in their spectra. These absorptions occurred between 7.8–7.9 (1288-1266 cm.$^{-1}$), 8.1–8.2 (1236-1220 cm.$^{-1}$), 8.87–8.97 (1128-1116 cm.$^{-1}$) and 10.4–10.5 μ (962-953 cm.$^{-1}$). The presence of a similar foursome was not observed in the published spectra of other steroids. Therefore these four bands cannot be utilized for identifying a substance as being a steroid. Many of these bands are reflections of the combined vibrations of the molecule (and thus of structural complexity). They may not appear consistently as one goes from relatively simple to more complex molecules.

Conclusions

At the moment the use of the 9–10 μ region for prediction of steric arrangements is not clearly established. The evaluation of spectra originating from structures containing hydroxyl groups at neighboring positions to C_3 is not complete. Fürst, *et al.*,[14] have demonstrated that cholestanol-2α gave rise to an intense doublet near 9.7 μ (1031 cm.$^{-1}$) while a weaker band occurred near 10 μ. The spectrum of cholestanol-2β had one band near 9.85 μ (1015 cm.$^{-1}$). Cholestanol-4α caused absorption near 9.6 μ and cholestanol-4β near 10 μ.

Since too many assumptions must be made in order to apply the 9–10 μ relationships to unknown steroid structures, it is best at present to postpone serious interpretation until additional pertinent compounds are investigated.[19] The assignment of an α/cis orientation would appear to be more selective than the other three possible arrangements.

We wish to express our sincerest gratitude to Dr. R. N. Jones for his suggestions.

(19) After this work was completed, A. R. H. Cole, R. N. Jones and K. Dobriner, THIS JOURNAL, **74**, 5571 (1952), confirmed the essential features of the present investigation.

SHREWSBURY, MASS.

[CONTRIBUTION FROM THE DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY AND THE DEPARTMENT OF MEDICINE, SCHOOL OF MEDICINE, UNIVERSITY OF CALIFORNIA AT LOS ANGELES]

Photoöxidation of Crystalline Estrogens in the Presence of Flavins[1]

BY JOSEPH F. NYC, HARRY B. FRIEDGOOD, JOSEPHINE B. GARST AND DOROTHY M. MARON

RECEIVED FEBRUARY 16, 1952

Spectrophotometric data have been presented as evidence of the fact that estrone, estradiol and estriol in alcoholic solution are destroyed at a rapid rate when exposed to light in the presence of riboflavin or lumichrome. This destruction of the three naturally occurring estrogens is inhibited by the addition of manganous chloride in a molar concentration of 10^{-2}. Experimental evidence indicates that the foregoing photoöxidation action causes the formation from estrone of several products. Zimmermann determinations indicate that the 17-ketone group of estrone is not affected during the photoöxidative process. Photoöxidation of estrone in the presence of riboflavin results in a large loss of its biological activity as determined by a modification of the Doisy vaginal smear method.

It is known that in the presence of visible light, riboflavin, lumichrome and various synthetic dyes such as eosine, methylol riboflavins and fluorescein initiate the oxidation of histidine, methionine, compounds containing an indole ring and a variety of proteins and enzymes.[2,3]

These observations suggested the desirability of investigating the photoöxidative destruction of estrogens by flavins for the purpose of determining whether or not it is a factor which can affect significantly the accuracy of current quantitative methods for the isolation and subsequent analysis of these steroids.

Part I. The Effect of Riboflavin on Estrogens in the Presence of Visible Light.—Five-tenths of a milligram of estrone, estradiol and estriol were dissolved, respectively, in 10 ml. of 95% ethanol.[4] Each tube of ethanol contained 80 micrograms of freshly dissolved riboflavin which had been protected from light. These solutions were exposed to visible light[5] for 90 hours. During this exposure ultraviolet spectral curves were determined at timed intervals on these solutions and also on a simultaneously irradiated riboflavin control solution which contained only the flavin at the same concentration used in the estrogen irradiation tubes. The results on estrone, corrected by subtracting the corresponding values for the flavin control, are recorded in Fig. 1.

