[CONTRIBUTION FROM THE DEPARTMENT OF MEDICAL RESEARCH OF THE UNIVERSITY OF TORONTO]

The Conjugation of Estrogens with Proteins. I[1a]

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The possibility of immunizing animals against hydrocarbon carcinogenesis by the injection of antigens prepared by the conjugation of carcinogenic hydrocarbons with proteins has been investigated by one of us.[2,3,4] Linkage of the isocyanate of the carcinogen evidently was effected chiefly through the free amino groups of the protein. Fieser and Creech[5] later conjugated hydrocarbons with amino acids in a similar manner. The possibility that, if an antigen were synthesized by combining a protein with an estrogenically active substance a much shorter time would be required for demonstrating any immunizing effect, led us to explore methods of preparing suitable derivatives of estrone and 4,4'-dihydroxy-α,β-diethylstilbene (diethyl stilbestrol).[6]

A means of conjugation that seemed promising was made available through the synthesis of certain ethers on the phenolic hydroxyl groups. The estrogen methyl ethers are stable and their physiological activity, though not so intense as that of the phenols, is in general more prolonged.[6,7] Other workers[8] have diazotized *p*-aminodiphenyl ether, coupled it with horse serum and found the conjugated protein to possess specific antigenic properties. We have prepared estrone *p*-nitrophenyl ether in 65% yield by a slight modification of Rarick's method for diaryl ethers.[9] Reduction of the nitro group proceeded smoothly at 20°, mainly according to Thiele[10] to give the *p*-aminophenyl ether. That the carbonyl group at C-17 was unaffected by the reduction was shown by the formation of a semicarbazone and a 2,4-dinitrophenylhydrazone. Both of these differed from the corresponding derivatives of estrone itself; estrone 2,4-dinitrophenylhydrazone was prepared for comparison. Acetylation of the amine gave estrone *p*-acetylaminophenyl ether. Estrone *p*-aminophenyl ether was readily diazotized in aqueous suspension and coupled with β-naphthol to give a scarlet compound and with *l*-tyrosine to form an orange product. Under similar conditions the diazotized amine appeared to couple with casein. The protein (6.5% tyrosine) was employed in slight excess over the amount required for reaction of one mole of tyrosine with one mole of amine, with the purpose of preventing as far as possible conjugation through both available nuclear positions of tyrosine and through other groups in the protein molecule.[11] A 92% by weight yield of azoprotein was obtained. The product was orange-yellow in color even after continuous extraction in the cold with organic solvents, whereas control casein remained colorless throughout. The solubility of the conjugated protein in dilute alkali was considerably less than that of the control protein.

Early trials indicate that the *p*-aminophenyl ether of estrone possesses physiological activity of a moderately high order (10 γ in olive oil produced estrus in more than 50% of the rats tested). An estrone phenyl ether *p*-azocasein preparation (aqueous solution) induced a more prolonged response in a dose of 830 γ but was inactive in 450 γ. On the basis of its estrone content the conjugate is about one-tenth as active as estrone *p*-aminophenyl ether. Other conjugated estrogens also possess a much lower activity than the free estrogens.[12]

The conjugation with casein of the simple compound phenyl *p*-aminobenzyl ether also has been investigated. Reduction of phenyl *p*-nitrobenzyl ether took place readily at 0° but the amine proved to be unstable. On attempted crystallization from cold organic solvents or even on standing in contact with the air the substance decomposed to phenol and a high-melting amorphous substance. Isolation of the pure ether finally was accomplished by selective adsorption of impurities on activated alumina. Phenyl *p*-aminobenzyl

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(1b) Eli Lilly Research Fellow, 1938–1940.

(2) Creech and Franks, *Am. J. Cancer*, **30**, 555 (1937).

(3) Creech and Franks, THIS JOURNAL, **60**, 127 (1938).

(4) Franks and Creech, *Am. J. Cancer*, **35**, 203 (1939).

(5) Fieser and Creech, THIS JOURNAL, **61**, 3502 (1939).

(6) Dodds, Golberg, Lawson and Robinson, *Proc. Roy. Soc.* (London), **B127**, 140 (1939).

(7) Girard, *Bull. soc. chim. biol.*, **15**, 562 (1933).

(8) (a) Erlenmeyer and Berger, *Biochem. Z.*, **252**, 22 (1932); (b) Jacobs, *J. Gen. Physiol.*, **20**, 353 (1937).

(9) Rarick, Brewster and Dains, THIS JOURNAL, **55**, 1289 (1933).

