the formation of these adducts. If these adducts are antigenic in

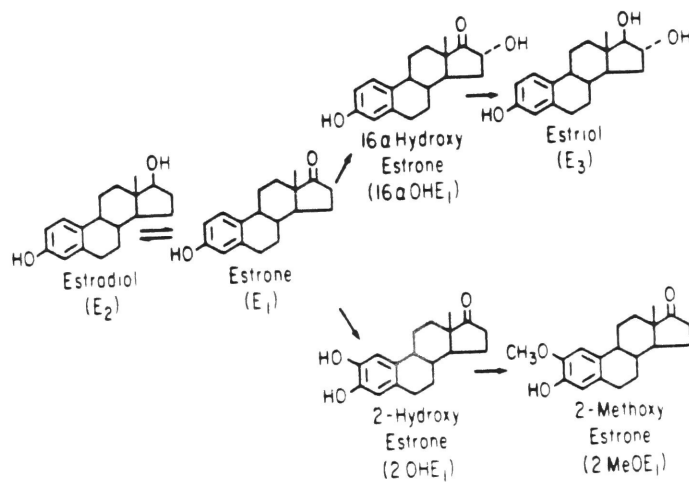


Figure 41. Metabolism of estradiol.

susceptible individuals, they might initiate or contribute to the immune complex manifestations of the disease in much the same way that penicillin-protein adducts produce penicillin allergy in sensitive individuals (102). Alternatively, 16 α OHE might covalently modify key proteins involved in immune regulation. The modification of lymphocyte surface proteins could disrupt cellular interactions and initiate the immune dysfunction which is characteristic of SLE.

The following chapter describes the nonenzymatic reaction of 16 α OHE with a number of proteins, the detection of these adducts in vivo, and studies of the occurrence of anti-estrogen antibodies.

B. The reaction between 16 α -hydroxyestrone and proteins in vivo.

As a first step toward analyzing the occurrence of 16 α OHE-protein adducts in vivo, 16 α -[³H]hydroxyestrone was incubated with whole blood in vitro and the distribution of covalent 16 α OHE-protein adducts determined. Membrane proteins were found to be labeled to the highest specific activity and the interaction of 16 α OHE and membrane proteins was studied using the erythrocyte as a model cell system. A method for the purification and detection of these adducts was developed and used to quantitate the formation of these adducts in vivo.

Methods

[³H]16 α OHE-whole blood incubations. For whole blood incubations, 2 μ Ci of [³H]16 α OHE was added to a 1.5 ml microfuge tube (Brinkmann) and the solvent evaporated under a flow of nitrogen. The residue was washed with 50 μ l ethanol and evaporated again. Whole blood from a normal volunteer was anticoagulated with EDTA and 0.6 ml added to the tube. Six microliters of a 1% solution of sodium merthiolate was included as a bacteriocidal agent. The mixture was incubated at 37 $^{\circ}$ with gentle shaking and 0.1 ml aliquots removed at intervals. Cells were separated from plasma by centrifugation for 30 sec in an Eppendorf microfuge (model 5415). The plasma was dialyzed against 50 mM potassium phosphate buffer pH 7.4/10% ethanol and then chromatographed on G-100 column as described on p. 15. Cells were washed three times with 50 ml phosphate buffered saline and the cell pellet lysed by the addition of 0.15 ml distilled H₂O. The lysate was dried under a heat lamp and oxidized in a Packard Tri-Carb sample oxidizer. Radioactivity was determined by

scintillation counting and recovery from the oxidation corrected with a tritium recovery curve. Background radioactivity was determined from the zero time point and subtracted for both plasma and cells.

For electrophoresis, 0.2 ml of a washed red cell suspension (45% v/v) in PBS with 0.5 mM EDTA was added to a microfuge tube containing 10 μ Ci [3 H]16 α OHE. In order to determine total Schiff base formation, a second mixture contained in addition, 2.4 μ mol of NaCNBH₃. At the end of a 24 hr incubation, the tubes were centrifuged, the supernatant removed, and the cells lysed by the addition of 12 ml of ice-cold 5 mM sodium phosphate buffer, pH 7.4 containing 1 mM phenylmethylsulfonyl fluoride and 0.5 mM EDTA. Red cell membranes were prepared following the method of Dodge et al. (103). Lysates were centrifuged at 29000 x g for 30 min at 4 $^{\circ}$ and the supernatant reserved for assessing [3 H]16 α OHE adduct formation with cytosolic protein. The membrane pellet was washed in buffer containing 10 mM sodium phosphate, pH 7.4 and 0.5 mM EDTA and centrifuged at 29000 x g for 30 min. This washing procedure was repeated three more times. The final membrane pellet was then dissolved by the addition of 50 μ l of 10 mM Tris-HCl buffer, pH 6.8 containing 4 SDS and 10 mM 2-mercaptoethanol. The solubilized membranes were dialyzed overnight against a buffer containing 10 mM Tris/HCl, pH 6.8, 10 mM 2-mercaptoethanol and 0.1% SDS. The membrane preparation was then subjected to electrophoresis or acid precipitation.

Polyacrylamide gel electrophoresis was performed on a 7% slab gel according to the method of Laemmli (72). Hemoglobin and a protein standard mixture were run in adjacent lanes. Gels were stained with Coomassie Blue and then fluorographed by the method of Laskey and Mills (73).

Acid precipitation of red cell lysates, membranes and cytosol was modified from the method of Kennel (83). Briefly, protein was precipitated with a final trichloroacetic acid concentration of 5% and incubated at 0° for 30 min. The precipitate was collected onto Millipore filters (0.45 μ m), washed successively with 10% trichloroacetic acid, and 70% ethanol, dried, and then measured for radioactivity.

Synthesis of 16 α -hydroxyestrone conjugates for antibody production.

Fifty microlitres of a 10 mg/ml solution of 16 α OHE in ethanol was added to 0.45 ml of a 20 mg/ml solution of HSA in 50 mM potassium phosphate buffer, pH 7.4. A second mixture was prepared which contained, in addition, 400,000 cpm of [³H]16 α OHE. Ten microlitres of a 1 M solution of sodium cyanoborohydride (NaCNBH₃) was then added and the solutions incubated for 6 days at 37°. For the synthesis of the 16 α OHE-albumin Heyns rearrangement product, the NaCNBH₃ was omitted.

At the end of the incubation, the solutions were dialyzed overnight against 1 litre of 50 mM potassium phosphate buffer, pH 7.4 containing 10% ethanol (v/v). The dialyzed material was then subjected to gel filtration over a column of Sephadex G-100 (1 x 29 cm), equilibrated with the same buffer. The flow rate was 3 ml/hr. The protein eluate was pooled and the total protein determined by the method of Bradford (71) utilizing bovine serum albumin as a standard and dye reagent supplied by Biorad Laboratories. The molar ratio of 16 α OHE to HSA was calculated from the second incubation by measuring the quantity of [³H]16 α OHE covalently bound to HSA.

16 α -hydroxyestrone-lysine (16 α OHE-lysine) was prepared as described on p. 35. 16 α OHE-albumin peptides were generated by incubating 60 μ l of NaCNBH₃ reduced 16 α OHE-albumin (1.2 mg/ml in 0.3 M potassium phosphate

buffer, pH 9.0) with 10 U of trypsin immobilized on agarose (Sigma) at 37° for 12 hr. Complete enzymatic hydrolysis was assessed by HPLC gel filtration utilizing an I-125 column (Waters Associates, Milford, MA) and monitoring at 280 nm.

Rabbit immunization. After lyophilization, the cyanoborohydride reduced 16 α OHE-HSA product was dissolved in sterile saline and the concentration adjusted to 2 mg/ml. One ml was emulsified with 1 ml complete Freund's adjuvant and injected into 2 New Zealand White rabbits. The immunization protocol, and antisera assay procedure followed the methods described on pp. 68-69.

Erythrocyte membrane preparation. Whole blood was obtained by venipuncture and was anticoagulated with EDTA. For the preparation of red cell membranes, 6-7 ml of blood was centrifuged for 10 min at 500 g and the plasma removed and stored at -80° for the later determination of free 16 α OHE. The buffy coat was discarded and the remaining red cells were washed three times with a ten-fold excess of phosphate buffered saline (PBS). For each 16 α OHE-lysine determination, 2 ml of packed red cells were lysed by the addition of 12 ml of 5 mM sodium phosphate buffer pH 7.4, which contained 1 mM phenylmethylsulfonylfluoride and 0.5 mM EDTA. This mixture was frozen and thawed once, and membrane proteins prepared using procedures described previously (103).

16 α OHE-lysine purification. A flow chart for the purification and quantitation of 16 α OHE-lysine is shown in Fig. 47. Crude membranes were isolated from cell lysates by centrifugation at 29,000 g for 30 min. After repeated washings, the pellet was suspended in 2 ml of 50 mM sodium phosphate buffer, pH 7.4. A 4 M solution of NaBH₃ was prepared in 1 mM NaOH and 250 μ l introduced into each pellet suspension. The

mixture was incubated for 10 min at room temperature followed by 50 min on ice. The reaction was quenched and the lipid soluble material removed by sequential extraction with 18 ml of acetone:ethanol (1:1, v/v), followed by 10 ml of acetone:ethanol and 2 ml of ethyl ether. The insoluble material was then collected by centrifugation (1000g x 20 min) and hydrolyzed with 5 ml of 6 N HCl (110° for 8 hr) in order to generate free 16 α OHE-lysine. The hydrolysate was evaporated in a Speed-Vac concentration apparatus (Savant Instruments). The pellet was redissolved in 0.5 ml distilled water, and the solution evaporated again.

Phenyl-Sepharose, SM-2, and HPLC Chromatography. The hydrolysate was dissolved in 2 ml of a buffer containing 5mM sodium phosphate, pH 7.4 and 4 M NaCl. The pH was adjusted by the addition of 35 μ l of 10 M NaOH and the solution extracted with 2 ml of ethyl ether. The layers were mixed on a rocking platform for 10 min and the ether layer cleared by centrifugation for 3 min at 500g. The ether was removed by aspiration and the extraction repeated two more times. The aqueous layer was then heated to 45° for 30 min to remove residual ether. The solution was layered onto a 1.1 x 2 cm column of phenyl-Sepharose (Pharmacia) equilibrated with a buffer containing 4 M NaCl and 5 mM sodium phosphate, pH 7.4 and flowing at a rate of 6 ml/hr. Elution was monitored at 280 nm with a Uvicord II (LKB Instruments). Unmodified amino acids, which were detected with a Beckman 119C amino acid analyzer (104), were found to elute in the first 10 ml of the starting buffer. The fraction containing 16 α OHE-lysine was eluted with 8 ml of 1 mM NaPO₄ buffer, pH 7.4. Subsequent elution with methanol/water (1:1, v/v) removed additional UV absorbing material and radioactivity from the

column. This latter material did not co-elute with [^3H]16 α OHE-lysine when analyzed with HPLC.

The 1 mM sodium phosphate buffer wash was evaporated in a Speed-Vac apparatus and the volume adjusted to 2 ml with distilled water. This material was applied to a column (0.8 x 4 cm) of SM-2 resin (Bio-Rad Laboratories) equilibrated with 1 mM sodium phosphate buffer, pH 7.4. The resin was washed previously with water, methanol, water, and 1 mM sodium phosphate buffer, pH 7.4. The column was eluted with 6 ml of 1 mM sodium phosphate buffer, pH 7.4 at a flow rate of 6 ml/hr. The eluting solvent was then changed to methanol/water (1:1, v/v). This latter fraction contained 16 α OHE-lysine.

A portion (50-200 μl) of the SM-2 eluate was injected into a Hewlett-Packard 1084B liquid chromatograph which contained an RP-8 (Hewlett-Packard) reverse-phase column and a μ Bondapak C₁₈ (Waters Associates) guard column. The mobile phase consisted of a gradient of 10 mM sodium phosphate, pH 7.0/50% methanol which increased linearly to 100% methanol from 0 to 20 min. The flow rate was 1 ml/min and solvent and column temperature was maintained at 40°. For each analysis, twenty 1 ml fractions were collected in 1.5 ml microfuge tubes (Sarstedt). The fractions were then evaporated in a Speed-vac and subjected to radio-immunoassay as described below. The entire HPLC elution therefore, was analyzed for each protein hydrolysate.

For the detection of radioactive flow, the column eluate was mixed with scintillation fluor (Flo-Scint II, Radiomatic Instruments) which flowed at a rate of 3 ml/min and analyzed with a Flo-One detector (Radiomatic Instruments, Inc.) Alternatively, 1 ml fractions were collected and counted in a scintillation spectrometer after the addition of 6 ml

of Hydrofluor (National Diagnostics).

Lymphocyte preparation. Forty ml of whole blood was withdrawn from each individual and anticoagulated with EDTA. The blood was then diluted 1:1 with PBS and divided into two portions. Each portion was centrifuged through a 15 ml layer of the density medium Ficoll-Paque (Pharmacia) following the procedures recommended by the manufacturer. The lymphocyte layer was withdrawn by aspiration and washed three times with PBS. The cell pellet was then lysed and membranes prepared using the same procedure described for red cells.

16 α OHE-lysine was purified from lymphocyte membranes using the method described above with the modifications that reduction was with 0.5 mmole of NaBH₄ and acid hydrolysis was with 2.5 ml of 6 N HCl.

Basement membrane preparation. Kidney tissue was obtained at autopsy from two women with SLE and two women without a history of autoimmune or kidney disease. Each specimen consisted of a coronal section of one-half of one kidney. Glomerular basement membrane was prepared according to the original procedure of Spiro (105) and employing the modifications of Beisswenger et al. (106) for human specimens. Each half-kidney was placed on ice and allowed to partially thaw. The kidney was divided into thin sections and the cortex sliced away from the medulla, which was discarded. Cortical slices were further sectioned into 1-2 mm pieces and forced through a 150 mesh sieve with the bottom of a small beaker. The disrupted tissue was collected in a receiving pan containing ice-cold saline (0.85 NaCl). Once the entire sample was processed, the suspended material was poured through an 80 mesh sieve which served to retain the tubular material but allowed the intact glomeruli to pass through and be collected on a 170 mesh sieve.

After a saline wash, the glomeruli were collected in a 50 ml conical centrifuge tube. Examination by phase contrast microscopy showed that of all the collected fragments, greater than 80% consisted of intact glomeruli. The glomeruli were then centrifuged, resuspended in 1 M NaCl at a concentration equivalent to 10 gm of cortex per ml of solution, and subjected to ultrasonic disruption. The insoluble material was then collected by centrifugation and washed 4 times with 50 ml of 1 M NaCl followed by 4 washes with glass distilled H₂O. After the last wash, the basement membrane material was lyophilized and then weighed.

For the preparation of 16 α OHE-lysine the dried material was resuspended in 2 ml of 50 mM sodium phosphate buffer (pH 7.4) and reduced with sodium borohydride at a concentration of 0.5 mmoles of NaBH₄ per mg of basement membrane. After extraction with acetone/ethanol and ethyl ether, the reduced basement membrane was acid hydrolyzed for 10 hours at a concentration of 1 mg per ml of 6 N HCl. Purification of 16 α OHE-lysine then followed the procedure described above and outlined in Figure 47.

Amino acid determination. The amino acid content after hydrolysis was measured by absorbance at 570 nm after reaction with ninhydrin (73). Samples were measured in triplicate and the leucine equivalent calculated by comparison to a leucine standard curve.

16 α OHE-lysine radioimmunoassay. For each purified protein hydrolysate, 20 HPLC fractions (1 ml) were collected. Each fraction was redissolved in 0.5 ml of a buffer containing 100 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.1% gelatin, and 0.01% NaN₃. One hundred microliters of anti-16 α OHE-albumin antiserum (final dilution 1:315,000), which contained 5,000 cpm of [2,4,5,7,16,17-³H]estradiol (142 Ci/mmole), was then

added to each tube. The samples were mixed and incubated at 4° for 12-16 hr. For each assay set, a standard curve was generated by incubating duplicate samples containing 0.001 to 1.380 pmoles of authentic 16 α OHE-lysine. The per cent bound was assayed from supernatants obtained after adding 0.6 ml of a solution of ammonium sulphate (saturated at 0°), vortexing, and centrifuging for 10 min in an Eppendorf microfuge (Model 5414). The amount of 16 α OHE-lysine present was calculated from the peak of activity which was found to co-elute with authentic [³H]16 α OHE-lysine (11 min on the HPLC chromatogram). The absolute content of 16 α OHE-lysine was calculated with an HP-97 calculator and a Hewlett-Packard program which performs a log-logit transformation (107) of the radioimmunoassay standard curve. Background values were obtained from radioimmunoassay of HPLC fractions after the injection of a buffer blank.

The recovery of 16 α OHE-lysine was estimated by adding [³H]16 α OHE-albumin (8×10^4 cpm, 4-5 μ Ci/mg) to a membrane preparation which was then purified concurrently with patient material. Radioactivity analysis of 5 purifications showed that $31.4\% \pm 3.0\%$ of the starting [³H]16 α OHE-lysine adducts were recovered in the SM-2 eluate. The recovery of 16 α OHE-lysine from red cell membranes was estimated in addition, by labelling red cells in vitro with [³H]16 α OHE. Both methods showed similar yields after purification. The recovery of 16 α OHE-lysine from HPLC chromatography was found to be 90% and was determined by radioimmunoassay of eluted fractions after the injection of 0.4 pmol of authentic 16 α OHE-lysine. Replicate assays of three red cell preparations from the same individual yielded an interassay variation of 24%.

Measurement of plasma 16 α OHE. The 16 α OHE content of human plasma

was assayed with modifications of a procedure described previously (100). Briefly, one milliliter plasma samples were extracted two times with 5 ml of freshly opened ether. The combined ether extracts were evaporated at 40° under a gentle stream of N₂ and the residue dissolved in 1 ml of ethanol. This solution was evaporated in a Speed-vac and redissolved in 0.5 ml of the radioimmunoassay buffer described above. A 0.1 ml solution of antisera (final dilution 1:21,000) which contained 5,000 cpm of [³H]16αOHE (50 Ci/mmol) was then added and the mixtures incubated at 4° for 12-16 hr. Antibody bound material was separated from free material by adding 0.6 ml of 100% saturated solution of ammonium sulphate and centrifuging for 10 min in an Eppendorf microfuge. A standard curve was calculated by assaying a duplicate series of eleven standard solutions containing a range of .006 to 1.75 pmole of 16αOHE.

Patient selection. Patients with SLE were studied at the Rockefeller University Clinical Research Center. Written informed consent was obtained and all procedures were approved by the Rockefeller Human Research Committee. The patients fulfilled the criteria of the American Rheumatism Association for the diagnosis of SLE. Normal volunteers were solicited from the Rockefeller University community. Normal subjects and patients with SLE were excluded if they were pregnant or had a history of liver or thyroid disease. Maternal samples were from women who were in the third trimester of pregnancy and within several days of term.

Results

The incubation of 16αOHE with whole blood leads, with time, to its

incorporation into both plasma proteins and cells (Fig. 42). In the case of plasma proteins, the formation of a stable adduct proceeds at a linear rate which reflects the rate-limiting Heyns rearrangement. The incorporation of 16α OHE into red cells initially was assessed by repeatedly washing the cells. After an early rapid uptake of steroid, incorporation was also linear with time and parallel to the rate for the incorporation of 16α OHE into plasma proteins. The initial uptake of 16α OHE into cells presumably is due to the distribution of the hydrophobic steroid into cell membranes, while the linear rate after 1 hr reflects the rate-limiting Heyns rearrangement of 16α OHE with protein amino groups.