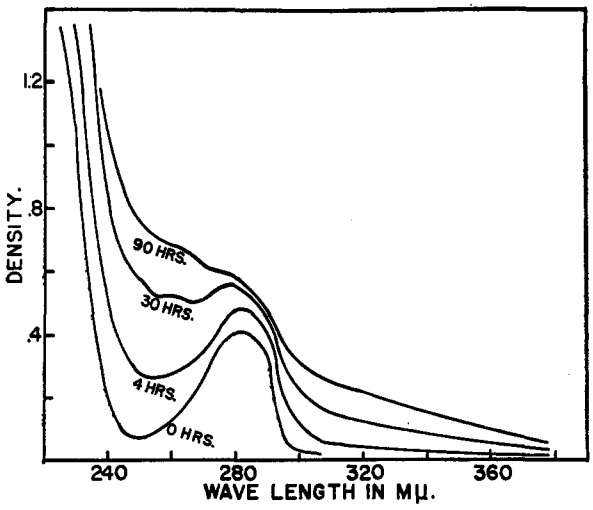

Fig. 1.—Change in ultraviolet spectrum of estrone following exposure to visible light at the time intervals noted. The data were obtained by subtracting the spectral curves of irradiated riboflavin controls from the additive spectra of the solutions containing both estrogen and riboflavin. The control and experimental solutions were irradiated simultaneously: density = log (I_0/I_x).

(1) These studies were supported by a generous grant from the California Institute for Cancer Research, Los Angeles, California. We are indebted also to Ayerst, McKenna and Harrison, Ltd., for estrone, to Ciba Pharmaceutical Products, Inc., for estradiol and estrone, and to Parke, Davis and Company for estriol.

(2) A. W. Galston, *Proc. Nat. Acad. Sci.*, **35**, 10 (1939).

(3) (a) A. W. Galston and R. S. Baker, *Science*, **109**, 485 (1949); (b) **111**, 619 (1950).

(4) All solvents were redistilled prior to use.

(5) All of the solutions in these experiments were exposed to about 100 foot candles of light from "daylight" fluorescent bulbs.

The qualitative changes in the ultraviolet spectra of estriol and estradiol were very similar to those observed for estrone.

On the basis of the spectrophotometric data which were obtained under the stated experimental conditions riboflavin has an observable effect on each estrogen spectrum which consists essentially of a marked increase in density at 250 mμ (minimal density of ultraviolet spectra for estrogens), and a smaller rise in density throughout the remainder of the curve. Almost identical results were obtained when lumichrome was substituted for riboflavin in the above experiment.

Part II. The Inhibitory Effect of Manganous Chloride on the Photoöxidative Action of Riboflavin and Lumichrome on Estrogens.—The experiments described in Part I were repeated with the addition of manganous chloride in a molar concentration of 10^{-2}. Under these experimental conditions the ultraviolet curve of each of the estrogens undergoes only a minute change even after being illuminated for 45 hours.

Spectrophotometric data, not shown here, reveal that the rate of decomposition of riboflavin *per se* in the presence of visible light (as measured by its ultraviolet curve) proceeds at the same rate and in the same direction whether or not it is exposed to $MnCl_2$.

Part III. Bioassay of Estrone after its Exposure to Light in the Presence of Riboflavin.—A solution of 1 mg. of estrone[6] and 0.1 mg. of riboflavin in 11 ml. of 95% ethanol was exposed to visible light for 135 hours. A control solution containing only 1 mg. of estrone in 11 ml. of 95% ethanol was exposed simultaneously to the same light source. These solutions were assayed through the courtesy of Dr. Oliver Kamm, Dr. A. C. Bratton and L. W. Rowe at Parke, Davis and Company.[7]

The results of these experiments indicated that the flavin-containing sample of estrone retained only 25% of its original biological activity, while the control solution exhibited 75% of its initial activity. The latter suggests that even dilute alcoholic solutions of estrone in the absence of sensitizing substrates undergo some destruction on prolonged exposure to strong visible light in the presence of oxygen. This finding is in accord with the observations of Engel, *et al.*,[8] who have reported that dilute alcoholic solutions of estrogens show significant changes in titer after simply being stored for a few weeks even at cold temperatures.

Part IV. The Oxidative Products of Estrone Resulting from the Action of Riboflavin on this Hormone in the Presence of Light.—Four hundred eighty-four milligrams of estrone[9] and 50 mg. of riboflavin were dissolved in 5 liters of 95% ethanol. After 110 hours exposure to light, the solution was taken to dryness under vacuum and put through the fractionating procedure outlined in Table I. All fractions were recrystallized to the point at which the quantity of material was too small for further purification. The chemical and physical data which were obtained on the various fractions are given in Table II under the respective numerical designations used in the work flow sheet (Table I).

All of the fractions isolated after exposure of estrone to photoöxidation give positive Zimmermann[10] reactions. This indicates that the 17-ketone group is still intact. The ultraviolet spectra of the purest fraction (IIB, Table I) in neutral and basic solvents are given in Fig. 2. The ultraviolet spectra, fundamental analyses and Zimmermann values for Fraction I (Table II) show that it consists primarily of unchanged estrone. This fraction does not give a mixed melting point depression with an authentic sample of estrone.