(10) Thiele and Dimroth, *Ann.*, **305**, 114 (1899).

(11) Marrack, "The Chemistry of Antigens and Antibodies," Medical Research Council, London, 1938, pp. 71–72.

(12) The sodium salt of estrone sulfuric acid is only one-fiftieth as active as estrone (Butenandt and Hofstetter, *Z. physiol. Chem.*, **259**, 222 (1939)). The sodium salt of the estriol-glucuronic acid complex is only one-seventeenth as potent as estriol on subcutaneous injection in mice (Marrian, *Symposia Quant. Biol.*, **5**, 16 (1937)).

ether could be diazotized readily and coupled with β-naphthol to form a scarlet azo compound and with *l*-tyrosine to give an orange product that soon turned brick red. Conjugation with casein produced a bright orange azoprotein the color of which was not extractable with organic solvents in the cold.

Estrone *p*-nitrobenzyl ether and diethyl stilbestrol di-(*p*-nitrobenzyl) ether were synthesized but neither could be reduced to the corresponding amino ether.

Estrone *p*-aminophenyl ether, treated in the customary way[5] with phosgene in benzene–toluene solution gave what appears to be estrone phenyl ether *p*-isocyanate in good yield. The crude product reacted rapidly with methyl and ethyl alcohol to give two distinct crystalline compounds analyzing correctly for the methyl and ethyl carbamates.

Attempts were made to bring about a coupling of the estrogens with diazotized *p*-nitroaniline and a subsequent reduction of the azo-compound. Marrian[13] noticed a purple color with diazotized *p*-nitroaniline and estrone, and Schmulovitz and Wylie[14] also obtained evidence of coupling. In our hands estrone and estrone methyl ether both appeared to react with the diazotized amine in acetic acid solution but reduction of neither product to an amino phenol could be effected. Diethyl stilbestrol and its dimethyl ether coupled much less completely.

Experimental

Estrone *p*-Nitrophenyl Ether.—Estrone (2.04 g.) was converted to its potassium salt with methanolic potassium hydroxide and the dry salt was heated for four hours at 200–210° with 200 mg. of freshly prepared copper catalyst[15] and 4 g. of *p*-nitrofluorobenzene. The latter was removed by steam distillation. The dark brown residue was dissolved in acetone, boiled with Norit and filtered. In order to remove any unreacted estrone an acetone–alcohol solution of the product was poured into 5 volumes of hot *N* sodium hydroxide solution and the mixture was filtered. The filtrate on acidification gave only 30 mg. of colorless solid. The alkali-insoluble substance was crystallized from acetone-methanol-water (1.90 g., 65%). Five more crystallizations from the same solvent gave glistening light-yellow plates, m. p. 192–194°.

Anal. Calcd. for $C_{24}H_{23}O_4N$: N, 3.60. Found: N, 3.62, 3.62.

Reaction at 160° yielded 1.2 g. of unreacted estrone (crystallized from alcohol and characterized by m. p. and mixed m. p.) which could be used over again at the higher temperature noted above. When *p*-nitrochlorobenzene was used the best yield (30% semi-pure) was obtained at 145°. Higher temperatures produced tars; at 205° the yield of nitro ether was zero.

Estrone *p*-Aminophenyl Ether.—Estrone *p*-nitrophenyl ether (1.60 g.) was dissolved in 40 cc. of the reducing solution.[10] The stannous chloride salt of the amine began to separate after ten minutes, but the mixture was allowed to stand with occasional shaking at 20° for twenty-four hours. The almost colorless needles (1.75 g., m. p. about 295° dec.) were filtered off, washed with acetic acid and dried *in vacuo*. The salt was dissolved in 30 cc. of methanol in the cold and the solution poured with stirring into an excess of *N* sodium hydroxide solution. It was sometimes necessary to repeat this operation in order to obtain complete decomposition of the salt. The amine separated in 70% yield (1.04 g.). Recrystallized as colorless plates from dilute methanol (Norit) it melted at 166.5–168.5°.

Anal.[16] Calcd. for $C_{24}H_{25}O_2N$: C, 80.19; H, 7.02; N, 3.90. Found: C, 80.23, 80.29; H, 7.17, 7.29; N, 3.80, 3.78.

The **picrate** separated from benzene as clusters of lemon-yellow microscopic crystals, m. p. about 160° dec. (darkening at 120°). Like estrone picrate[7] it appears to be readily dissociable since it could not be recrystallized without considerable decomposition.

Anal. Calcd. for $C_{30}H_{28}O_8N_4$: N, 9.52. Found: N, 8.90, 8.85.