Table 7 shows the distribution of acid precipitable radioactivity at 24 hr in the major protein components of the blood. Fractionation of the red cells into cytosolic and membrane components demonstrated that 68% of the precipitable [3 H] 16α OHE was associated with cytosolic protein and 32% with membrane protein. Membrane proteins were found to be labelled to the highest specific activity, over 15x higher than albumin. Evidently, the membrane protein species which are modified by 16α OHE are very reactive toward the Schiff base and rearrangement reactions. The hydrophobic membrane environment probably contributes to this reactivity by increasing the local concentration of the steroid in the vicinity of the membrane proteins.

Figure 43 shows the gel electrophoresis and fluorography profile of plasma which had been incubated with [3 H] 16α OHE. All of the detectable adducts are present on albumin, even when NaCNBH₃ is included in the incubation mixture. Electrophoresis of cytosolic proteins revealed that all the acid-precipitable radioactivity is due to adduct formation

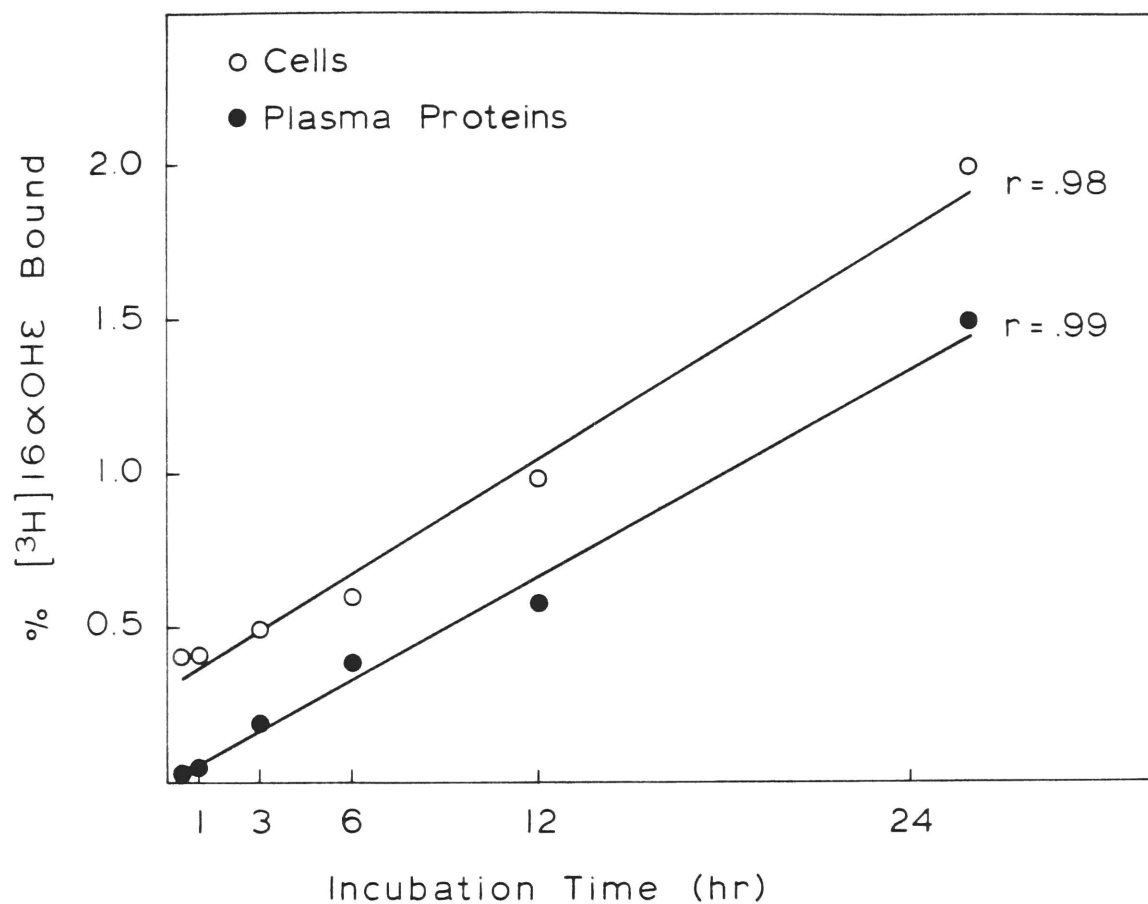


Figure 42. Incorporation of $[^3\text{H}]16\alpha$ -hydroxyestrone into cells and plasma proteins after incubation with whole blood in vitro.

Figure 43. Electrophoresis and fluorography of whole plasma labeled with [^3H]16 α -hydroxyestrone. Left: Coomassie Blue Staining. Right: tritium incorporation.

Plasma Proteins

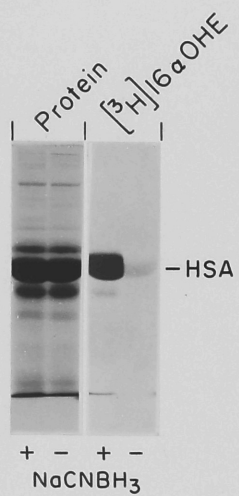
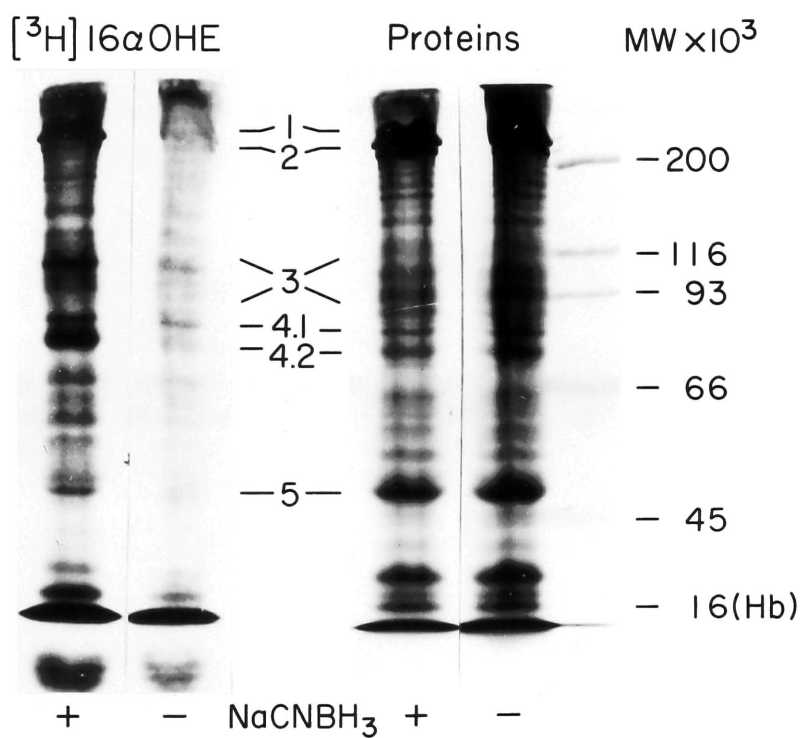


Figure 44. Electrophoresis and fluorography of erythrocyte membrane proteins isolated from erythrocytes incubated with [^3H]16 α -hydroxyestrone for 48 hr. Right: Coomassie blue staining. Left: Tritium incorporation. The numbers in the center refer to the major erythrocyte membrane protein components (108).

Erythrocyte Membrane Proteins



<u>Protein</u>	<u>% Bound</u>	<u>[³H]16αOHE</u>	<u>fmo1 16αOHE/mg protein</u>
Plasma - Albumin	1.5		4.3
Cells - Hemoglobin	0.6		2.7
Membrane Protein	0.3		63.8

Table 7. Distribution of acid-precipitable tritium in the major protein components of blood after incubation with [³H]16 α -hydroxyestrone.

with hemoglobin (data not shown). Figure 44 shows the gel electrophoresis pattern for erythrocyte membrane proteins. Proteins modified by [^3H]16 α OHE in the absence of NaCNBH $_3$ can be assigned by comparing this electrophoresis pattern with previously published studies (108). These include band 1 and 2 (the alpha and beta subunit of spectrin), proteins in the band 3 region, a protein near or identical to band 4.1, and membrane-associated hemoglobin. Several minor protein species are also labeled. Small differences in the electrophoretic pattern of Figure 44 from previously published descriptions of red cell membranes - particularly the poor resolution of some bands - are most likely due to proteolytic artifacts generated in the course of the 24 hr incubation at 37 $^\circ$.

In order to examine which membrane proteins form reversible Schiff bases with 16 α OHE, an aliquot of red cells was incubated with [^3H]16 α OHE in the presence of NaCNBH $_3$. All potential 16 α OHE-protein adducts are trapped at the level of the Schiff base intermediate and the amount of [^3H]16 α OHE incorporation is increased. Additional proteins which are labeled under these conditions include band 4.1 and band 5 (actin).

The modification of a small quantity of red cell membrane proteins by 16 α OHE suggested that these proteins might be readily analyzed for 16 α OHE adduct formation in vivo. Furthermore, the long half-life of the red cell (60 days as opposed to 19 days for albumin) would permit a greater accumulation of adducts with time on erythrocytes than on albumin. The availability of large quantities of red cells from peripheral blood also would facilitate the analysis of 16 α OHE adduct formation in normal and diseased states. For these reasons, our efforts focused on the red cell as a model cell for the study of 16 α OHE adduct

formation in vivo. Since free 16 α OHE is normally present in small amounts; [6 to 15 pg/ml of plasma (100)] only an immunochemical method could provide the required sensitivity for the detection of 16 α OHE modified proteins. As a first step toward this goal, we prepared antisera to albumin modified by the nonenzymatic addition of 16 α OHE.

Figure 45 shows a family of standard curves obtained for four different antigens utilizing [3 H]estradiol as the competitor. The use of tritiated estradiol-17 β as the competitor (available at a 3-fold higher specific activity than [3 H]16 α OHE), produced an increase in assay sensitivity. As expected, the greatest inhibition of binding was with the immunogen: cyanoborohydride reduced 16 α -hydroxyestrone-albumin (16 α OHE:HSA = 2.7:1). The antisera is probably recognizing neighboring amino acid determinants in addition to the 16 α OHE hapten. Estradiol and 16 α OHE showed identical inhibition curves. 16 α OHE-lysine (the cyanoborohydride product) showed a similar binding curve to that of 16 α OHE but with a slight decrease in inhibition at lower concentrations. Scatchard analysis for the binding of 16 α OHE revealed a K_a of $5.75 \times 10^9 \text{ M}^{-1}$ (Fig. 46). The minimum detectable amount of antigen at the 95% confidence limit was calculated to be 3.5 pmoles for 16 α OHE-albumin, 8 pmoles for 16 α OHE, and 20 pmoles for 16 α OHE-lysine.

Table 8 summarizes cross-reactivity studies with several steroids and 16 α OHE derivatives. The complete cross-reactivity of the major estrogens and 16 α OHE-lysine indicates little specificity for structural changes in ring D. The antisera is most specific for the estrogen A ring as all of the nonestrogens exhibited negligible cross-reaction ranging from <.001% to .004% relative to 16 α OHE. The high specificity for the phenolic A-ring is further demonstrated by the poor competition

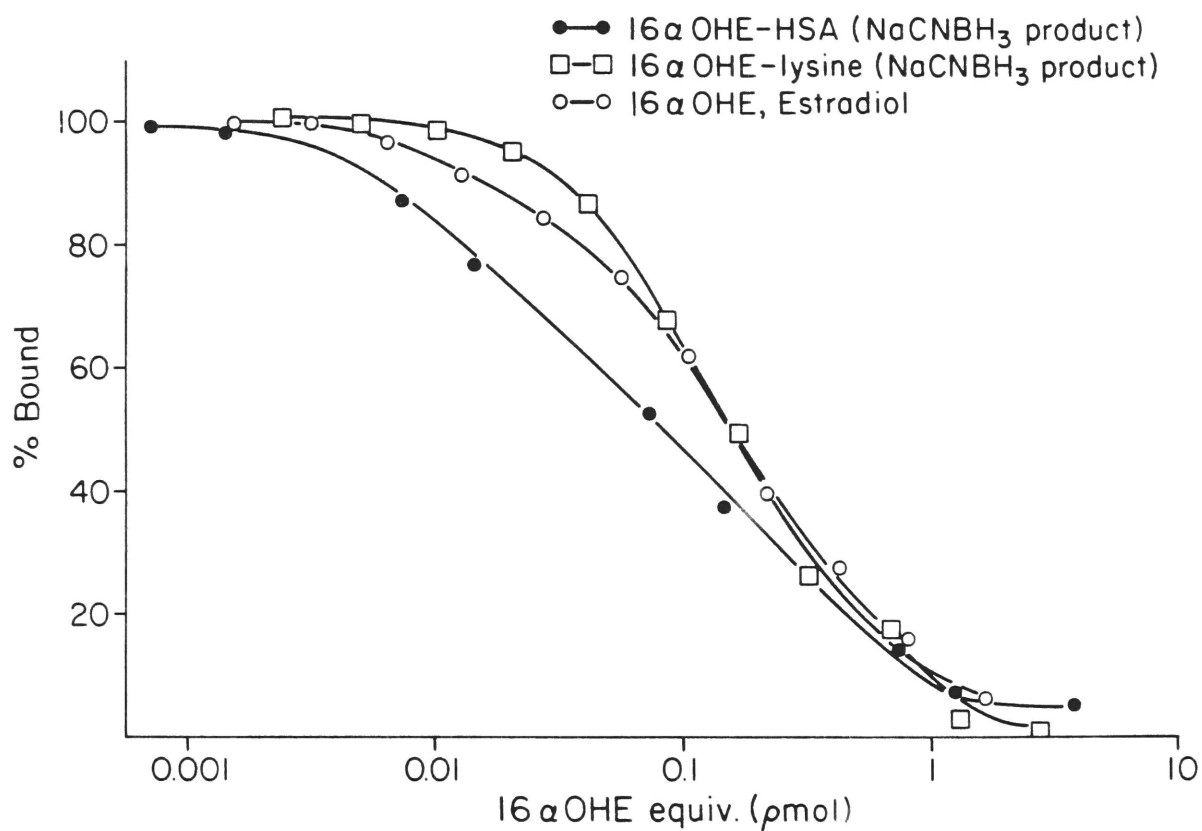


Figure 45. The relative percentage bound versus log dose curves for anti-16 α -hydroxyestrone-albumin antiserum with four antigens utilizing [³H]estradiol as the competitor. Each point represents the mean of duplicate determinations.

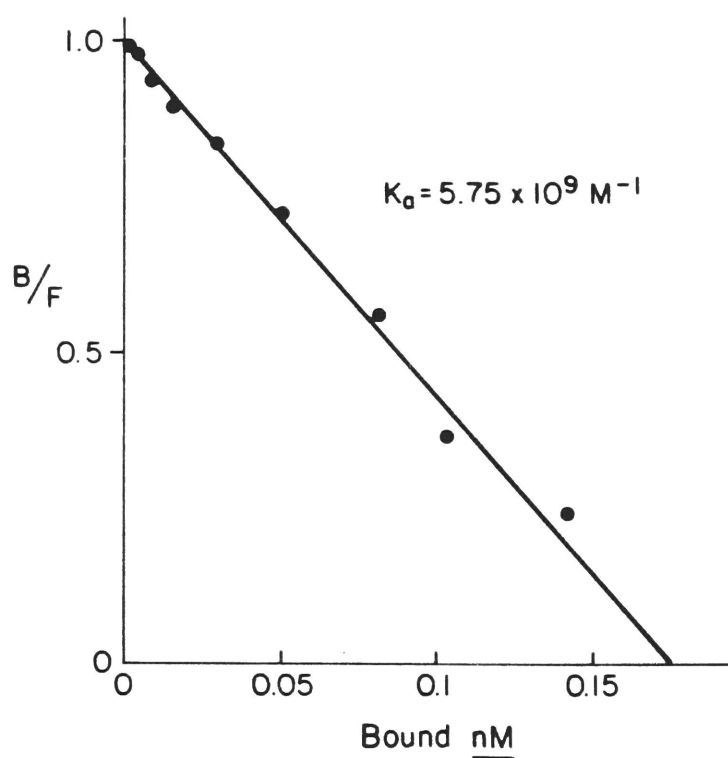


Figure 46. Scatchard plot of anti-16 α -hydroxyestrone-albumin antiserum for the binding of 16 α -hydroxyestrone.

COMPOUND	% CROSS REACTIVITY AT 50% BINDING
16 α -Hydroxysterone (16 α OHE)	100
17 β -Estradiol	100
Estriol	100
Estrone	108
2-Hydroxyestradiol	0.008
16 α OHE-lysine (NaCNBH ₃ product)	100
16 α OHE-peptides (NaCNBH ₃ product)	99
16 α OHE-albumin (NaCNBH ₃ product)	124
16 α OHE-albumin (Heyns product)	52
Dehydroepiandrosterone	0.004
Progesterone	0.003
Etiocholanolone	0.002
Cortisol	< 0.001
Prednisone	< 0.001

Table 8. Percent cross-reaction relative to 16 α -hydroxysterone of anti-16 α -hydroxysterone-albumin antiserum with selected compounds.

of 2-hydroxyestradiol, which differs from estradiol in possessing an additional hydroxyl group at position 2.

Tryptic peptides prepared from NaCNBH_3 reduced $16\alpha\text{OHE}$ -albumin showed the same degree of inhibition as $16\alpha\text{OHE}$ -lysine, but less than $16\alpha\text{OHE}$ -albumin, evidently reflecting the loss of tertiary structure determinants adjacent to the $16\alpha\text{OHE}$ hapten. Interestingly, the $16\alpha\text{OHE}$ -albumin Heyns product, which had 1 mole of $16\alpha\text{OHE}$ per mole of albumin, inhibited binding less than did NaCNBH_3 reduced $16\alpha\text{OHE}$ -albumin. This may reflect the fact that the reactive lysine involved in the Heyns rearrangement lies in an obscured pocket and the $16\alpha\text{OHE}$ adduct which forms with it is not completely accessible to binding by the antibody.