During the separation by crystallization of the irradiation products of estrone, it was observed that solutions of irradiated estrone discolored rapidly on standing and turned brown when heated for short periods of time. Since there is apparently some alteration of these irradiation products during procedures involving recrystallization from various solvents, attempts were made to study them by chromatographic techniques. For these studies the solutions defined in Table III were exposed to light for 72 hours.

At the termination of the irradiation period, 50 mg. of estrone was added to solution No. 2 (Table III) and the four solutions were taken to dryness under reduced pressure. During this evaporation and all succeeding manipulations the contents of these solutions were protected from light.

Each of the residues from the four solutions in Table III was dissolved in 25 ml. of methyl alcohol and 0.2-ml. ali-

TABLE I

ISOLATION OF THE PRODUCTS OF THE REACTION OF ESTRONE AND RIBOFLAVIN IN THE PRESENCE OF LIGHT

485 mg. estrone and 50 mg. riboflavin in 5 liters 95% EtOH exposed to light for 110 hr., taken to dryness under vacuum, shaken with 85 ml. $CHCl_3$, and filtered

- 28.7 mg. undissolved material[a]
- $CHCl_3$ filtrate washed 3 × 35-ml. vol. of H_2O
  - $CHCl_3$ taken to dryness; residue (525.7 mg.) extracted with 50 ml. ligroin (b.p. 60–90°)
    - Residue refluxed 5 min. with 8.5 ml. C_6H_6, cooled to room temp. and filtered
      - Filtrate taken to dryness, and recryst. from min. vol. 100% EtOH
        - Ppt. (58.3 mg.) recryst. from 80% EtOH[b]
          - Filtrate Fraction I (32.4 mg.)
        - Filtrate (315 mg.) recryst. twice from 80% EtOH[b]
          - Filtrate Fraction III (11.0 mg.)
          - Fraction IIA (96.9 mg.) recryst. from 12 ml. 60% EtOH
            - Filtrate
            - Fraction IIB (13.9 mg.)
      - Benzene-insoluble fract. recryst. from 80% EtOH[b]
        - Ppt. (29.3 mg.) recryst. from 80% EtOH[b]
          - Filtrate Fraction III (11.0 mg.)
        - Filtrate (57.1 mg.) recryst. from 80% EtOH[b]
          - Filtrate Fraction IV (17.5 mg.)
    - Ligroin filtrate
  - H_2O extracts

[a] Primarily lumichrome. [b] The minimum volume of the solvent (8 parts EtOH to 2 parts H_2O by volume) which would dissolve the sample at the boiling point of the solvent.

(6) Ciba Pharmaceutical Products, Inc., m.p. 260–262°.

(7) Estrogen assays were done with an improved modification of the Doisy vaginal smear method.

(8) L. L. Engel, W. R. Slaunwhite, P. Carter and I. T. Nathanson, *J. Biol. Chem.*, **185**, 255 (1950).

(9) U. S. P. Ayerst, McKenna and Harrison, Ltd., softens 253.5°, melts 256.5–259.8°. *Anal.* Calcd. for estrone: C, 80.0; H, 8.40. Found: C, 80.2; H, 8.55.

(10) W. W. Bngstrom and H. L. Mason, *Endocrinology*, **33**, 229 (1943).

TABLE II

PHYSICAL AND CHEMICAL PROPERTIES OF THE PRODUCTS OF THE REACTION OF ESTRONE AND RIBOFLAVIN IN THE PRESENCE OF LIGHT

Substance	M.p., °C. (cor.)	Zimmermann[a]	Analyses C, %	H, %	O, %	N, %
Calcd. for estrone $C_{18}H_{22}O_2$		100	79.96	8.20	11.83	....
Fraction I	249–255	98	79.88	8.50	10.86	0.76
Calcd. for $C_{18}H_{22}O_3$			75.49	7.74	16.76	....
Fraction IIA	173–178	94	74.79	7.64	17.21	0.36
Fraction IIB	206–209	83	75.02	7.30	17.68	Trace[b]
Calcd. for $C_{18}H_{22}O_4$			71.50	7.34	21.16	....
Fraction III	298–310 dec.	70	70.69	7.27	21.06	0.98
Fraction IV	236–241	82	77.30	8.22	12.34	2.14

[a] Reading at 520 mμ relative to that given by an equal weight of estrone rated at 100. [b] Less than 0.1%.