The amine does not form a trinitrobenzene complex.

Estrone *p*-Aminophenyl Ether Semicarbazone.—Using Butenandt's procedure for estrone,[17] with methanol as solvent instead of ethanol, we obtained minute colorless needles, m. p. about 295° after recrystallization from pyridine–dioxane.

Anal. Calcd. for $C_{25}H_{28}O_2N_4$: N, 13.46. Found: N, 13.65.

Estrone *p*-Aminophenyl Ether 2,4-Dinitrophenylhydrazone.—This derivative, prepared according to the general method of Allen,[18] was recrystallized as clusters of very small, orange-yellow crystals, m. p. 238–240° dec., from pyridine–alcohol–water.

Anal. Calcd. for $C_{30}H_{29}O_5N_5$: N, 12.99. Found: N, 12.96.

Estrone 2,4-Dinitrophenylhydrazone.—This substance separated from dioxane–alcohol as minute yellow crystals, m. p. 278–280° dec. (darkening at 268°).

Anal. Calcd. for $C_{24}H_{24}O_5N_4$: N, 12.50. Found: N, 12.30.

Estrone *p*-Acetylaminophenyl Ether.—The amine could be acetylated by boiling for five minutes in acetic acid–anhydride or by boiling for fifteen seconds in pure acetic anhydride. The crude product in both cases melted at 166–169° (mixed m. p. with the amine, 145–160°). Crystallization from aqueous methanol gave hydrated colorless needles, m. p. 201–202° (softening at 100° and again at

(13) Marrian, *Biochem. J.*, **24**, 1021 (1930).

(14) Schmulovitz and Wylie, *J. Biol. Chem.*, **116**, 415 (1936).

(15) "Organic Syntheses," John Wiley and Sons, Inc., New York, N. Y., 1934, Vol. XIV, p. 66.

(16) Carbon–hydrogen analyses by Ayerst, McKenna and Harrison, Ltd., Montreal.

(17) Butenandt, Störmer and Westphal, *Z. physiol. Chem.*, **208**, 168 (1932).

(18) Allen, THIS JOURNAL, **52**, 2955 (1930).

170°, then resolidifying). When dehydrated over phosphorus pentoxide the substance melted at 201–202° (softening at 170°). Crystallization of the crude product from benzene–ligroin gave colorless needles, m. p. 202–204° (softening at 170–172°, then solidifying).

Anal. Calcd. for $C_{26}H_{27}O_3N$: N, 3.49. Found: N, 3.39.

Phenyl *p*-Aminobenzyl Ether.—The nitro ether, prepared according to Reid[19] in 86% yield (pure), melted at 91.5–93.5° (Reid, m. p. 91°). Morgan's method[20] of reducing nitro ketones to amino ketones gave a pale yellow amorphous substance (27%), m. p. above 340°. Reduction of the nitro ether (2.0 g.) with stannous chloride and hydrogen chloride in glacial acetic acid at 0° yielded 3.72 g. of the stannous chloride salt of the amine as glistening pale yellow plates. The amine was produced (1.1 g., 63%) on twice triturating the salt with 4% sodium hydroxide solution at 20°. Attempts to crystallize the substance resulted in decomposition to phenol and a colorless solid, m. p. about 200°. The crude amine therefore was dissolved in 15 cc. of methanol (acetone-free) in the cold, stirred with Norit and filtered through a tower of activated alumina. An impurity fluorescing blue-violet in ultraviolet light remained on the filter. Dilution of the filtrate with water gave lustrous colorless plates, m. p. 71–73° (evacuated capillary).

Anal.[16] Calcd. for $C_{13}H_{13}ON$: C, 78.34; H, 6.58; N, 7.02. Found: C, 78.74, 78.83; H, 6.62, 6.77; N, 6.94, 6.83.

The **picrate** precipitated from cold benzene as clusters of small yellow plates, m. p. 80.5–82.5°.

Anal. Calcd. for $C_{19}H_{16}O_8N_4$: N, 13.08. Found: N, 12.28 (first preparation), 12.17 (second preparation).

On warming in organic solvents the picrate was converted rapidly into an amorphous orange solid. Similar decomposition occurred within a few days when the dry solid was allowed to stand in a test-tube.