The detection of $16\alpha\text{OHE}$ -lysine adducts formed in vivo necessitated the analysis of a large quantity of protein. Therefore, a method was developed for the purification of $16\alpha\text{OHE}$ -lysine adducts away from free amino acids and non-polar steroids. This scheme is outlined in Figure 47. Erythrocyte membranes are prepared and reacted with an excess of NaBH_4 . Sodium borohydride reduces the 16-keto group of the Heyns product, generating an adduct which is stable to acid hydrolysis and structurally similar to the NaCNBH_3 product. The reduction is then quenched and the membrane lipids extracted in a single step by adding nine volumes of acetone/ethanol (1:1). The membrane protein pellet is then subjected to acid hydrolysis in order to generate free amino acids and their derivatives, including $16\alpha\text{OHE}$ -lysine. Ether extraction after acid hydrolysis removes any additional lipid soluble material which interferes with the succeeding hydrophobic chromatography step. Estrogens, the only known molecules which cross-react significantly with

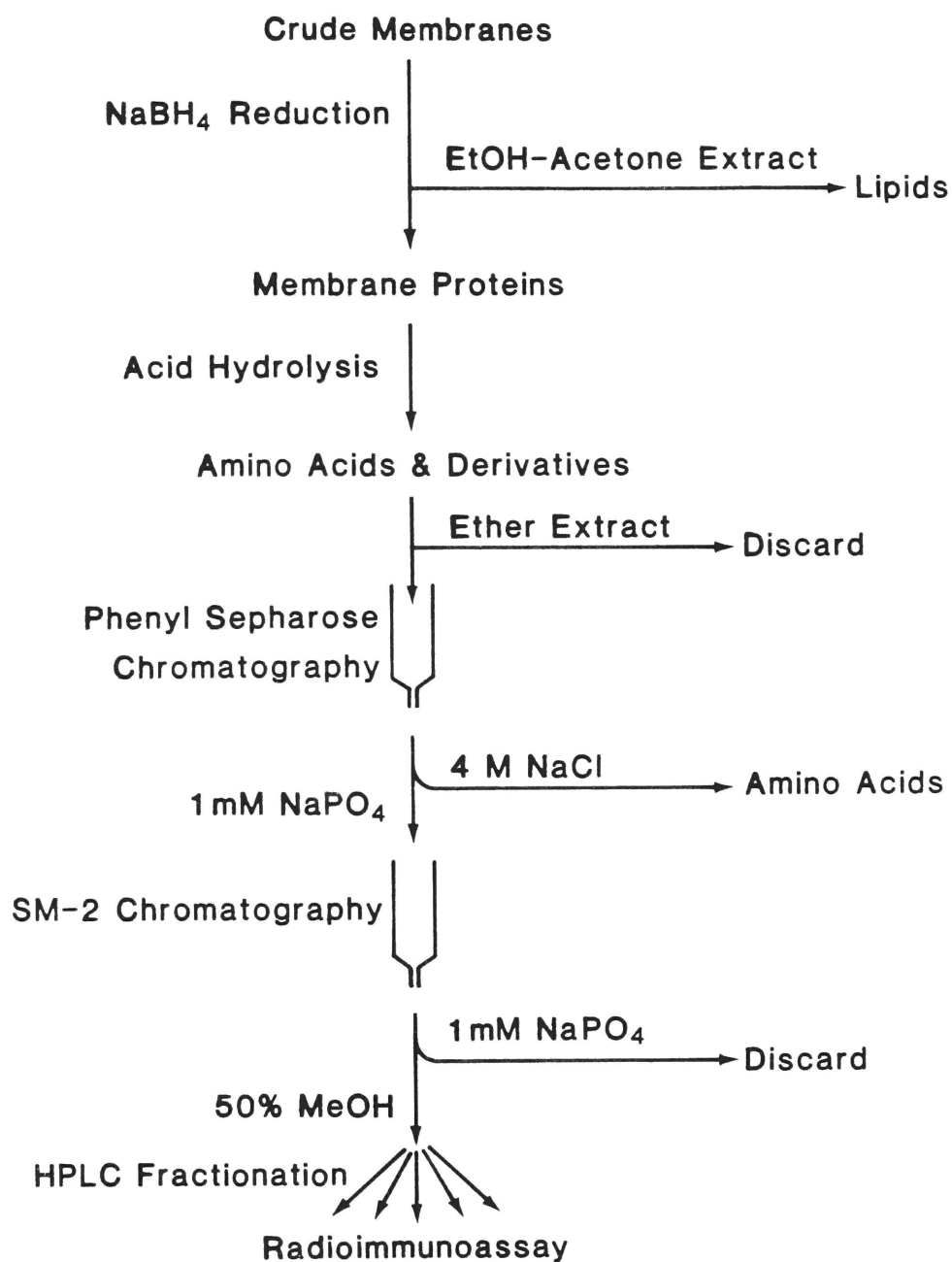


Figure 47. The flow scheme for the purification of 16 α -hydroxyestrone-lysine from membrane proteins.

the antibody, are removed by the two organic extractions.

Utilizing synthetic [^3H]16 α OHE-lysine as a standard, several chromatographic methods were attempted in an effort to purify 16 α OHE-lysine from the hydrolysate. 16 α OHE-lysine absorbed to charcoal (Norit-A), but could not be eluted with either methanol or ethanol containing 2 M NH_4OH or 2 M pyridine. Chromatography in the amino acid system of Moore et al.(104) utilizing a Beckman Type W-2 resin was unsuccessful as 16 α OHE-lysine was eluted only with the 0.2 M NaOH regeneration step. Similarly, cation-exchange chromatography on a sulfonic-acid-polystyrene resin (Bio-Rad AG50W-X8) was hampered by the nonspecific absorption of 16 α OHE-lysine onto the solid support.

Specific elution of 16 α OHE-lysine under mild, aqueous conditions was finally achieved with a chromatography system employing phenyl-Sepharose. The pH of the hydrolysate is neutralized and made 4M in NaCl. At this ionic strength, 16 α OHE-lysine absorbs to phenyl-Sepharose while amino acids are eluted. 16 α OHE-lysine is then removed by lowering the ionic strength. Fig 48 shows the elution profile of a hydrolysate of membrane proteins modified by incubation with [^3H]16 α OHE. Chromatography of a hydrolysate of [^3H]16 α OHE-albumin produces an identical pattern. Tyrosine and other amino acids elute as shown and [^3H]16 α OHE-lysine elutes with 1 mM sodium phosphate buffer. From an analysis of three [^3H]16 α OHE-albumin hydrolysates, it was found that $32 \pm 4\%$ of the starting [^3H]16 α OHE adducts survive acid hydrolysis and are recovered in this fraction. The identity of this [^3H]16 α OHE-lysine product was confirmed by its co-elution under reverse-phase HPLC with authentic [^3H]16 α OHE-lysine. Of the total tritium applied to the phenyl-Sepharose column, an additional 20% can be

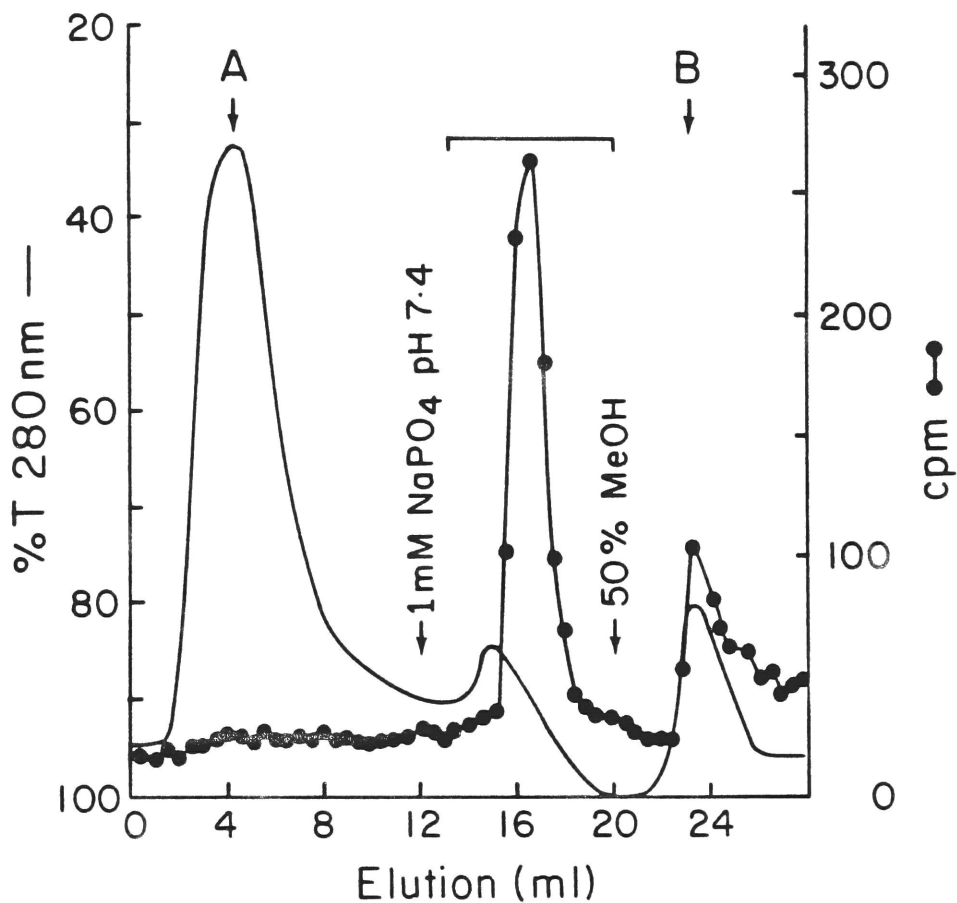


Figure 48. The elution profile of a membrane protein hydrolysate from red cells incubated with [^3H]16 α OHE and chromatographed on phenyl-Sepharose. A and B show the elution positions of tyrosine and 16 α OHE, respectively. The horizontal bar indicates the fractions pooled for SM-2 chromatography.

eluted with 50% methanol. Further washing with 10% SDS removes almost all the remaining radioactivity (38%). Analysis of these later fractions by HPLC showed several radioactive peaks, none of which coeluted with 16 α OHE-lysine.

The 16 α OHE-lysine containing fraction is then rechromatographed over a column of SM-2 resin (Fig. 49). The majority of the material absorbing at 280 nm is removed in the initial 1 mM sodium phosphate buffer wash. 16 α OHE-lysine is eluted with methanol/water (1:1, v/v) and is now present in a volatile solvent with a minimum of solute. The recovery of 16 α OHE-lysine from this resin is 100%, as assessed by chromatography of the [3 H]16 α OHE-albumin hydrolysate.

The purified material was subjected to radioimmunoassay and measurable binding activity was detected in all the red cell extracts tested. Fractionation of the purified hydrolysates by reverse-phase HPLC showed that all the erythrocyte preparations had antibody binding activity which co-eluted with the 16 α OHE-lysine standard. Figure 50 shows representative antibody binding profiles of samples from three individuals. These were generated by injecting 0.1 ml of the SM-2 eluate (equivalent to 0.2 ml of packed cells) and collecting 1 ml column fractions for radioimmunoassay. Figure 50B is the chromatogram of a [3 H]16 α OHE-membrane hydrolysate fractionated in the same manner as in Figure 50A and counted in a liquid scintillation counter. Fig 50C shows the integrated radioactive flow profile of an authentic [3 H]16 α OHE-lysine standard. The radioactivity of the [3 H]16 α OHE-membrane hydrolysate co-chromatographs with the [3 H]16 α OHE-lysine standard, indicating that 16 α OHE-lysine is the only acid-stable adduct generated by the modification in vitro of erythrocytes with 16 α OHE. The three

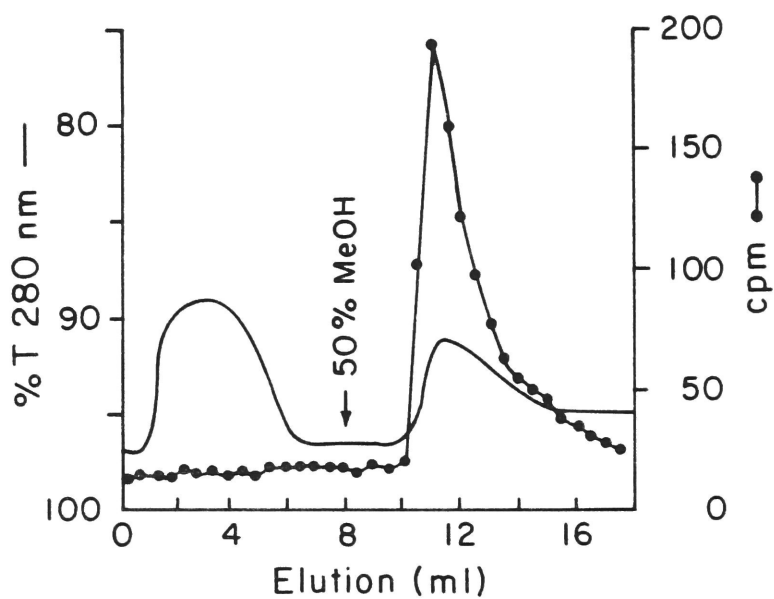


Figure 49. The SM-2 chromatographic profile of the [^3H]16 α OHE-lysine containing fraction isolated previously by phenyl-Sepharose chromatography.

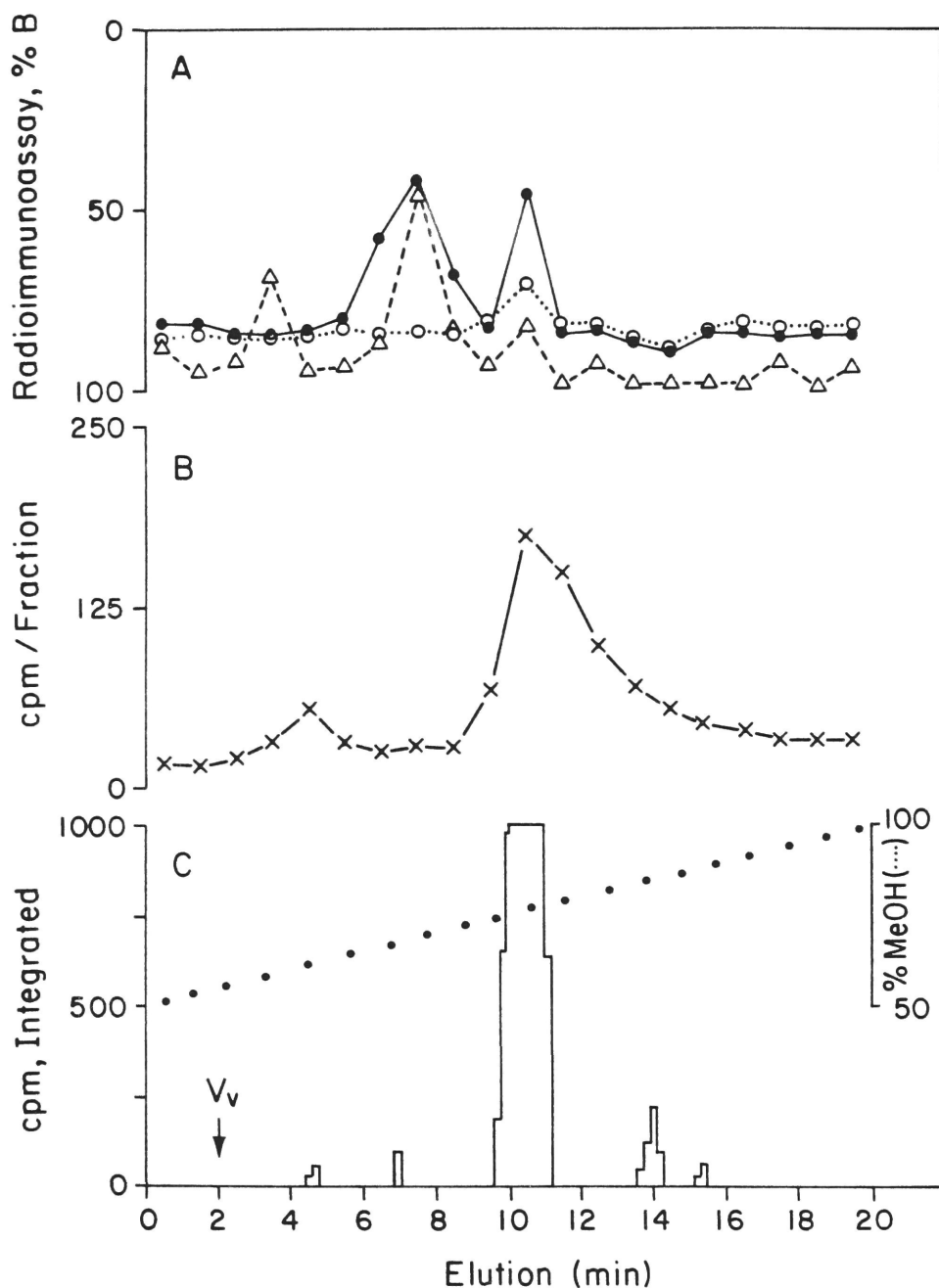


Figure 50. Reverse-phase HPLC fractionation of purified membrane protein hydrolysates. A: The relative percentage bound as measured by radioimmunoassay for 1 ml fractions of three hydrolysates. B: Fractionation of a hydrolysate of cells incubated with $[^3\text{H}]16\alpha\text{OHE}$. C: Authentic $[^3\text{H}]16\alpha\text{OHE}$ -lysine.

erythrocyte preparations shown in Figure 50A all have antibody-binding peaks which co-elute with $16\alpha\text{OHE}$ -lysine. Additional products with binding activity are present in two of the extracts and elute in a more polar region of the chromatogram. The identity of these products is unknown. It is unlikely that $16\alpha\text{OHE}$ is modifying an additional amino acid since the incubation of $[^3\text{H}]16\alpha\text{OHE}$ with erythrocytes in vitro produces only $[^3\text{H}]16\alpha\text{OHE}$ -lysine. Perhaps, once attached to protein, $16\alpha\text{OHE}$ is oxidized or metabolized in the course of the red cell life, producing an adduct which is more polar and continues to cross-react with the antisera. Covalent estrogen lysine adducts might also result from the reaction of the minor estrogens 16β -hydroxyestrone and 16 -ketoestradiol.

Figure 51 shows the $16\alpha\text{OHE}$ -lysine values obtained for erythrocyte membrane proteins from the different groups of individuals studied. On the left abscissa, the values are expressed as a specific activity of $16\alpha\text{OHE}$ -lysine per leucine equivalent of hydrolyzed membrane protein. The right abscissa expresses this value in terms of the average amount of $16\alpha\text{OHE}$ -lysine adducts on a single erythrocyte. Table 9 lists the mean value and standard error of the mean for each group shown in Figure 51. The data show that there is a significant elevation in $16\alpha\text{OHE}$ -lysine content in maternal and SLE women when compared to normal women. The mean level of $16\alpha\text{OHE}$ -lysine in the maternal red cell is higher than in the SLE patient but, as a group, the individual points also show a wider distribution. The mean $16\alpha\text{OHE}$ -lysine value in the male SLE patients also appears to be higher than in the control male group, but this mean reflects only four points and is not statistically significant ($P < .15$).

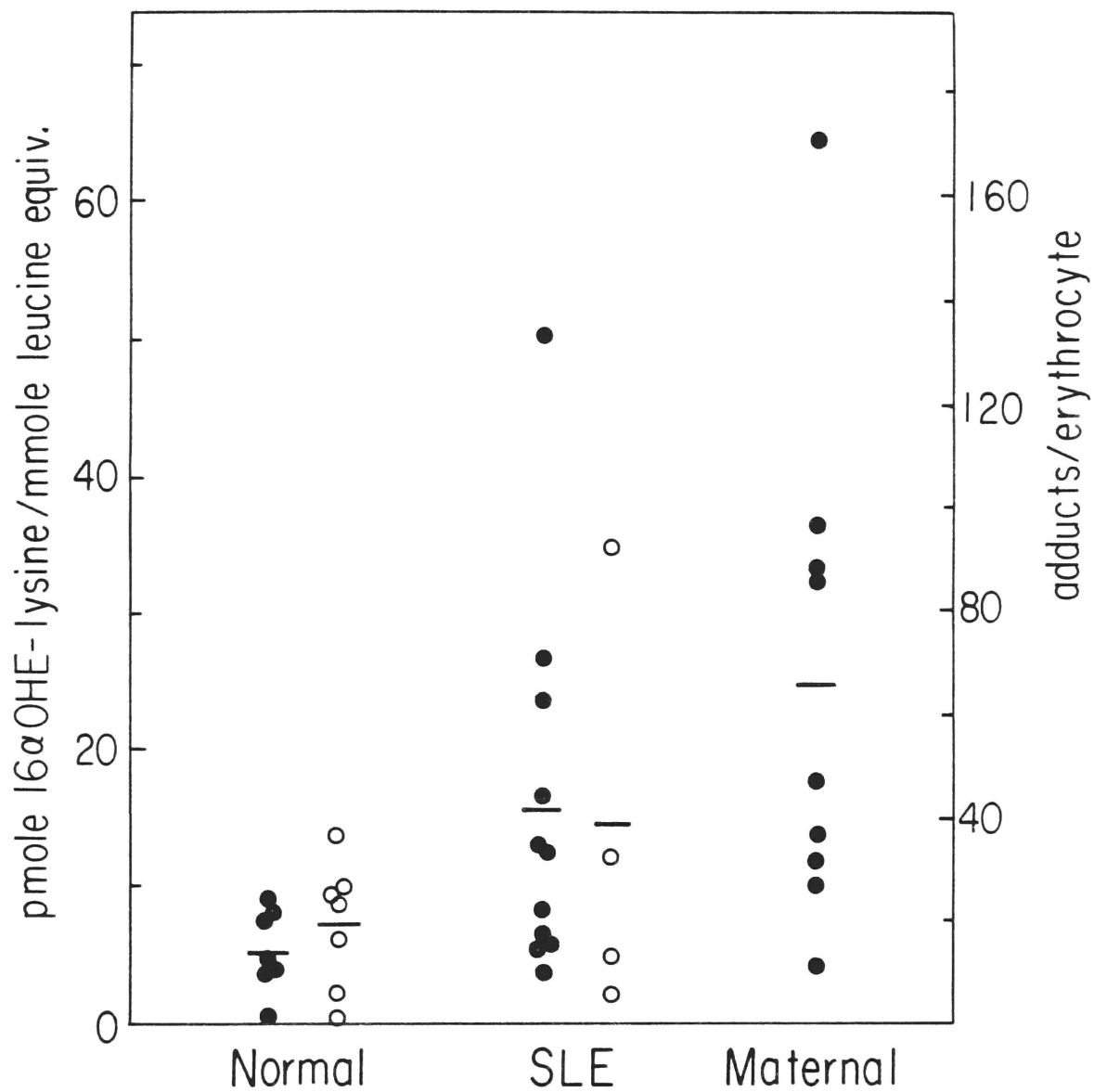


Figure 51. Erythrocyte membrane 16α-hydroxyestrone-lysine levels in normal, SLE, and Maternal subjects. The horizontal bar indicates the mean value within each group.
 (●) : females. (○) : males.

16 α OHE-lysine levels in erythrocyte membrane proteins

	<u>Number</u>	<u>16αOHE-lysine*</u>	<u>P</u>
Normal female	7	5.2 \pm 1.1	-
SLE female	11	15.7 \pm 4.1	< .05
Maternal	9	24.9 \pm 6.2	< .01
Normal male	7	7.1 \pm 1.8	-
SLE male	4	14.7 \pm 8.6	< .15

*pmoles 16 α OHE-lysine/m mole leucine equivalents,
Mean \pm 1 S.E.M.

Table 9. Summary of Figure 51 data.

Women with SLE and women in the third trimester of pregnancy therefore, have significantly higher levels of red cell 16 α OHE-lysine adducts than normal controls. The maternal state is associated with a gradually increasing plasma 16 α OHE level which peaks during the third trimester at about 2.8 pmole/ml and then rapidly falls off (109). The red cell associated 16 α OHE-lysine adducts presumably accumulate during this rapid elevation of plasma 16 α OHE. The SLE females show a red cell 16 α OHE-lysine value which falls between the normal and maternal population means. Plasma 16 α OHE levels are only moderately elevated in most patients and fluctuations in these levels have been observed to occur with time (110).

Since the life of the red cell is 120 days, this cell would be expected to act as an integrator to provide, over an extended period, a time average of the 16 α OHE status of a person. It is apparent from the red cell data however, that there is some variation in the levels of 16 α OHE-lysine present. In the case of the maternal data, this could be due to several reasons. Variations in the absolute plasma content of 16 α OHE or its rate of increase during the second trimester would effect the cumulative exposure of the cell to 16 α OHE. Lysine adducts involving the minor estrogens 16 β OHE and 16-keto-estradiol may also be present during pregnancy and might not be distinguishable from 16 α OHE-lysine in the assay. In the case of the SLE group, the presence of a low-grade hemolysis would decrease the red-cell lifetime and cause variability in the level of 16 α OHE-lysine. The susceptibility of red cell proteins to modification also may vary in individuals, perhaps as a result of genetic differences in the composition of reactive proteins in the membranes.

The patients with SLE which were studied had a varied clinical history. There was no statistical correlation between the 16 α OHE-lysine values in the red cell and clinical parameters such as erythrocyte sedimentation rate, hemolytic complement, and anti-nuclear or anti-DNA antibodies. Furthermore, there was no relation between 16 α OHE-lysine and patient age or the degree of glucocorticoid therapy.

An attempt was made to correlate the extent of protein modification by 16 α OHE in the red cell membrane with the level of plasma 16 α OHE measured at the time the blood was sampled. A regression plot of plasma 16 α OHE versus red cell 16 α OHE-lysine is shown in Figure 52 for normal and SLE patients. There is a linear correlation ($r = 0.55$, $P < 0.0025$) for this data although a number of points fall outside the 95% confidence limits. Maternal values are not plotted since the high estriol values associated with pregnancy interfere significantly with the radio-immunoassay for free 16 α OHE (100).

The fact that a number of points fall outside the 95% confidence interval however, indicates that the measurement of one parameter is only of limited value in predicting the value of the second parameter. Since the plasma 16 α OHE level represents one measurement in time, while the 16 α OHE-lysine content is an integral value reflecting a long time period, variation in the correlation may be the result of periodic fluctuations in the plasma content of 16 α OHE. This has been observed to occur, particularly in SLE patients (110). In addition, recent experiments indicate that significant levels of 16 α OHE are present in the plasma in the form of the 3-sulphate (111). Lysine addition products could form with this metabolite and would be detected in 16 α OHE-lysine assay. The level of reactive plasma 16 α OHE however,

would be underestimated because the 16 α OHE radioimmunoassay does not detect the sulfate derivative.

We next applied the assay for 16 α OHE-modification to proteins obtained from a membrane preparation of lymphocytes isolated from peripheral blood by Ficoll density gradient centrifugation. The lymphocyte is of interest because it is a much longer lived cell than the erythrocyte (112) and an intriguing possibility exists that increased plasma levels of free 16 α OHE might lead to an increased modification of lymphocyte cell surface proteins and cause immune dysfunction. Figure 53 shows the levels of 16 α OHE-lysine measured in lymphocyte membranes from normal controls and patients with SLE. There is a significant elevation of 16 α OHE-lysine in women with SLE when compared to normal women (Table 10). The measurement of a single male SLE value which was obtained is also shown. Of significance is the fact that the level of protein modification within the lymphocyte is three times higher than for the red cell in each of the groups studied. Within each group, there is also a greater variation in the lymphocyte 16 α OHE-lysine values than in the red cell. In the SLE group, there was no correlation between the level of lymphocyte protein modification and any objective clinical parameter.

The mean level of 16 α OHE-protein modification within lymphocytes was three times higher than in the red cell for the normal and the SLE subjects studied. These values most likely reflect the longer circulating life of the lymphocyte; for example, a mean lifespan of 530 ± 64 days has been reported (112). In addition, women with SLE were found to have significantly higher levels of 16 α OHE-lysine adducts than normal women. The levels of 16 α OHE-lysine also showed a wider distribution

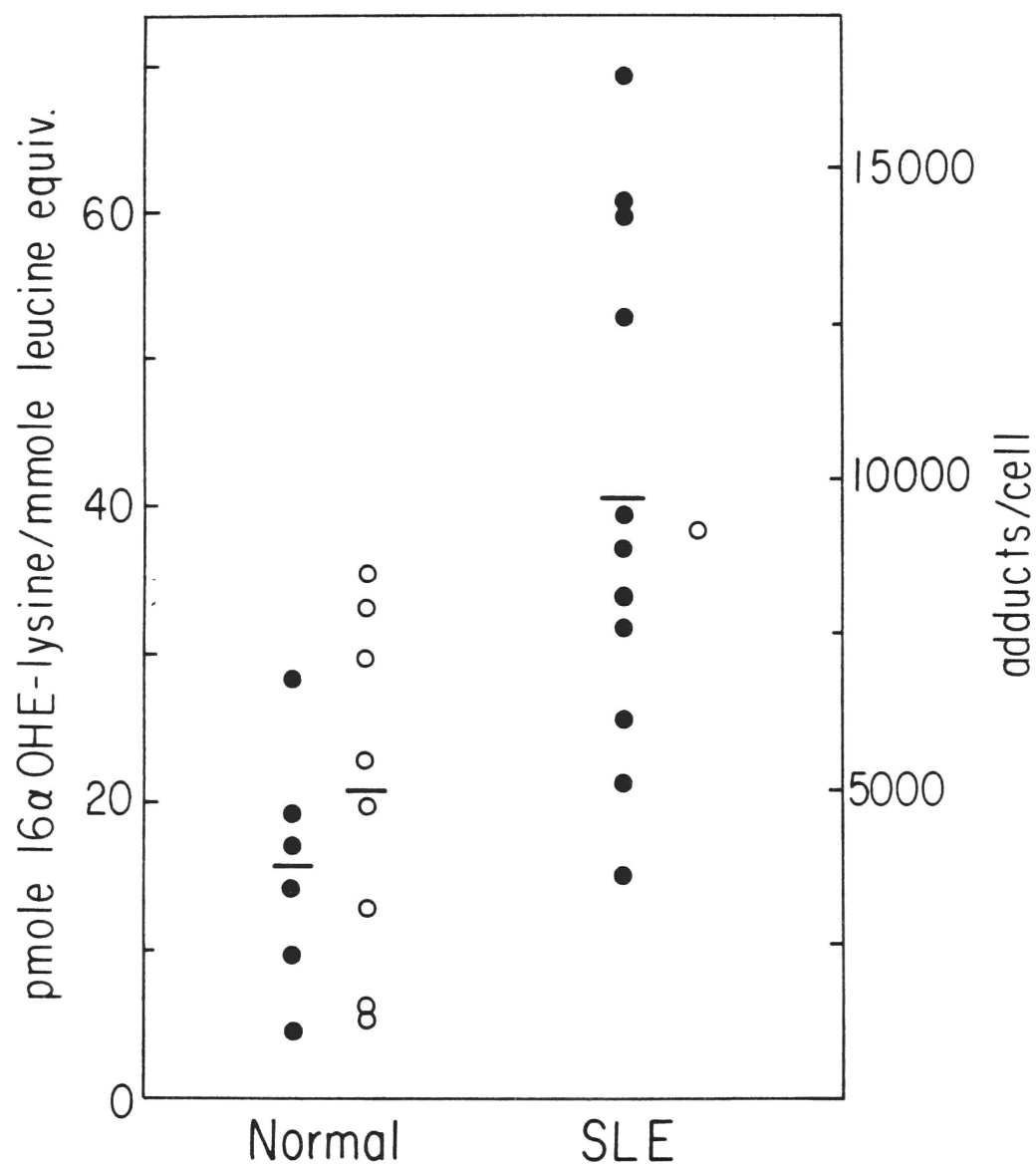


Figure 53. Lymphocyte membrane 16 α -hydroxyestrone-lysine levels in normal and SLE subjects. The horizontal bar indicates the mean value within each group. (●) : females. (○) : males.

16 α OHE-lysine levels in lymphocyte membrane proteins

	<u>Number</u>	<u>16αOHE-lysine*</u>	<u>P</u>
Normal female	6	15.6 \pm 3.4	-
SLE female	11	40.5 \pm 5.4	<.005
Normal male	8	20.7 \pm 4.2	
SLE male	1	38.4	

*pmoles 16 α OHE-lysine/mmmole leucine equivalents,
Mean \pm 1 S.E.M.

Table 10. Summary of Figure 53 data.

than in the red cell. Variation in the turnover of membrane proteins or in the lifespan and content of different lymphocyte subsets, particularly with the SLE group, might be a factor contributing to the wide distribution of 16 α OHE-lysine values.

An extremely long-lived extracellular protein which has been found to accumulate the products of nonenzymatic glycosylation over the age of the whole organism is collagen (113). Reasoning that 16 α OHE-adducts might accumulate similarly on collagen, the presence of these adducts was assayed on four preparations of glomerular basement membrane. The results of these determinations are shown in Table 11. Samples were selected that were close in age and sex-matched. The values for both of the normal individuals are over one order of magnitude higher than the mean 16 α OHE-lysine value found in the normal red cell. This is presumably due to the extremely low turnover rate of collagen. Of the SLE kidneys, sample A was from a patient with a 12 year history of SLE and a 9 year history of glomerulonephritis. Sample B was obtained from a patient with a thirty-year history of SLE and an 8 year history of nephritis.

The SLE kidneys were found to have higher levels of basement membrane 16 α OHE-lysine than age and sex-matched normal controls. Data on either the plasma 16 α OHE level or the rate of 16 α -hydroxylation was not available for the patients in this group. It is noteworthy, nevertheless, that the patient with the highest levels of 16 α OHE-lysine (256 ± 61) had SLE for 30 years and an 8 year history of nephritis.

It is clear from this data that the level of protein modification by 16 α OHE increases with the lifespan of the protein. Table 12

16 α OHE-lysine values in the glomerular basement membrane

		<u>Age</u>	<u>16αOHE-lysine*</u>
Normal female	A	50	86 \pm 20
	B	60	129 \pm 31
SLE female	A	49	102 \pm 24
	B	50	256 \pm 61

*pmoles 16 α OHE-lysine/mole leucine equivalents,
Mean \pm 1 S.E.M.

Table 11.

<u>Protein</u>	<u>16αOHE-lysine*</u>		<u>Mean Lifespan</u>
	<u>Normal</u>	<u>SLE</u>	
Erythrocyte membrane	5.2	15.7	120 d.
Lymphocyte membrane	15.6	40.5	\approx 530 d.
Basement membrane collagen	107	179	many years

*mean, pmoles/ μ mole leu. equiv.

Table 12. Summary of 16 α OHE-lysine levels in different proteins.

summarizes the mean values of 16 α OHE-lysine measured in different proteins in both normal individuals and patients with SLE. For each protein, the level of modification appears proportional to its biological lifespan. In relative terms, the level of modification for each type of protein is increased in SLE patients. This is consistent with the idea that 16 α OHE adducts accumulate over a long time period, and that the degree of protein modification is a time integral of the free 16 α OHE level.

C. Studies of Anti-estrogen Autoantibodies.

Since SLE patients produce a diversity of antibodies to self-antigens, a reasonable possibility is that autoantibodies may also be present against endogenous 16 α OHE-lysine adducts. The existence of autoantibodies would also indicate that 16 α OHE-modified proteins are recognized by components of the immune system.

In order to detect the occurrence of these anti-estrogen antibodies, an enzyme-linked immunoassay was developed. The following section describes this assay, the detection of anti-estrogen antibodies in oral contraceptive users, and preliminary studies of the anti-estrogen antibody activity in patients with SLE.

Methods

Reagents. Agarose immobilized 17 β -estradiol, 17-hemisuccinate (100 ml) was custom synthesized by Pierce Chemical Co. using 6% crosslinked diaminopropylamine agarose as the solid support. Estradiol content was found to be 26 μ mole/ml resin and was derived from the extinction at 280 nm of acid hydrolyzed resin (1 N HCl at 60 $^{\circ}$ for 5 min). The amount of linked estradiol was then calculated by comparison to a standard curve generated from the acid hydrolysis of unmodified agarose to which free estradiol had been added. The resin was washed extensively with a buffer containing PBS, 0.05% Tween-20 (Biorad Laboratories), and 3 mM NaN₃. Alkaline phosphatase linked anti-human IgG (H & L chain) was from Cappel Laboratories (Cochranville, PA).

Assay Procedure. All buffers were filtered through a 0.45 μ m Millipore filter. Prior to analysis all plasma samples were centrifuged

for 1 min in an Eppendorf microfuge (Model 5414) to remove insoluble material. The plasma supernatant was then diluted 1:40 in a buffer containing PBS, 0.05% Tween-20, and 3 mM NaN₃ and filtered through a 0.22 µm Millex filter unit. 0.4 ml was then added to 0.1 ml of a 25% solution of estradiol-agarose and incubated at room temperature with gentle rocking for 12-16 hr. At the end of this time, the resin was washed 4 times with 1 ml of PBS-Tween. 0.5 ml of a solution of anti-human IgG (diluted 1:500 in a buffer containing 0.9 M NaCl, 10 mM NaPO₄, pH 7.4, 0.05% Tween-20, and 3 mM NaN₃) was then added and the mixture incubated with gentle rocking for 6 hr. The second antibody was then removed and the resin washed 4 times with 0.9 M NaCl, 10 mM NaPO₄, pH 7.4, 0.05% Tween-20, and 3 mM NaN₃; and 2 times with 10 mM diethanolamine, pH 9.0, and 3 mM NaN₃. 0.5 ml of p-nitrophenol phosphate (Sigma), dissolved in 0.1 M diethanolamine pH 9.8 with 3 mM NaN₃, was then added and the mixtures incubated at room temperature. At the end of 30 min, the reaction was terminated by adding 0.2 ml of 1 M NaOH. The resin was then removed by centrifugation and the absorbance of the supernatant recorded at 400 nm.

Patient Selection. Patients with SLE were selected by the same criteria as described on p. 111. The non-SLE patients included males and females with rheumatoid arthritis (n = 7), osteoarthritis (n = 4), primary biliary cirrhosis (n = 11), scleroderma (n = 3), Sjogren's syndrome (n = 1), mixed connective tissue disease (n = 1), polymyositis (n = 1), tenosynovitis (n = 1), hypergammaglobulinemia (n = 1), agammaglobulinemia (n = 1), and multiple sclerosis (n = 1).

Results

An outline of the immunoassay for anti-estrogen antibodies is shown in Figure 54. Analysis of a number of control plasmas at arbitrary dilutions revealed that many disease-free normal women had antibodies which bound to estrogen-linked-agarose beads. This result was puzzling until it was realized that all of the women who displayed this activity had at some time been users of oral contraceptives. Figure 55 summarizes results obtained for normal plasma assayed at a final dilution of 1:50. Approximately 30% of women with a history of oral contraceptive use displayed activity of greater than 0.2 O.D. on this assay.

The occurrence of anti-estrogen antibodies in oral contraceptive users has been reported previously by Beaumont et al. (114-116). Using an assay based on the specific binding of 17α -[^3H]ethinylestradiol (the estrogen component of oral contraceptives), they observed circulating anti-ethinylestradiol antibodies in oral contraceptive users, but not in non-users. In a number of cases these antibodies were present years after the discontinuation of birth control medication (114). Among the positive values shown in Figure 55, several were from women who had not been on birth control medication for 2 to 8 years.