Coupling Reactions of the Diazotized *p*-Aminopheny Ether of Estrone.—The amine (15 mg.) dissolved readily to give a pale green-yellow solution when diazotized at 0° with sodium nitrite and dilute hydrochloric acid. One-third of the solution was added to a dilute sodium hydroxide solution of 5 mg. of β-naphthol (*p*H 9–10) giving a bright scarlet precipitate moderately soluble in alcohol, more soluble in acetone. Another third of the diazo solution added to an aqueous alkaline solution of 5 mg. of *l*-tyrosine produced an immediate orange precipitate. In the case of *l*-tryptophan, however, the precipitate formed was no deeper in color (pale yellow) than that produced in a blank experiment.

Conjugation with casein was tried first in 0.1 *N* sodium carbonate solution. An ice-cold solution of the diazotized amine was added with stirring to a freshly prepared aqueous solution of casein (British Drug Houses light white soluble passed through 100-mesh screen) kept at 20°. An orange color appeared almost at once and coupling seemed complete after ten minutes, since there was no further deepening of color. The azoprotein was precipitated by adding dilute acetic acid to *p*H 4.5, the mixture was centrifuged and the product washed in the cold, first with water and then with acetone until color could no longer be removed. The extracted azoprotein, bright orange in color, did not dissolve completely in dilute sodium carbonate solution even after several hours, although it became gelatinous in appearance. When pyridine was added up to 30% by volume the protein dissolved readily.

In an effort to obtain a more soluble estrone-azoprotein the following method was adopted. Casein (850 mg.), dissolved by gentle stirring in dilute sodium hydroxide solution (40 cc.) at *p*H 9–10, was cooled to 0° and treated over a half-hour period with a diazo solution prepared from estrone *p*-aminophenyl ether (100 mg.), water (15 cc.), concentrated hydrochloric acid (3 drops) and sodium nitrite (25 mg.). Occasional addition of dilute alkali kept the *p*H at about 8–10 (measured colorimetrically). The solution turned yellow at first then orange after fifteen minutes. After this time the *p*H could not be determined with any accuracy by the colorimetric method; however, the same rate of addition of reagents was maintained throughout. The solution was then stirred for one-half hour at 20° and the azoprotein was precipitated and extracted as before. Further washing with cold dioxane and with ether removed no color. On extraction for six hours with acetone in a Soxhlet at room temperature, no color was dissolved out after the first siphoning of liquid. The orange-yellow azoprotein, dried *in vacuo* (880 mg., 92%) swelled somewhat in sodium carbonate solution but had not dissolved completely after twenty hours at 20°. The azoprotein dissolved almost entirely in cold, dilute sodium hydroxide and the "solution" could be readjusted within a few minutes with dilute acetic acid to *p*H 8 without reprecipitating the protein. The bulk of the azoprotein again separated at *p*H 4.5–5. Control experiments showed that, in the absence of estrone *p*-aminophenyl ether, neither the color nor the solubility of the casein was noticeably affected by any of the reagents used: the colorless control protein dissolved in dilute sodium carbonate solution within a few minutes.

Coupling Reactions of Diazotized Phenyl *p*-Aminobenzyl Ether.—The procedures followed were identical with those given for estrone *p*-aminophenyl ether. Coupling did not seem to occur with *l*-tryptophan, the product being almost colorless. Although the solubility of the orange azocasein in dilute alkali was noticeably lower than that of control casein, the azoprotein could be dissolved in dilute alkali and the *p*H readjusted to 8 without further decreasing the solubility of the product.

Reaction of Estrone *p*-Aminophenyl Ether with Phosgene.—Estrone *p*-aminophenyl ether (200 mg.) in dry benzene (15 cc.) was boiled under reflux for one-half hour with tenfold excess of phosgene (in toluene). All but 5 cc. of the solvent was then distilled off in an atmosphere of nitrogen under reduced pressure. The remaining solvent was removed in a vacuum desiccator. The residue was dissolved in dry carbon tetrachloride (Norit), the filtrate was evaporated to a small volume and then treated with 10 volumes of petroleum ether. After standing for an hour at 5°, colorless plates (m. p. 138–143°) separated in hemispherical clusters. The yield was 155 mg. (72%). The substance was extremely soluble in carbon tetrachloride and benzene. Recrystallizations from benzene–ligroin and

(19) Reid, THIS JOURNAL, **39**, 304 (1917).

(20) Morgan and Hickinbottom, *J. Chem. Soc.*, **119**, 1883 (1921).

carbon tetrachloride-petroleum ether gave products of less purity than the above and the substance has not as yet been obtained pure. It was boiled in absolute methanol for ten minutes under reflux, most of the solvent was then removed and a large volume of petroleum ether added. Lustrous colorless needles soon separated. Recrystallized from ligroin, the **carbamate** melted at 210–212° (softening at 207°).