In further studies, the Beaumonts observed a significant correlation between circulating immune complexes, the presence of anti-ethinylestradiol antibodies within these complexes, and the occurrence of vascular complications among oral contraceptive users (115). Although a definitive role for immune complexes in thrombosis has not been established, they propose that these antibodies and their associated immune complexes may, over a long period of time, be the factor contributing

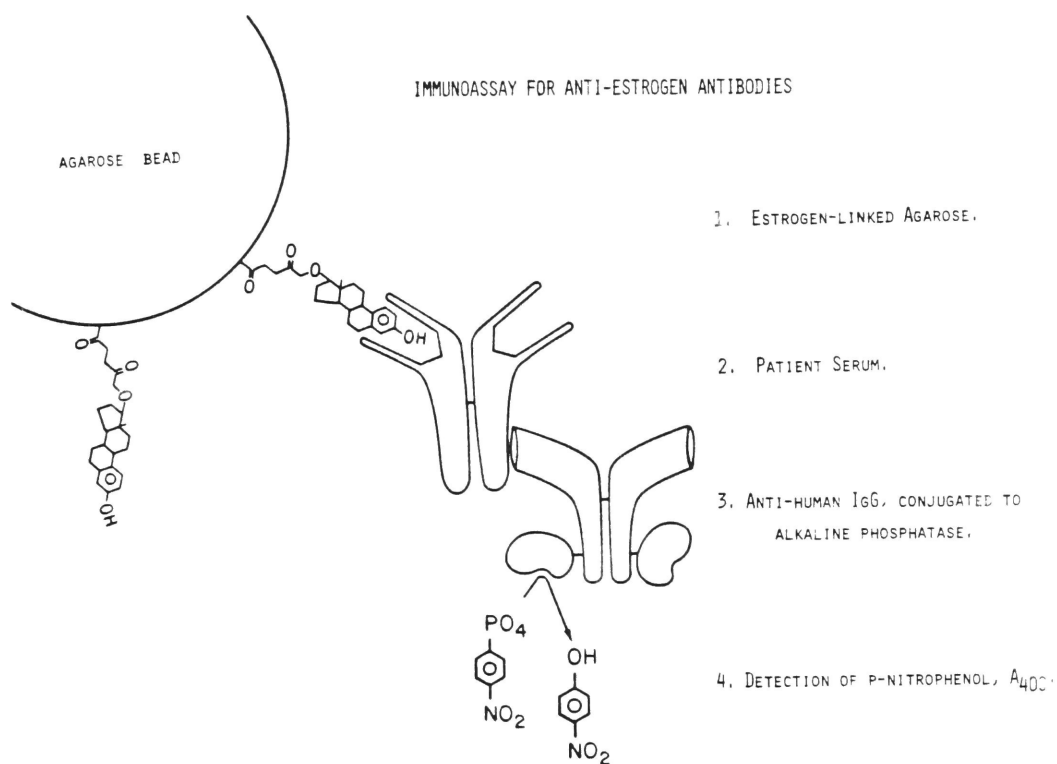


Figure 54. Schematic outline of the enzyme-linked immunoassay for anti-estrogen antibodies.

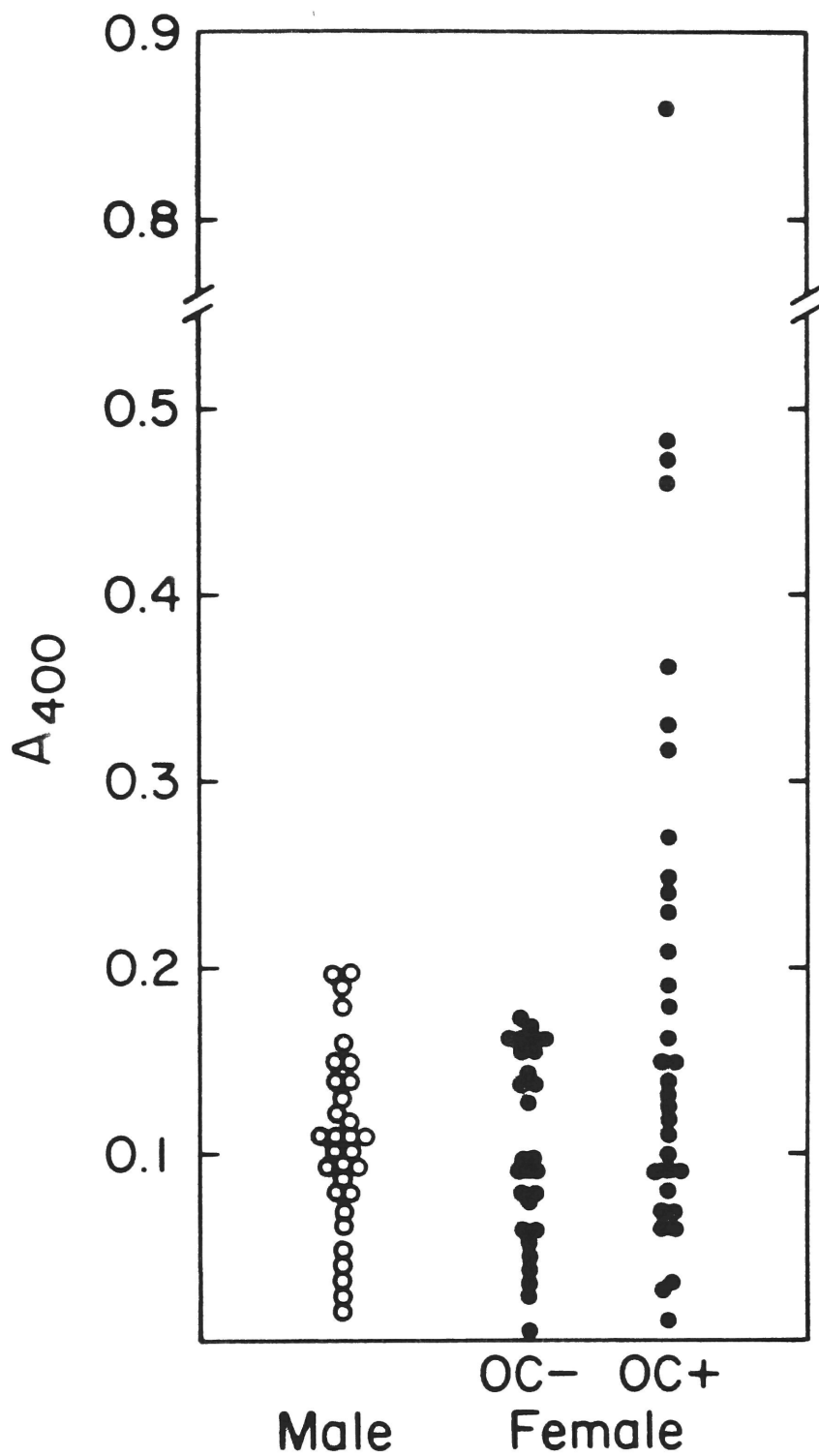


Figure 55. Anti-estrogen antibody activity in normal, disease-free males and females. OC+ : denotes a history of oral contraceptive use.

to the increased risk of thrombosis and vascular disease present in women who have been on birth control pills for many years.

The estrogen which is common to all estrogen containing birth control pills is the synthetic analog, 17α -ethinylestradiol. The ethinyl function serves to increase the oral potency of this derivative by inhibiting oxidation and conjugation of the estrogen D-ring. Ethinylestradiol can undergo enzymatic conversion to metabolites which form covalent adducts with proteins; leading to haptens which could stimulate antibody production. Figure 56 shows two possible routes of enzymatic activation. The 2-hydroxylase pathway is believed to be the major metabolic route because 16-hydroxylation, the alternate pathway, is blocked by the presence of the ethinyl function. The resulting catechol derivatives can undergo oxidation to yield semiquinones and quinones which readily add to protein amino or sulfhydryl groups. A number of studies have indicated that this route results in the formation of covalent estrogen-protein adducts in the liver (117, 118). The pathway produced by the action of cytochrome P-450 has been partially defined. There is evidence for the formation of an unsaturated epoxide at the ethinyl function, which probably leads to the binding and inactivation of P-450 and the generation of hepatic "green pigment" (119). Epoxide formation is also believed to be the step in the generation of the metabolite D-homoestrone (120). It is likely that the unsaturated epoxide also would react with nucleophilic groups in proteins to yield estrogen adducts linked in the steroid D-ring. Since the D-ring conjugated product is antigenically similar to the agarose immobilized estrogen, the P-450 product is probably the one which induced the anti-estrogen antibodies detected in the immunoassay.

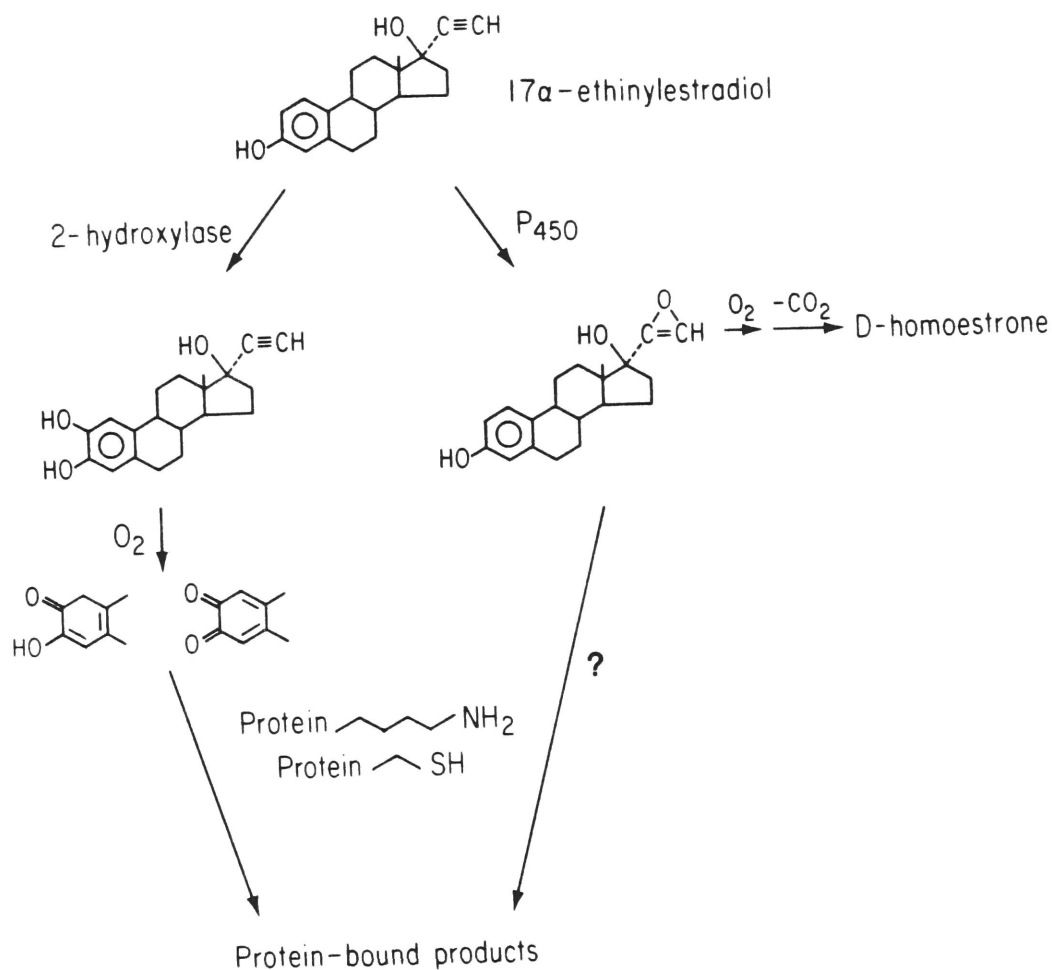


Figure 56. Enzymatic conversion of 17 α -ethinylestradiol to reactive intermediates which can form irreversible protein-bound products.

Figure 57 shows the preliminary results of SLE plasma assayed for the presence of anti-estrogen antibodies. The left side shows male and female values obtained from patients with autoimmune or rheumatic disease other than SLE. A few male and female SLE patients display activity on this assay without ever having been on oral contraceptives. This activity may represent an antibody formed to the endogenous 16 α OHE-lysine adduct or alternatively, an autoantibody elicited in response to another cross-reactive antigen. The pathological significance of these estrogen reactive antibodies awaits further study.

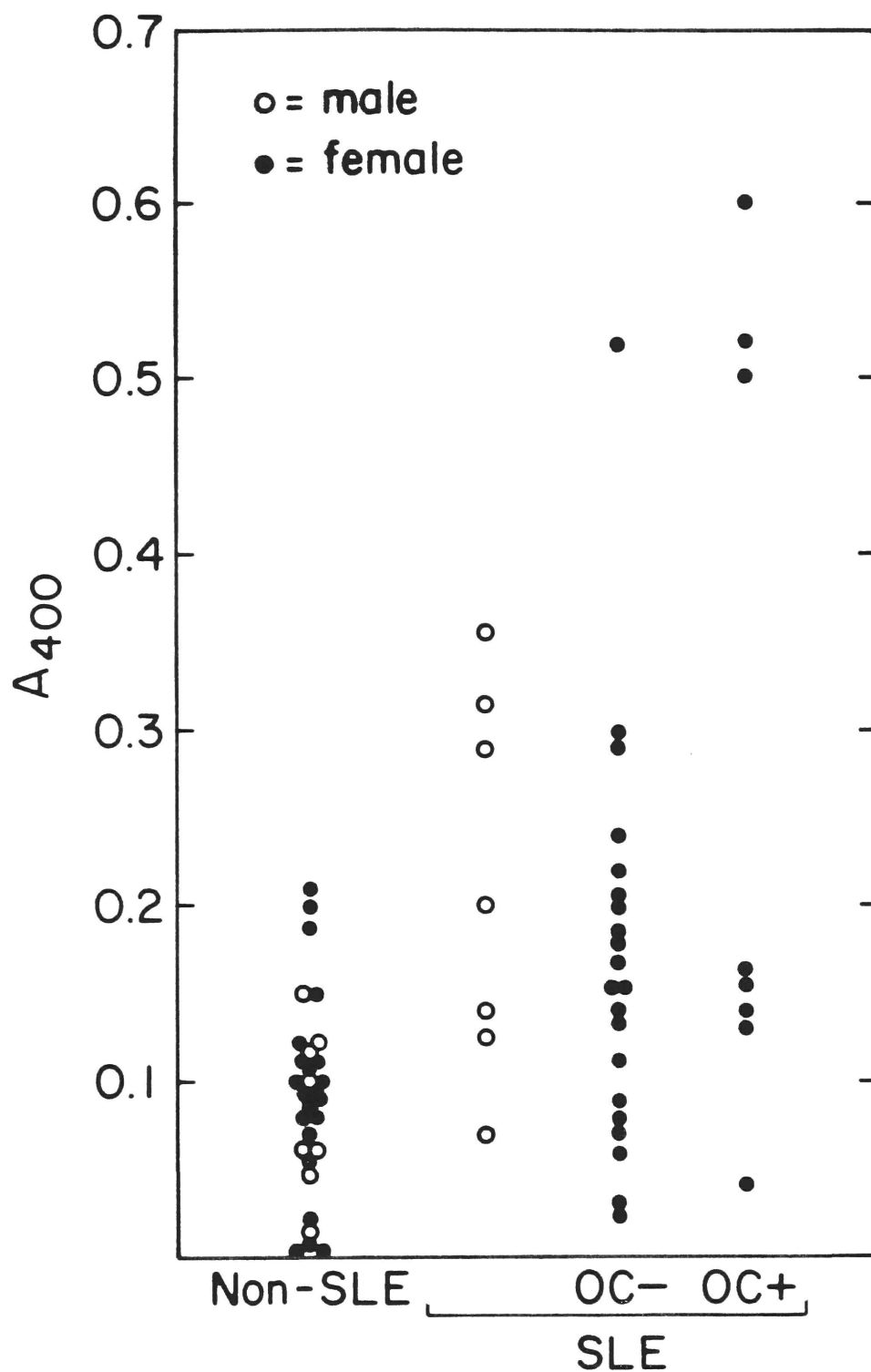


Figure 57. Anti-estrogen antibody activity in patients with autoimmune disease other than SLE (non-SLE) and in SLE patients. OC+ : denotes a history of oral contraceptive use.

D. Conclusion.

In analogy to cortisol and the glucocorticoids, the ketolic estrogen 16α -hydroxyestrone can react with the lysine residues of proteins to form stable, covalent addition products. These adducts can be detected in vivo on the membrane proteins of the erythrocyte and the lymphocyte and the long-lived structural protein collagen. The level of protein modification by 16α OHE increases with the lifespan of the protein. Furthermore, conditions such as pregnancy and SLE, in which increased levels of free 16α OHE occur, also are associated with increased protein modification.

Proteins modified by the nonenzymatic addition of 16α OHE can be antigenic, and 16α OHE modified albumin was used to prepare the antisera used in the radioimmunoassay of 16α OHE-lysine. In vivo, it appears that one example of a protein bound estrogen, produced by the metabolism of 17α -ethinylestradiol, leads to anti-estrogen antibodies in women receiving this steroid for oral contraception. Some patients with SLE also display an anti-estrogen antibody activity. These antibodies may represent a cross-reactive autoantibody or perhaps, be produced in response to the increased levels of endogenous 16α OHE-protein adducts which are present in these patients.

The role of 16α OHE-protein adducts in either the etiology or the pathology of SLE has not yet been defined. These adducts may in fact stimulate autoantibody production in some individuals. It is also possible that 16α OHE can trigger immune dysfunction by reacting with immunoregulatory membrane proteins and disrupting cellular interactions.

5. Nonenzymatic Modification of DNA by Reducing Sugars.

A. Biochemical and Genetic Studies of DNA modification in Prokaryotic Systems.

The demonstration that proteins undergo nonenzymatic glycosylation in vivo and that protein-bound Maillard products occur on long-lived structural proteins led to the hypothesis that nucleic acids might also undergo chemical modification by reducing sugars. Because nucleic acids are long-lived molecules in the resting cell, the accumulation of stable addition products with time would affect the viability of the genetic material. Current theories of aging emphasize the concept of "error catastrophe" and that the functional decrements characteristic of cellular senescence are due to the progressive accumulation of unrepaired genetic lesions (121, 122). Age-dependent changes in the genetic material are well-documented and include increased tumorigenesis (123), chromosomal aberrations (124), DNA strand breaks (125), and decreases in DNA repair (126), replication (127), and transcription (128). Interestingly, there are two age-related phenomena which are exacerbated by the diabetic state; the decreased replicative capacity of the aged fibroblast and the increased frequency of abnormal births seen with the increasing age of the mother (129, 130).

The following studies show that the primary amino groups of nucleotides can react nonenzymatically with sugars to produce spectral changes, abnormalities in DNA template function, and mutagenesis.

Methods

Spectroscopic studies. Solutions (1 ml) were prepared that contained 10 mM deoxyribonucleotide (Sigma) and 150 mM glucose-6-

phosphate (G6P, Calbiochem) or glucose (Baker) in 50 mM Hepes/0.5 mM EDTA, pH 8.0, adjusted with NaOH. All solutions were sterilized by filtration through a Millex filter prior to incubation at 37° in the dark. Control solutions contained sugar alone or nucleotide alone. DNA incubation mixtures contained calf-thymus DNA (Sigma) at 2 mg/ml. Single-stranded DNA (ssDNA) was generated by boiling DNA for 5 min and then rapidly cooling the solution on ice. At the end of 4 days, the solutions were diluted 1:1 with distilled H₂O and the visible and UV spectra recorded on a Hewlett-Packard model 8450A sepectrophotometer.

Fluoresence measurements were made on a Perkin-Elmer model 204 fluorescence spectrophotometer. Incubation mixtures showing absorbance changes were diluted 1:6 with distilled H₂O, and the excitation and emission maxima were determined by preliminary scanning. Final recordings were made at an excitation wavelength of 315 nm and an emission wavelength of 420 nm. Fluorescence spectra of modified nucleotides and DNA were determined by subtraction of the background fluorescence generated by G6P degradation (131), which occurs during prolonged incubation. DNA and nucleotides incubated without G6P did not exhibit measurable fluorescence. The magnitude of the quantum yield was calculated with reference to the emission at 442 nm of a standard solution of quinine (10 µM in 0.05 M H₂SO₄) excited at 315 nm (132).

DNA Transfection. Bacteriophage f1 DNA was isolated by phenol extraction of concentrated phage particles. Each transfection assay mixture contained 2 µg of DNA in 0.1 ml of sterile solution of 50 mM Hepes buffer, pH 8.0, and 0.5 mM EDTA and either glucose or G6P. Control incubation mixtures contained either DNA or sugar alone.

After incubation at 37° in the dark, duplicated aliquots were transfected into 0.3 ml of a suspension of calcium chloride-treated *Escherichia coli* (strain K38) (133). Plaque-forming units were quantified after an overnight growth.

DNA Electrophoresis. DNAs were electrophoresed in 1% agarose gels under alkaline conditions (30 mM NaOH) sufficient to separate linear from covalently closed DNA (134). After neutralization, the gel was stained with ethidium bromide at 1 µg/ml. For detection of G6P incorporation, 4 µCi of D-[1-¹⁴C]glucose 6-phosphate (50 mCi/mmol; New England Nuclear) was incubated with 16 µg of purified fl DNA for 2 weeks as described above. Radiochemical purity was assessed by HPLC using a C₁₈ column (Waters Associates) and a mobile phase of 20 mM potassium phosphate buffer (pH 5.5) containing 2% methanol that increased linearly to 20% methanol from 0 to 20 min (135). At the end of the incubation period, 8 µg of DNA was loaded per lane and electrophoresed, and the gels were impregnated with EN³HANCE (New England Nuclear). Fluorography was for 4 weeks at -80°.

Mutagenesis Assay. Purified pBR322 DNA (1 µg) was incubated at 37° under sterile conditions with 150 mM to 400 mM G6P in 0.2 ml of buffer containing 50 mM Hepes (pH 8.0) and 0.5 mM EDTA. Control incubations lacked G6P. At the indicated times, 0.3 ml of a CaCl₂ treated *E.coli* strain K38 was transformed with 5-20 µl aliquots of the incubated solutions. Cells were plated onto ampicillin supplemented TY plates (50 µg/ml) and colonies enumerated after overnight growth. Colonies selected for amp^r were then replicated onto tetracycline containing media (20 µg/ml) and the presence of tet^r assessed.

Southern Hybridization. A stab from an isolated single colony was

inoculated into 40 ml of tryptone broth and harvested after bacterial growth reached an OD₆₆₀ of 1-1.4. Whole E. coli DNA was prepared by the method of Smith (136), and DNA content determined by absorbance at 260 nm. Six μ g of DNA was electrophoresed in each lane of a 0.6% agarose gel (TBE and ethidium bromide, (137)), and run until the bromophenol blue marker dye had migrated 14 cm. The gels were washed in 0.25 N HCl, processed for Southern transfer, and blotted onto Gene Screen Plus (New England Nuclear) by capillary transfer following the methods recommended by the manufacturer. Staining of the transferred gels with ethidium bromide demonstrated that all of the DNA had transferred to the hybridization membrane. The membranes were then washed by shaking at 37° with a pre-hybridization solution containing 50% formamide, 5x SSC, 1x Denhardt's solution (137), 1% SDS, and 0.1 mg/ml denatured salmon sperm DNA. After 6 hr, 1×10^7 cpm (Cerenkov) of a nick-translated (137) ³²P-labelled probe (2.4×10^8 cpm/ μ g) was added and hybridization allowed to proceed for 12 hr. The hybridization membranes were then washed three times at room temperature in 0.2x SSC, 1% SDS, and 0.1% sodium pyrophosphate followed by two washes at 65° in 0.5x SSC and 1% SDS. The membranes were then air-dried and exposed to x-ray film for 4 hr at -80° in the presence of intensifying screens.

Results

Although the Maillard reaction is characteristically a slow process, studies with proteins have shown that modification occurs as an integral function of time and sugar concentration (138). As a first step toward the study of DNA modification by sugars therefore, individual nucleotides and purified DNA were incubated at 37° with high concentrations of

either glucose or G6P. As shown in Figure 58, 4 days of incubation with G6P was sufficient to produce absorbance changes in the 300 - 400 nm range that are similar to those described for the nonenzymatic browning of proteins (139, 140). No changes were detected in the 220 to 300 nm region. The enhanced browning effect of G6P - in contrast to glucose, which did not show any effect under these conditions - is consistent with the observation that phosphorylated sugars glycosylate proteins more readily than nonphosphorylated ones (141), and at a rate which is a function of their reducing action (142). Of significance is the fact that negligible spectral changes occurred with TMP, the only nucleotide lacking a primary amino group, and with double-stranded DNA (dsDNA), presumably because hydrogen bonding hinders the reaction of the amino groups with G6P.

Flourescence spectra were obtained for the chromophores produced by incubation of ss DNA and the nucleotides AMP, CMP, and GMP with G6P; excitation and emission spectra (uncorrected) are shown in Figure 59. Excitation in the 250 to 270 nm region, where nucleotides absorb strongly, produced neither new nor significant emission. Instead, the chromophores produced had excitation maxima at 315 nm with calculated quantum yields of 2.37×10^{-2} for GMP, 1.15×10^{-2} for AMP, and 1.9×10^{-3} for CMP. AMP showed in addition, a large second excitation maximum at 360 nm. CMP and ssDNA had smaller excitation maxima in the 330 to 350 region. As shown in Figure 59, excitation at 315 nm produced emissions that ranged from 350 to 600 nm.

We next investigated the biochemical effects of glucose and G6P on DNA from the E. coli bacteriophage $\phi 1$. $\phi 1$ DNA is a single stranded, covalently closed circle composed of 6470 nucleotides (143). The

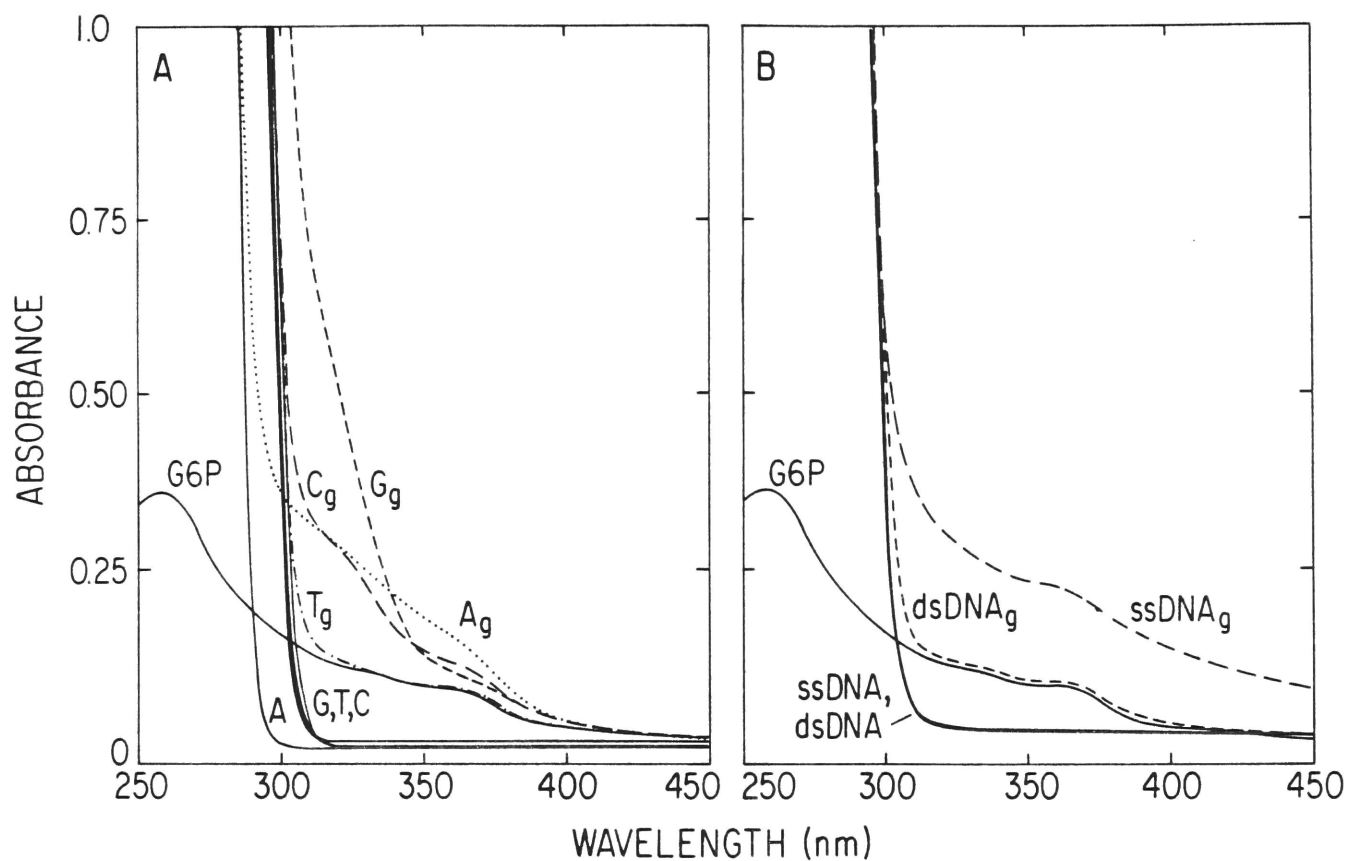


Figure 58. UV and visible absorbance spectra of nucleotides and single- and double-stranded DNAs after 4 days of incubation at 37⁰. (A) G6P: glucose-6-phosphate alone; A,C,G,T: nucleotides alone; A_g,C_g,G_g,T_g: nucleotides incubated with G6P. (B) G6P alone; ss and ds DNA alone; ss DNA_g and ds DNA_g: ss and ds DNA incubated with G6P.

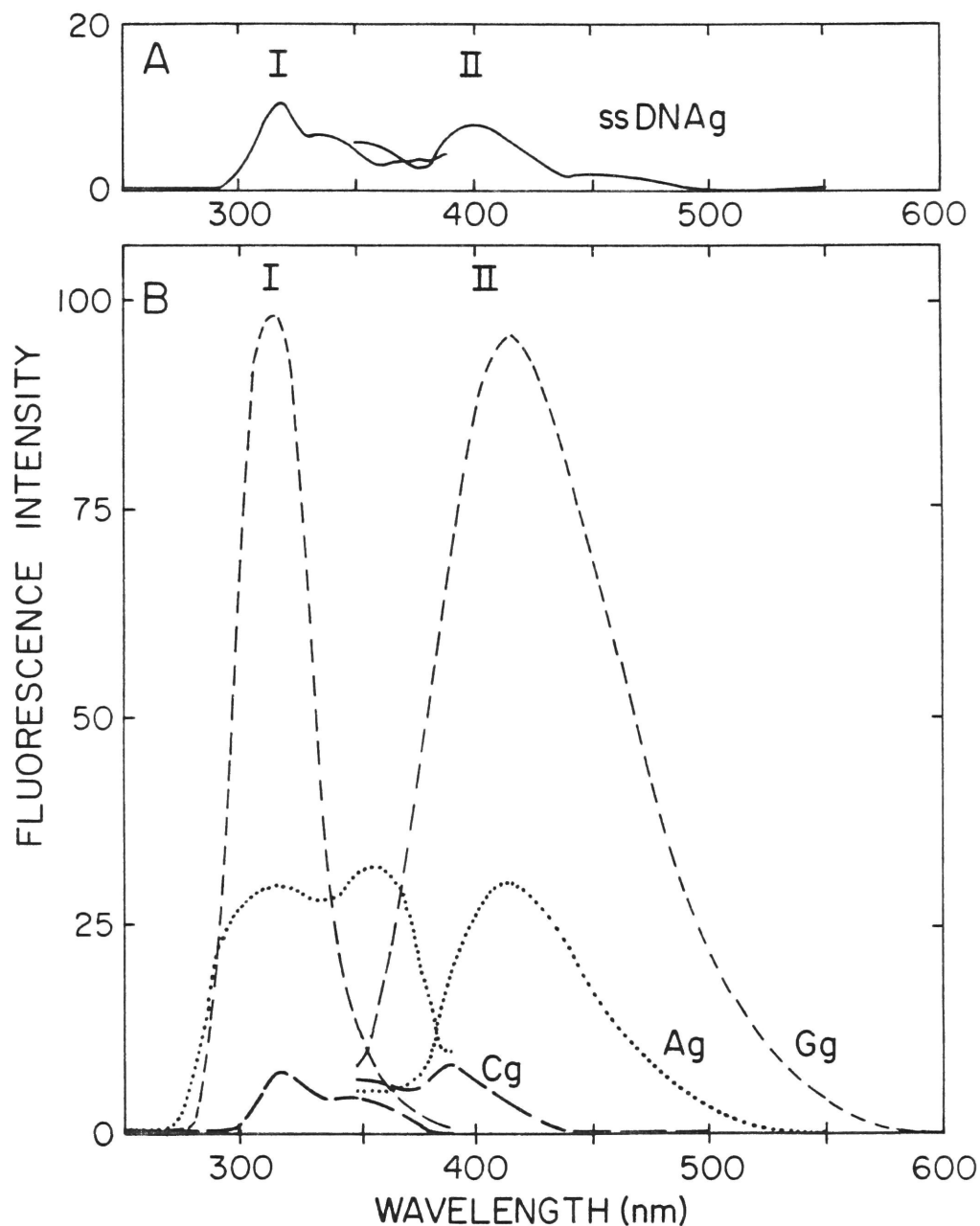


Figure 59. Excitation (I) and emission (II) difference spectra (uncorrected) for ss DNA and G6P modified nucleotides. Fluorescence intensity is expressed in arbitrary units. (A) ss DNA_g: G6P modified ss DNA. (B) A_g, C_g, G_g: G6P modified nucleotides.

properties of the DNA examined included its transfection potential into the host strain K38 and its structural integrity as analyzed by alkaline gel electrophoresis. DNA was isolated from the virus by phenol extraction and incubated with different concentrations of sugars for eight days at 37°. At the end of this time, aliquots were removed and transfected into the calcium chloride-treated E.coli.

The percentage decrease in the transfection efficiency of glucose and G6P treated DNA relative to that of DNA incubated without sugar is shown in Figure 60. DNA exposed to G6P showed a dramatic decrease in transfection efficiency; incubation with 150 mM G6P decreased the value by 4 orders of magnitude. The rate of loss of biological activity was first order with respect to sugar concentration. Glucose caused a much smaller decrease in transfection efficiency; at 150 mM, efficiency was reduced to ~33% of control.

The following experiment was carried out to test for the possibility that the carbohydrate alone, or the products of spontaneous carbohydrate degradation, could inhibit expression of the viral genome. Glucose and G6P (150 mM) were incubated for 8 days and equivalent amounts were added to calcium chloride-treated E. coli together with untreated fl DNA. As shown in Figure 60, these conditions produced only a small (<50%) inhibition of transfection.

Controls for the G6P inhibitory effect were also carried out. The possibility that trace metals might be present in the G6P solution and catalyze DNA degradation was tested by treatment with the resin Chelex 100. Similarly, the presence of any contaminating DNA nucleases was diminished by filtering the G6P solution through a dialysis membrane (M_r cutoff 3,500). The effects of these treatments were insignificant

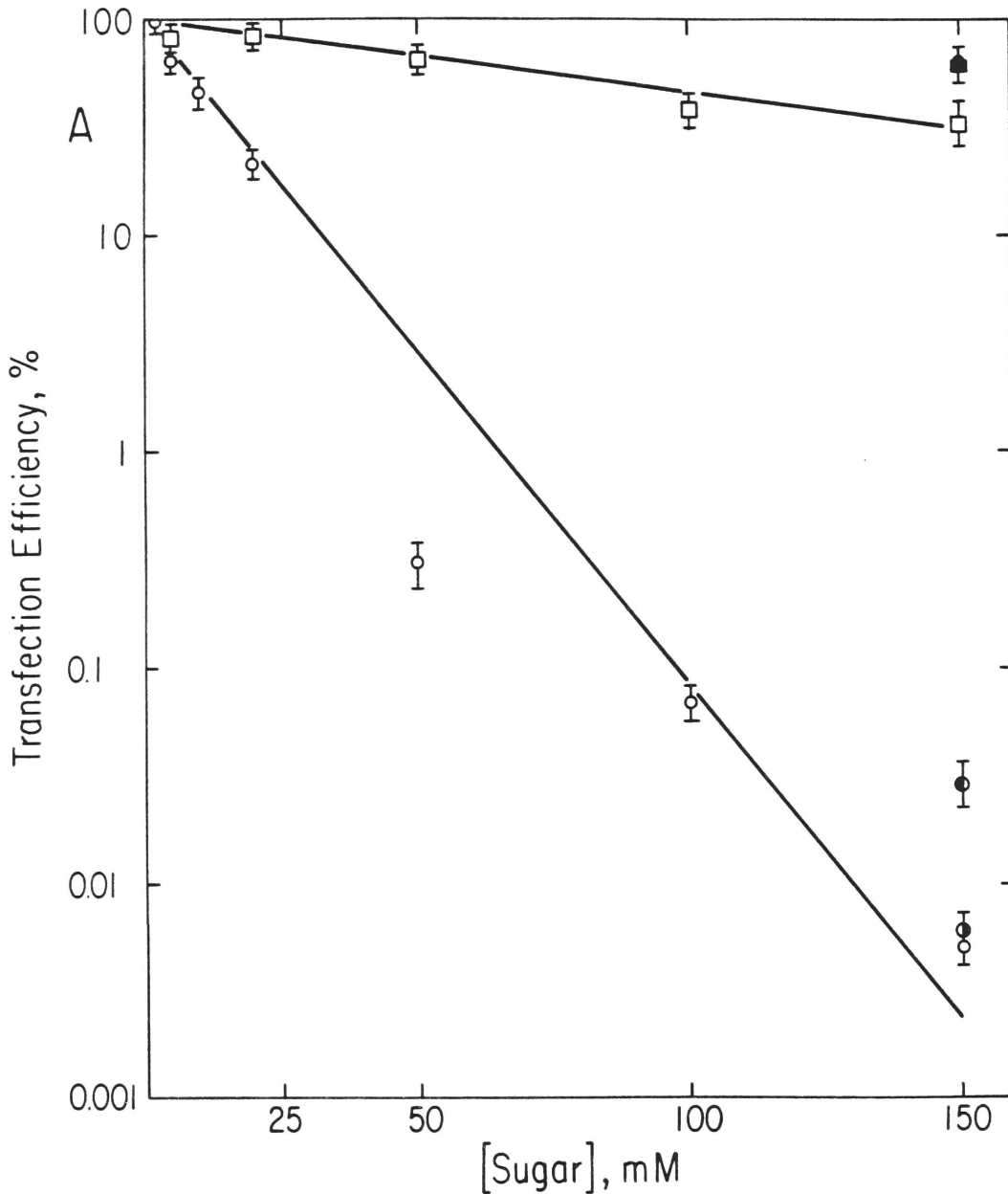


Figure 60. Transfection efficiency of f1 DNA after 8 days of incubation with different sugar concentrations. (□): glucose; (○): G6P; (■,●): incubated sugars added to the transfection assay with unincubated DNA; (●): G6P purified by ultrafiltration; (●): G6P treated with Chelex 100. Results show the mean \pm S.E.M. of duplicate experiments.