Anal. Calcd. for $C_{26}H_{37}O_4N$: N, 3.36. Found: N, 3.25.

The **ethyl carbamate** was prepared similarly. It separated from ethanol–petroleum ether as very small, colorless crystals which, after recrystallization from ligroin, melted at 163–165° (softening at 160°). Both of these carbamates are very soluble in all organic solvents but petroleum ether.

Anal. Calcd. for $C_{27}H_{39}O_4N$: N, 3.25. Found: N, 3.24.

Estrone *p*-Nitrobenzyl Ether.—Reid's method[19] was modified somewhat. A solution of estrone (400 mg.) in 8.3 cc. of warm 90% ethanol containing the requisite amount of dissolved sodium, was treated gradually with the theoretical amount (325 mg.) of *p*-nitrobenzyl bromide. The ether began to precipitate almost at once. The suspension was heated on the water-bath for one hour and then diluted with 50% alcohol. The ether was filtered off, washed with alcohol and crystallized from acetone–alcohol–water (Norit) as pale green-yellow needles (407 mg., 68%). The recrystallized substance melted at 176.5–178.5°.

Anal. Calcd. for $C_{25}H_{25}O_4N$: N, 3.48. Found: N, 3.52.

Butenandt's procedure[17] for estrone semicarbazone yielded a **semicarbazone** which crystallized from dioxane–ethylene chloride as microscopic colorless crystals, m. p. 273–275°.

Reduction of the nitro ether in alcoholic solution[20] yielded estrone as the main product. On reduction at 0° in acetic acid[10] no reaction was observed after thirty hours; at 20° estrone was recovered almost quantitatively.

4,4′-Di-(*p*-nitrobenzyloxy)-α,β-diethylstilbene.—From 250 mg. of diethyl stilbestrol we obtained 335 mg. (72.5%) of the ether, which was recrystallized from acetone as lustrous colorless plates, m. p. 183–185°. Like estrone *p*-nitrobenzyl ether, it is practically insoluble in alcohol. In common with other derivatives of diethyl stilbestrol, the ether gives a purple-red color reaction with cold concentrated sulfuric–nitric acid.

Anal. Calcd. for $C_{32}H_{30}O_6N_2$: N, 5.20. Found: N, 5.21, 5.23.

Thiele's reduction[10] at 0 and 20° gave a complete recovery of unreacted nitro compound; at higher temperatures decomposition to highly colored water-soluble products occurred. Morgan's method[20] produced an amorphous product, m. p. above 350°.

Diazotized *p*-Nitroaniline and Estrogens.—The diazonium salt solution added to estrone (35 mg.) in glacial acetic acid, according to the coupling method used by Fieser,[21] produced a rapid color change of green to dark brown and the solution when poured into water gave 39 mg. of a red-brown amorphous substance, m. p. 145–155° dec. On reduction of the crude product with stannous chloride and hydrochloric acid in absolute alcohol[21] no amine could be isolated. Estrone methyl ether gave a greenish-brown product from which no amine was obtained on reduction. Diethyl stilbestrol and diethyl stilbestrol dimethyl ether exhibited a very slow color change and yielded tarry products.

The Micro Kjeldahl Analyses.[22]—The estrone derivatives were particularly difficult to analyze, even by Friedrich's method[23] at a temperature of 360°. Low results usually were obtained unless 0.05–0.1 g. of red phosphorus was introduced into the bomb tube together with 1 cc. of hydriodic acid previously decolorized by heating with red phosphorus.

Summary

Estrone *p*-nitrophenyl ether was prepared and reduced to estrone *p*-aminophenyl ether. The latter on diazotization coupled with phenolic compounds, and with casein to form an orange-yellow azoprotein. Phenyl *p*-nitrobenzyl ether was reduced to phenyl *p*-aminobenzyl ether and the diazotized amine was coupled with casein giving an orange azoprotein. Reduction of estrone *p*-nitrobenzyl ether and 4,4′-di-(*p*-nitrobenzyloxy)-α,β-diethyl-stilbene failed to yield the corresponding estrogen amino ethers.

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(21) (a) Fieser and Campbell, THIS JOURNAL, **60**, 1142 (1938); (b) Fieser and Hershberg, *ibid.*, **61**, 1565 (1939).

(22) Carried out by L. F. K.

(23) "The Quantitative Organic Microanalysis of Pregl," H. Roth, Third English Edition, J. and A. Churchill, Ltd., London, 1937, p. 91.