(Fig. 60).

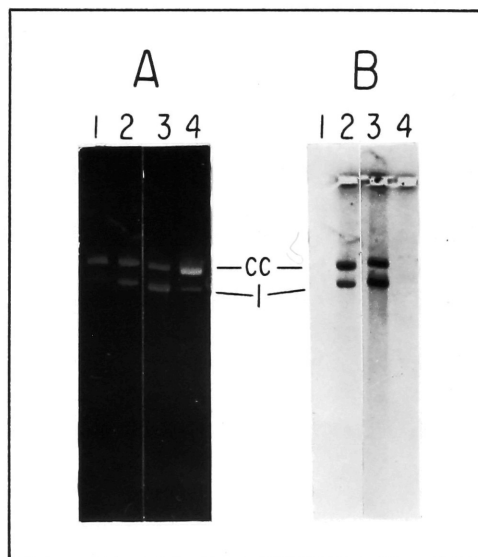
f1 DNA from the incubation mixtures was subjected to gel electrophoresis under conditions that separate covalently closed DNA from DNA in a linear form. Linear DNA would result from a single nick in the phosphodiester backbone of the circular DNA. Figure 61 shows that there is a concentration-dependent strand scission that occurs in the course of incubation with G6P. With increasing amounts of G6P (lanes 3-6), the proportion of DNA in the circular form decreases as the amount of linear form increases. At very high G6P concentrations (lanes 5 and 6), the closed circular DNA disappears and increasing amounts of low molecular weight DNA fragments become visible. Control incubations without sugar (lane 2) or with glucose (lanes 7 and 8) show only a slight increase in linear DNA when compared with unincubated DNA (lane 1). A similar progression of strand scission was observed with G6P purified by ultrafiltration.

Electrophoresis and fluorography of f1 DNA incubated with [^{14}C]G6P showed that ^{14}C is incorporated both into intact closed circular DNA as well as into linear DNA (Fig. 62). Thus, the formation of a G6P addition product precedes the event which leads to strand breakage. Figures 60 and 61 shows that stand scission also must occur at a rate slower than the rate of transfection inhibition. Lane 1 (Fig. 61), containing 8 μg of unincubated f1 DNA, shows that 80-90 of the starting DNA is in intact closed circular form. Incubation with 50 mM G6P (lane 4) decreases the amount of intact DNA by about one-half when compared with the starting material, so that the amount of intact circles is roughly equal to the amount of linear DNA present. If DNA breakage events follow a Poisson distribution, then about one-third of the

Figure 61. Alkaline gel electrophoresis of f1 DNA that had been incubated with 10, 50, 100, or 150 mM G6P (lanes 3-6) or 50 or 150 mM glucose (lanes 7 and 8). Lane 1: unin-cubated DNA. Lane 2: DNA incubated without sugar.



Figure 62. Alkaline gel electrophoresis of f1 DNA that had been incubated with [^{14}C]G6P for 6 days (lane 2) or 14 days (lane3). Lane 1: unincubated f1 DNA. Lane 4: the same DNA treated, immediately prior to electrophoresis, with [^{14}C]G6P that had been incubated alone for 14 days. (A): Ethidium bromide staining. (B): Fluorography. cc: closed circular DNA. l: linear DNA.



original molecules should remain unnicked when the number of linear and circular molecules are equal. Under the same incubation conditions however, transfection efficiency was decreased by more than two orders of magnitude (Fig. 60). While we cannot exclude contaminating nuclease activity, it alone cannot account for the observed decrease in transfection efficiency. The initial G6P adduct may be sufficient to inhibit template activity and, with time, further reaction or rearrangement occurs that destabilizes the phosphodiester backbone and causes DNA degradation.

The time dependent decrease in transfection efficiency of fl DNA after incubation with 25 mM G6P or glucose is shown in Figure 63. Inhibition was also first order with respect to incubation time. Figure 63 in addition, shows the inhibition kinetics for these sugars in the presence of 5 mM N- α -t-Boc-lysine. The ϵ -amino group of lysine should be far more reactive toward reducing sugars than the primary amino groups of nucleotides which, because of tautomerization, possess partial secondary character. The presence of lysine in the incubation mixture should thus accelerate the Maillard process. Studies of the nonenzymatic browning of proteins have shown that products of the later stages of the Maillard process are highly reactive, can crosslink proteins, and may even be mutagenic (53, 144).

As shown in Figure 63, the rate of transfection inhibition is initially less in the presence of Boc-lysine. With longer incubation times, transfection efficiency decreases, reaching a rate that is more rapid than the rate for either glucose or G6P alone. This pattern leads to the hypothesis that the amino group of the lysine initially consumes enough of the sugar to inhibit its reaction with DNA, and that with

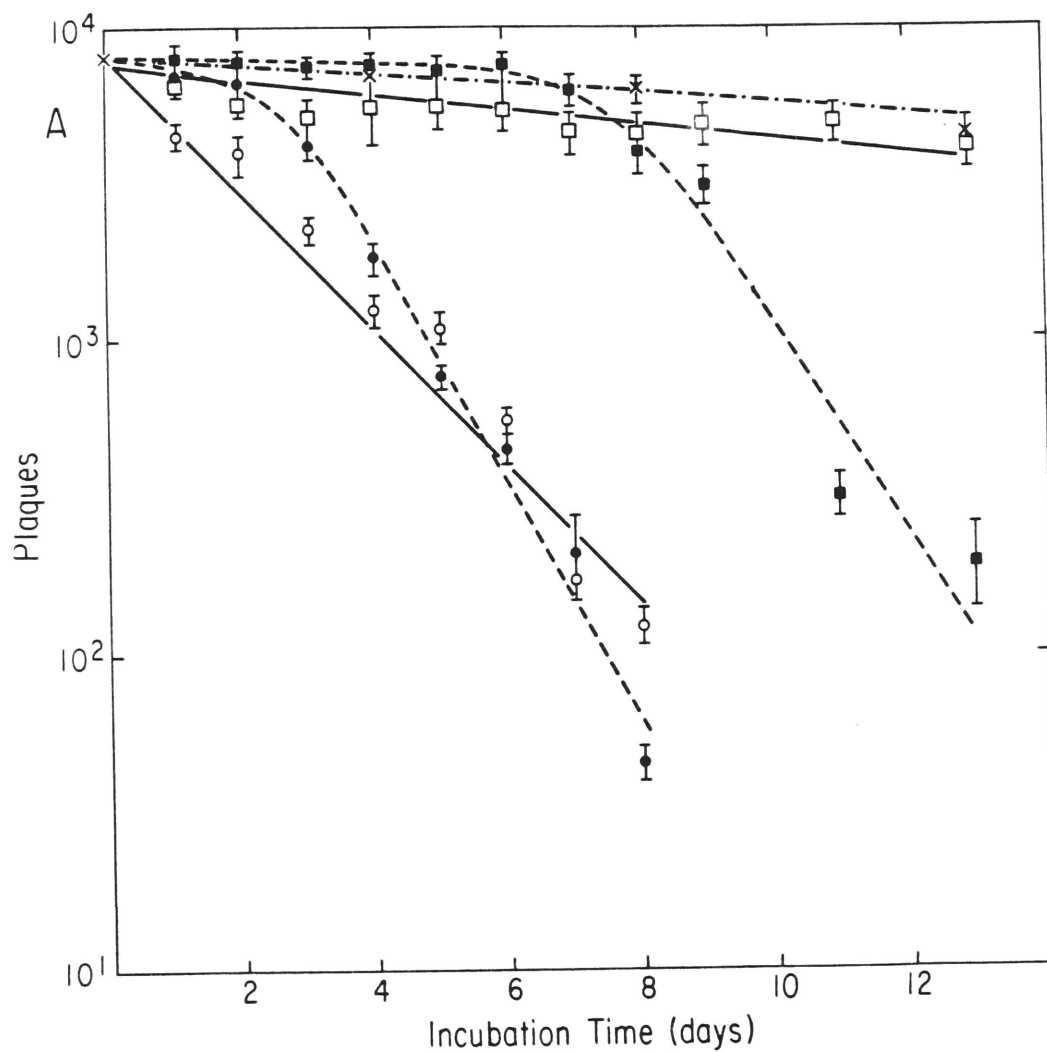


Figure 63. Rate of f1 DNA inactivation by 25 mM G6P (○), 25 mM glucose (□), 25 mM G6P/5 mM Boc-lysine (●), and 25 mM glucose/5 mM Boc-lysine (■). X: Control DNA incubated with Boc-lysine alone.

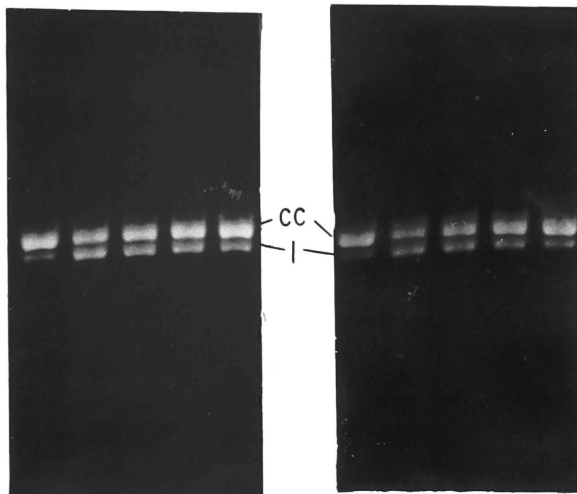
Figure 64. Alkaline gel electrophoresis of DNA incubated with 25 mM G6P (left) or 25 mM G6P/5 mM Boc-lysine (right). DNA was incubated for 0, 2, 4, 6, or 8 days. cc: Closed circular DNA. l: linear DNA.

B

G6P

G6P +
BOC-lysine

0 8 6 4 2 :days: 0 8 6 4 2



time, reactive Maillard intermediates are generated by the reaction of G6P with Boc-lysine. An alternative explanation is that lysine promotes the rate of chain breakage at apurinic sites, as has been previously been shown for a number of amines (145). This is unlikely under our experimental conditions because gel electrophoresis at various times of DNA incubated with G6P or with G6P/Boc-lysine showed no discernible difference in the progressive decrease of closed circular DNA (Fig. 64).

Products formed from the reaction of nucleic acids with simple alkylating agents often vary depending on whether the polynucleotide is in a double- or a single-stranded form. This occurs in cases in which modification affects amino positions that are hydrogen bonded in the double-stranded molecule (146). These sites are less reactive, although some reaction at these sites will occur as a result of thermal denaturation (147). *f1* exists as a ds DNA during its replicative phase. Purified Rf1 (*f1*, replicative form) was incubated with 150 mM G6P under the conditions described previously for single-stranded *f1*. Transfection analysis of Rf1 modified by incubation with G6P for 1 and 33 weeks showed inhibitory effects of 0.5 and 4 orders of magnitude, respectively. Gel electrophoresis of samples incubated for 3 weeks showed substantial (90%) chain breakage (data not shown).

We next examined the mutagenicity of G6P induced lesions in the double-stranded DNA plasmid pBR322. This plasmid contains two selectable genes, amp and tet, which confer resistance to the antibiotics ampicillin and tetracycline respectively, on an E. coli host (148). Plasmid DNA was incubated with G6P in vitro and aliquots transformed into the E.coli strain, K38. As shown in Figure 65, a total

of 17 Amp^rTet^S mutants (designated pBRGM1-pBRGM17) were isolated. Appreciable numbers of mutants were detected only after the transforming capacity of the plasmid DNA had been reduced by over 4 orders of magnitude.

The isolated mutants were tested for their level of antibiotic sensitivity. Utilizing both microbial susceptibility discs (149) and growth on antibiotic gradient plates (150), it was found that each mutant displayed a level of ampicillin resistance equivalent to that of cells bearing wild-type pBR322 and a level of tetracycline sensitivity equivalent to that of the uninfected K38 host. Mutants also were tested for Tet^S by positive selection on medium containing fusaric acid (151). This lipophilic chelating agent inhibits the growth of Tet^r cells via its toxic interaction with a tet^r gene product. Unexpectedly, one of the Amp^rTet^S strains (pBRGM13) was found to be sensitive to fusaric acid. This plasmid evidently bears an unusual mutation which inactivates Tet^r function but does not affect the toxic effect of fusaric acid.

In order to investigate structural changes in the mutant plasmids, low molecular weight DNA was prepared from subclones of the original isolates which had been maintained by serial transfer on ampicillin supplemented media (50 µg/ml). In the case of 13 of the 17 mutants, DNA yields were found to be far below expectation, even for single-copy DNA plasmids. Surprisingly, antibiotic sensitivity analysis of colonies derived from single cells of these subclones revealed that in these isolates, the Amp^r phenotype persisted but that resistance had decreased from a wild-type pBR322 level of greater than 200 µg/ml to about 50

[G6P]	Incubation time	Inactivation log	Colonies tested	Amp ^r Tet ^s	Percent mutation of survivors
0mM	25d	.08	250	0	< 0.4
150mM	"	3.2	172	2	1.1
200mM	"	4.2	46*	0	< 2.2
0mM	14d	.05	300	0	< 0.3
300mM	"	5.1	344	5	1.3
400mM	"	5.9	304	10	3.3

*only 46 colonies recovered.

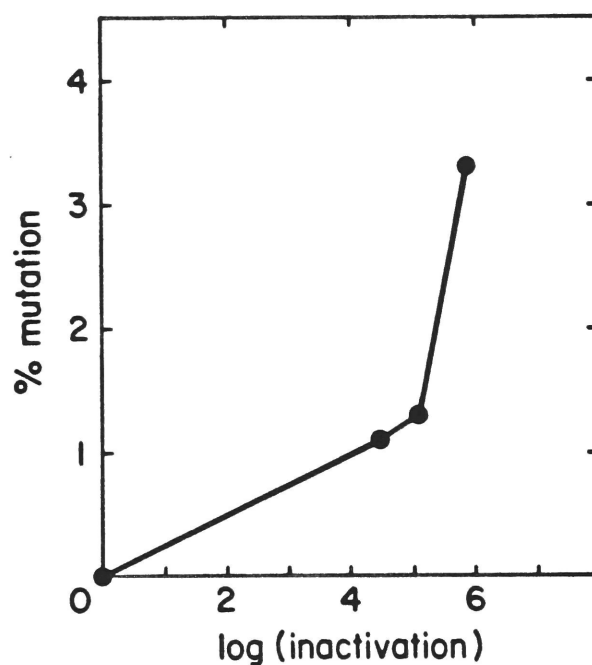


Figure 65. Summary of transformed single colonies assayed on selective media. The graph shows mutation frequency versus the log of plasmid inactivation. Inactivation, or the loss of transformation capacity, was calculated from the number of Amp^r colonies appearing after transformation with control DNA minus the number appearing with G6P-treated DNA.

μg/ml (Fig. 66). Thus, ampicillin resistance in these subclones must have been the result of intense selection for chromosomal resistance in the E. coli. host. This hypothesis was confirmed by Southern hybridization analysis of total DNA from these Amp^r subclones. Utilizing both a ³²P-pBR322 probe and a single-copy gene probe as a control, no sequence homology between pBR322 and whole cellular DNA was detected (Fig. 67).

The fraction of cells remaining Amp^r in overnight liquid cultures which had been inoculated from the original transformed colonies was then determined. A varying loss of the Amp^r phenotype was observed, presumably as the result of plasmid segregation. Therefore, the mutation in 13 of the plasmids leads not only to a loss of tetracycline resistance but also to the inactivation of function(s) required for stable replication. Furthermore, maintenance of these slowly segregating Amp^rTet^S plasmid bearing strains on ampicillin supplemented media resulted, in each case, in the emergence of E. coli colonies which had developed chromosomal resistance to high levels of ampicillin.

DNA from the plasmid bearing strains was then analyzed by Southern hybridization using, as substrate, whole E.coli DNA prepared from a liquid culture inoculated from the original transformed colony. A chimeric plasmid consisting of pBR322 and an E. coli DNA fragment containing the thioredoxin gene (152) was labeled by nick-translation and used as a probe. Since the thioredoxin gene is present in only a single copy in the E.coli chromosome, comparison of the hybridization intensity of this sequence with plasmid sequences would indicate loss of plasmid copy number due to rapid segregation. Figure 68 shows the hybridization pattern obtained for whole E.coli DNA derived from 16 of

Figure 66. Ampicillin resistance level measured with ampicillin gradient plates. K38•Amp^r1 and K38•Amp^r2 denote two subclones isolated from the original mutant-plasmid bearing colonies. pBRGM1-pBRGM17 denote the resistance phenotype of the original mutant-plasmid bearing colonies.

AMPICILLIN RESISTANCE LEVEL

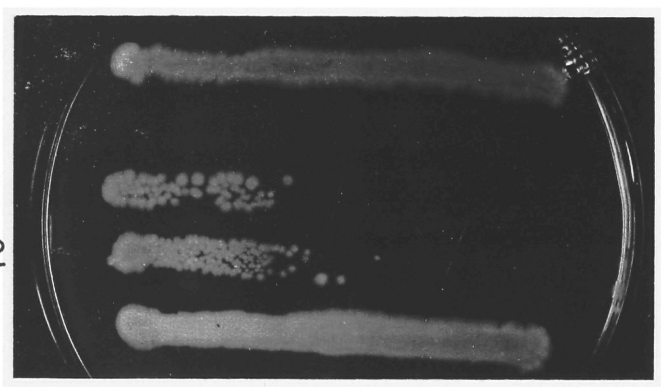
pBR322

K38

K38·Amp^r 1

K38·Amp^r 2

pBRGM1 –
pBRGM17



[ampicillin]
 $\mu\text{g/ml}$

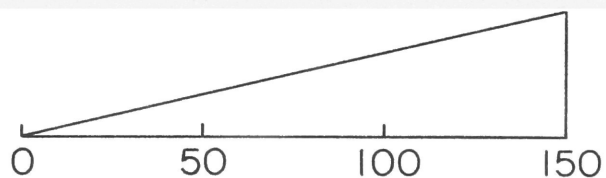
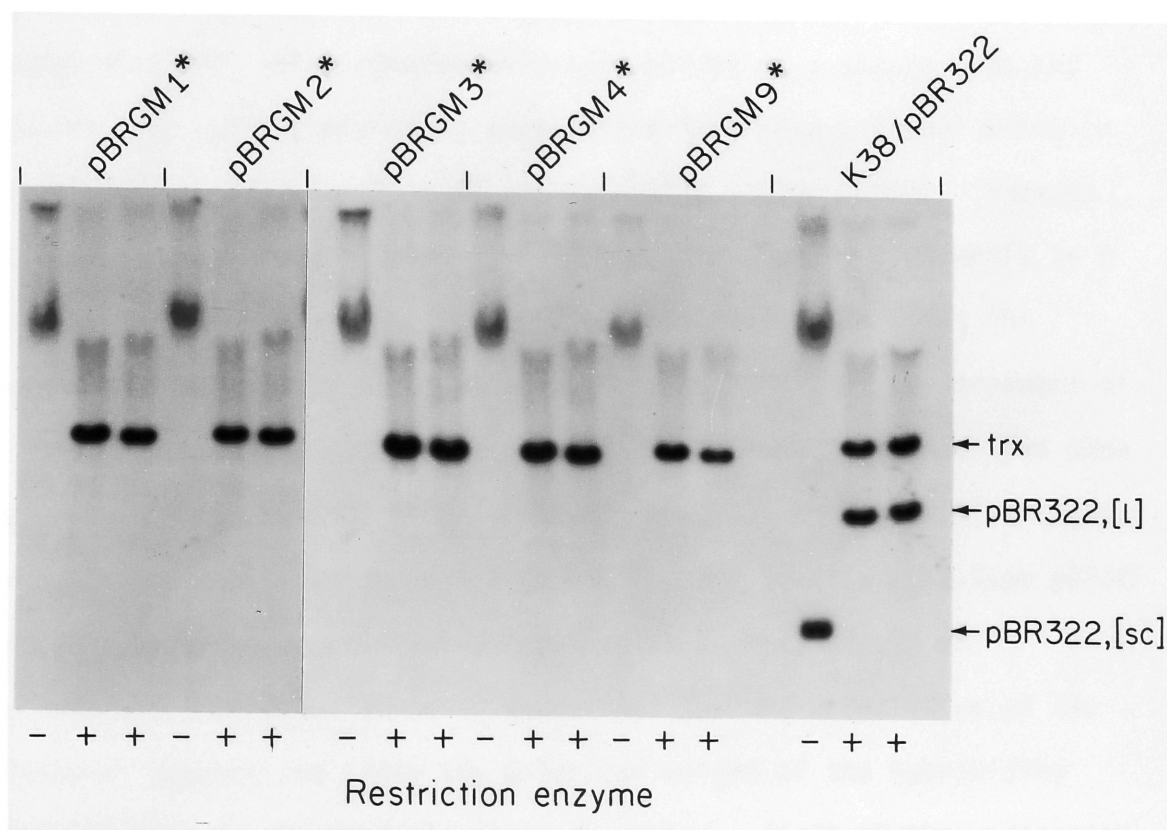


Figure 67. Southern hybridization analysis of whole *E. coli* DNA derived from the amp^r subclones (pBRGM1* - pBRGM9*) and pBR322 bearing K38. DNA was probed with a chimeric ³²P-pBR322-thioredoxin gene plasmid, both with (+) and without (-) prior digestion with the restriction enzymes Hind III or Sal I. trx: the position of the host, single-copy thioredoxin gene. l: linear plasmid sequences. sc: supercoiled plasmid sequences.



the 17 mutants. The hybridization intensity of pBRGM13, for example, is very low relative to wild-type pBR322 and roughly equivalent to the intensity of the thioredoxin gene (Fig. 68A and B). Thus the copy number of this plasmid has substantially decreased relative to wild-type pBR322 and a significant amount of plasmid segregation had occurred during growth in liquid culture.

Alternating lanes in Fig. 68 show DNA digested with the restriction enzyme Hind III, which cleaves wild-type pBR322 at a single site and converts the rapidly migrating supercoiled form into a linear molecule which migrates as a function of its molecular weight (153). Cleavage also serves to reduce multimeric but otherwise identical plasmids to a single species. Plasmid multimers occur spontaneously during the replication of unmutagenized pBR322. Four mutants show the presence of a large insertion (pBRGM1, pBRGM4, pBRGM12, and pBRGM15), and five show large deletions (pBRGM2, pBRGM7, pBRGM8, pBRGM13, and pBRGM14). Eight of the 17 mutant plasmids appear to be the same size as wild-type pBR322 and presumably bear mutations which involve a small number of nucleotides (<50 bp). Table 13 summarizes the characteristics of the different mutants and lists the molecular weight of the hybridizing bands present in the Hind III treated samples. Eight of the undigested lanes appear to contain small amounts of additional supercoiled plasmids which are larger in size than the major hybridizing species. In the case of four of these mutants (pBRGM5, pBRGM6, pBRGM10, and pBRGM12), Hind III cleavage gives rise to DNA fragments whose sizes suggest that they are linear monomers and dimers (Table 13). Thus, the highest molecular weight Hind III fragment is most likely the product of incomplete digestion of the parent dimer. In the case of four other

Figure 68. (A) Southern hybridization of whole E. coli DNA from cells containing the mutant plasmids (pBRGM1-pBRGM16), wild type plasmid (pBR322), and no plasmid (K38). The lane marked STDs includes base-pair markers obtained from an Eco RII restriction digestion of pBRGM15 plus a sample of pBRGM15 cleaved with Hind III. [sc]: denotes supercoiled pBR322. [l]: denotes linear form pBR322. thrd.: indicates the chromosomal DNA fragment containing the thioredoxin gene.

(B): Autoradiogram of pBRGM13 lane exposed for longer than in (A) in order to demonstrate the comparable hybridization intensities of pBRGM13 and the thioredoxin gene.

[illegible]

Western blot analysis of pBRGM13. The blot shows two lanes labeled '-' and '+'. An arrow points to a band in the '+' lane labeled 'thrd.'

Plasmid	Segregation Phenotype	Fusaric Acid Sensitivity	DNA (bp)
pBR322	-	-	4363
pBRGM1	+	+	5200
pBRGM2	+	+	3500 ^m , 9800
pBRGM3	+	+	4360
pBRGM4	+	+	4700 ^m , 9800
pBRGM5	+	+	4360 ^m , 9800 ^d
pBRGM6	-	+	4360 ^m , 9800 ^d
pBRGM7	+	+	3600
pBRGM8	+	+	3500 ^m , 9800
pBRGM9	+	+	4360
pBRGM10	+	+	4360, 9800 ^d
pBRGM11	+	+	4360
pBRGM12	+	+	5400 ^m , 7400, 11,000 ^d
pBRGM13	+	+	3000
pBRGM14	+	+	3500 ^m , 9800
pBRGM15	-	+	5131
pBRGM16	-	-	4360
pBRGM17	-	+	4360

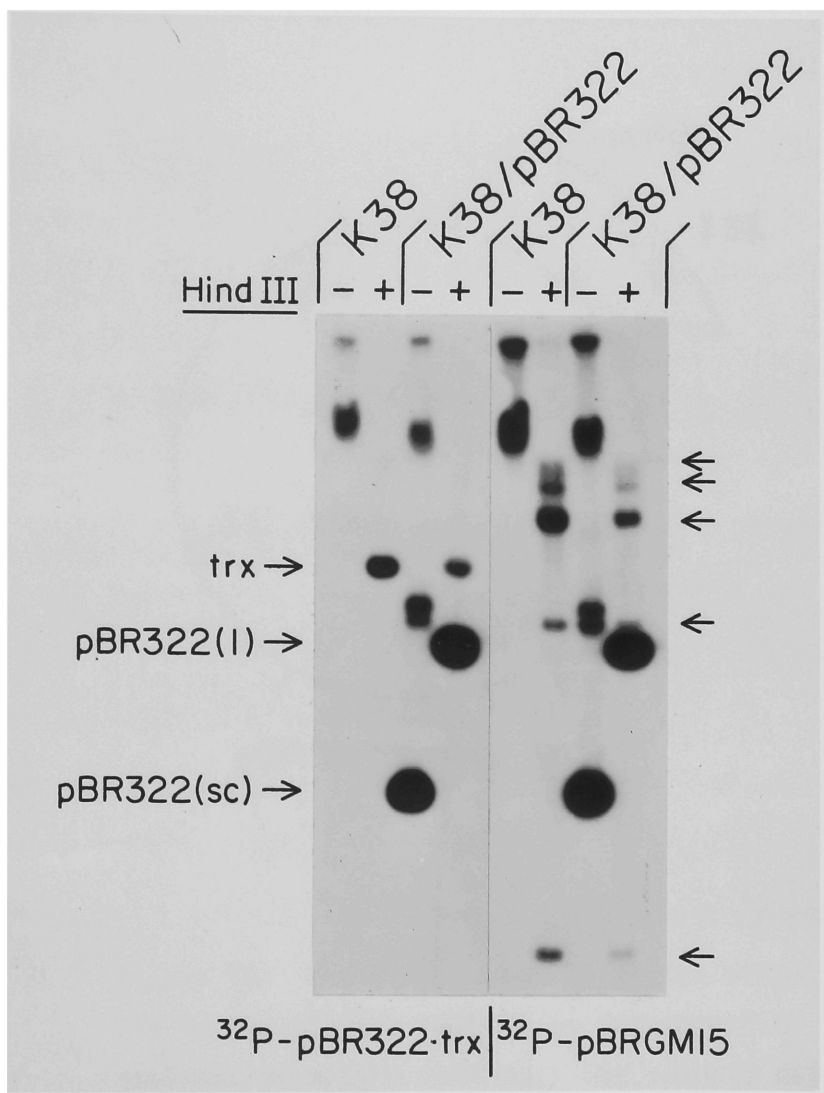
Table 13. Summary of mutant characteristics. DNA size is derived from Hind III digested samples and is estimated from a plot of $\log[\text{bp}]$ versus R_f and the 6 DNA standards described in Figure 2. The DNA size of pBRGM17 was determined in separate experiments not displayed in Figure 2. (m):denotes the major species, (d):denotes a probable dimer form.

mutants, (pBRGM2, pBRGM4, pBRGM8, and pBRGM14), Hind III treatment produces fragments whose sizes do not correspond to products of a single plasmid species plus an associated multimer. Therefore, cells derived from these clones contain several plasmid species; a predominant low-molecular weight plasmid and small amounts of a pBR322-dimer size molecule.

A mutant displaying stable replication (pBRGM15) was further analyzed in order to ascertain the origin of its large insertion. Southern hybridization with a ^{32}P -pBRGM15 probe of E.coli DNA from a strain lacking plasmid demonstrated the presence of host sequences complementary to pBRGM15 (Fig. 69). Sequential restriction mapping of pBRGM15 with the enzymes EcoR II and Sal I demonstrated that the insertion is located within the tet^r gene, is 770 bp in length, and contains an Eco RII site about one-sixth of the distance from one end (Figure 70). These data are consistent with the interpretation that pBRGM15 has acquired the insertion element IS1; a mobile element present in the E.coli genome which is 768 bp long and contains a single Eco RII site 655 bp downstream from its origin (154).

To inquire into the possible role of DNA repair mechanisms in the expression of G6P induced DNA lesions, pBR322 DNA modified by incubation with G6P was transformed into a uvrC⁻ strain (K704). This strain lacks one subunit of the uvrABC excision nuclease, an enzyme which has been shown to remove a number of bulky DNA adducts (155, 156) Transformation of modified DNA into this strain was found to result in no detectable mutagenesis (0 of 648 colonies screened). Transformation of DNA from the same incubation mixture into strain K38 produced mutations at the expected frequency. This result would appear to implicate the uvrABC

Figure 69. Southern hybridization of whole E. coli DNA from host strain K38 and strain K38 bearing the plasmid pBR322. (left): DNA probed with the chimeric thioredoxin gene containing plasmid, ^{32}P -pBR322·trx. (right): DNA probed with the mutant plasmid ^{32}P -pBRGM15. trx: position of the chromosomal thioredoxin gene. l: linear form pBR322. sc: supercoiled pBR322. The right arrows denote sequences present in K38 which are complementary to pBRGM15.



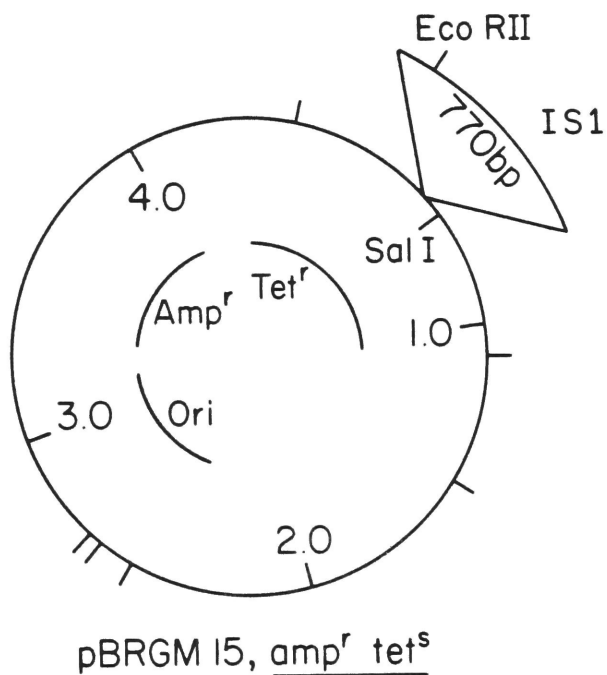


Figure 70. Position of the insertion element IS1 in the plasmid pBRGM15 as determined by restriction enzyme mapping. The numbers denote kb. units. The outside lines show the position of Eco RII sites.

nuclease in the induction of mutagenic lesions. The gross chromosomal alterations observed in a number of the pBRGM mutants however, do not appear to be characteristic of the simple excision repair activity described for this enzyme and may be indicative of more extensive damage which is only partially excised.

The in vitro inactivation of double-stranded pBR322 allows one to predict the frequency of G6P damage which may be occurring in a larger genome. Although one naturally must be circumspect in this type of estimation, it does signify possible biological relevance. Figure 71 shows the loss of the capacity of pBR322 to transform E.coli to Amp^r with time. This data is derived from the results of Figure 65. Loss of transformation is linear with time and proceeds at a rate which is 4.5 orders of magnitude slower than the rate of single-stranded phage DNA (Figure 60). Assuming that DNA damage is proportional to G6P concentration (Figure 60) and that the intracellular G6P concentration is 0.25 mM (159), then a 4.2 log inactivation (25 days for 200 mM G6P, Fig. 71) for the ampicillin gene would indicate that the human genome on the average might suffer 2.7 G6P "hits" per day. Additional damage would be anticipated from the reaction of other reducing sugars, e.g. glucose, glyceraldehyde-3-P₀₄, with DNA.

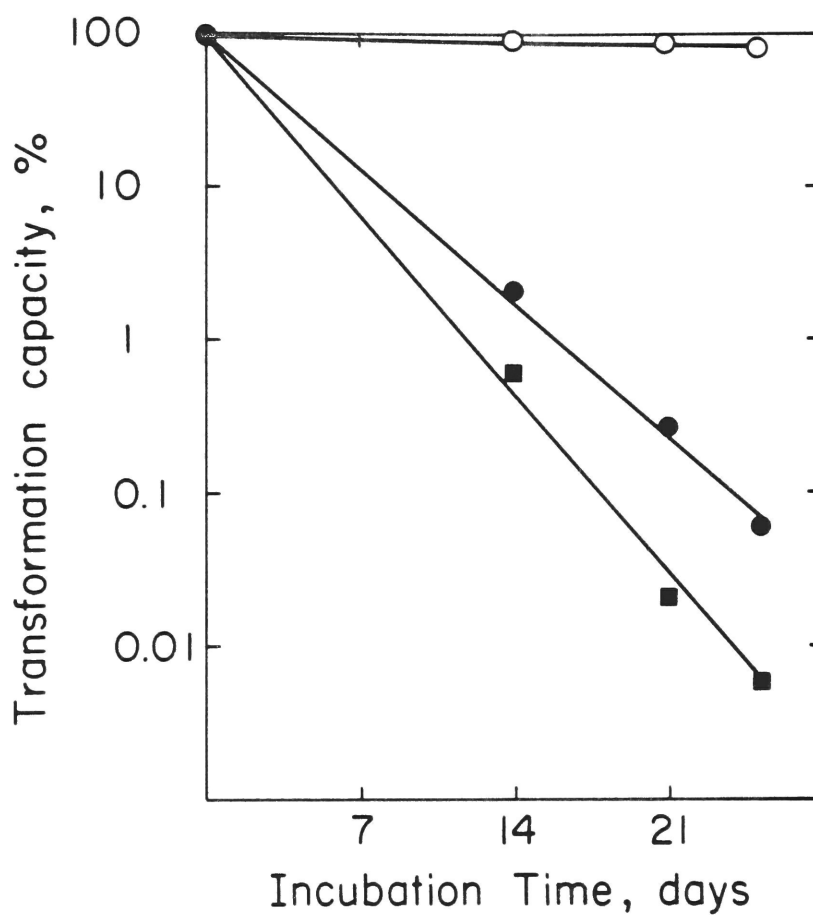


Figure 71. The loss of the ability of pBR322 to transform E.coli to Amp^r with time at 0 mM G6P (○), 150 mM G6P (●), and 200 mM G6P (■).

B. Conclusion.

The incubation of DNA or single nucleotides leads to absorbance and fluorescence changes which suggest that DNA can be glycosylated and undergo nonenzymatic browning. Spectral changes were not observed with TMP, which lacks a primary amino group, nor with ds DNA, in which hydrogen bonding may hinder the amino groups from reacting. Incubation of single-stranded viral DNA with these sugars causes a time and concentration dependent decrease in transfection efficiency. G6P also was observed to induce strand scission at a rate slower than the rate of transfection inhibition.

These results suggest that G6P, and glucose at a much slower rate, forms adducts with the primary amino groups of bases that inhibit template function. With time, these adducts undergo chemical rearrangement that in a manner analogous to DNA alkylating agents, can labilize the glycosidic bond between the purine and the deoxyribose. This leads to depurination and, after still more time, β -elimination and strand scission.

DNA which has been nonenzymatically modified by G6P in vitro also leads to mutations in a prokaryotic host. Analysis of these mutants provided two surprising observations. The first is that the majority of the isolated mutants (76%) are unstable and undergo segregation. This result was unexpected but probably is not specific for G6P induced damage. It is possible that DNA damaging agents in general, lead to a high percentage of unstable mutants, but that progeny from these mutants are rapidly lost and escape detection.

The second unusual finding is the striking degree of DNA alteration

which had occurred in several of the isolated mutants. The one stable plasmid which was analyzed appears to contain the common E. coli insertion element IS1, but other mutant plasmids most likely have undergone more dramatic structural changes. The mechanism by which these DNA alterations arise is unknown, but is presumably the result of single, and perhaps multiple deletions, insertions, or duplications. Preliminary experiments implicate the uvrABC pathway in the expression G6P induced mutations, but the DNA rearrangements observed appear not to be characteristic of simple nucleotide adducts and may be the consequence of extensive damage such as interstrand cross-linkage.

Products formed from the in vivo reaction of reducing sugars with nucleic acids have not yet been isolated. The occurrence of these products however, seems likely in view of the reactivity of DNA toward reducing sugars, the long-life of nucleic acids in the resting cell, and much evidence indicating that proteins undergo nonenzymatic glycosylation in vivo. The observation that an intracellular metabolite can modify DNA suggests a mechanism for the accumulation of genetic lesions that may lead to cellular senescence. Free amines within the cell might further contribute to DNA damage by nonenzymatic browning, as the experiments with the simultaneous incubation of Boc-lysine suggest. The occurrence of Maillard products on nuclear proteins might account for the increased crosslinking of proteins to DNA that has been observed in the chromatin of aged organisms (157). Alternatively, basic proteins, which are bound to at least 50% of the mammalian genome (158), may play a protective, shielding role. In any event, the induction of nucleic acid damage by endogenous sugars implies that nonenzymatic reactions of this type may contribute to the decreased genetic viability and increased tumorigenesis observed in the aged organism.

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