TABLE III

Solution	Estrone,[a] mg.	Riboflavin, mg.	$MnCl_2$, g.	95% ethanol, cc.
1	50	5	..	500
2	..	5	..	500
3	50	5	0.99	500
4	..	5	..	500

[a] Parke, Davis and Company, m.p. 259–262°.

quots of these alcoholic solutions were chromatogrammed simultaneously on four columns of pulverized rubber according to the method described by Nyc, Maron, Garst and Friedgood.[11] The 0.2-ml. aliquots of solutions 1, 2 and 3 correspond to a mixture of 400 γ of estrone and 40 γ of riboflavin based on the original non-irradiated materials. The 0.2-ml. sample of solution 4, which corresponds to 40 γ of riboflavin prior to irradiation, was used as a control to make background corrections for the flavins present in the other three solutions. Twenty ml. each of 20, 40 and 60% methanol (v./v.) was passed successively through the rubber columns at a rate of 0.4 ml. per minute at 25°. They were collected in 5-ml. fractions. All of the foregoing 12 fractions for each chromatogrammed sample were taken to dryness, and the residues were dissolved in 4 ml. of 95% ethanol. The values for the ultraviolet absorption of these fractions at 280 mμ were obtained in a Beckman spectrophotometer by reading each fraction from solution 1, 2 and 3 (Table III) against the corresponding control fraction from solution 4.

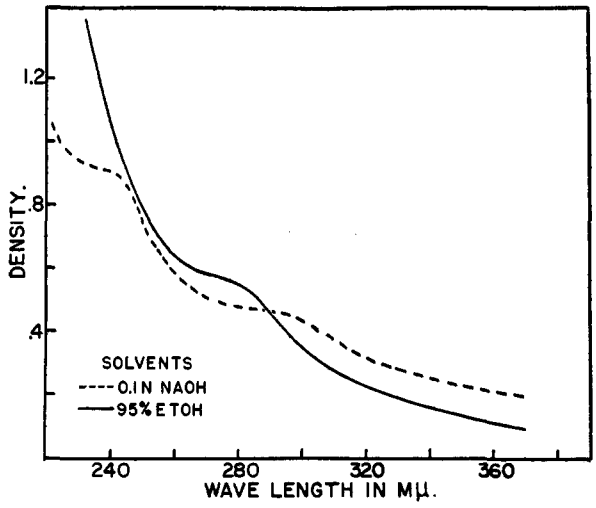

Fig. 2.—Ultraviolet spectra of fraction IIB at a concentration of 50 micrograms per ml. of 95% ethanol and 0.1 *N* NaOH, respectively.

Estrone added to irradiated riboflavin (Fig. 3) gave the normal chromatogram expected for a crystalline sample of estrone.[11] The result of the chromatogram of estrone which had been exposed

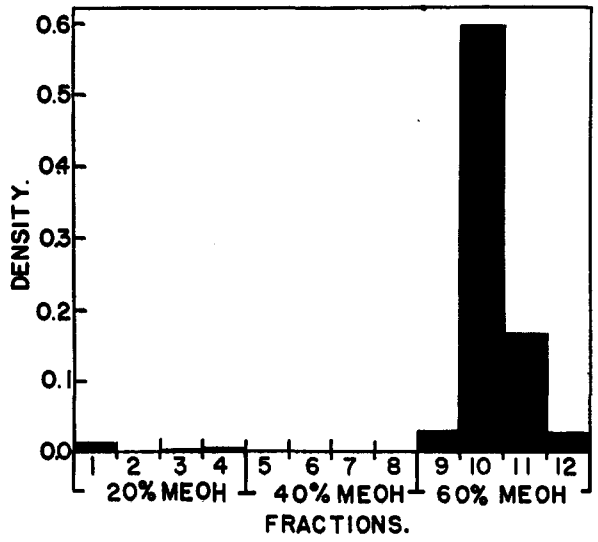

Fig. 3.—Corrected densities at 280 mμ for fractions obtained when an aliquot of sample No. 2 (Table III) was passed through a 11.5-cm. chromatographic column. Each fraction represents 5 ml. of eluant.

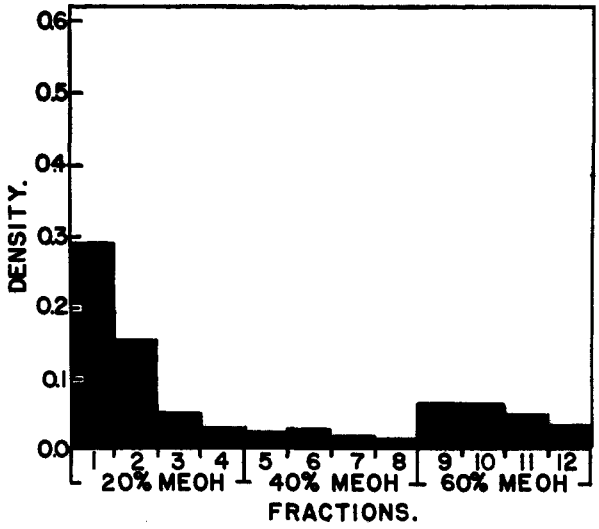

Fig. 4.—Corrected densities at 280 mμ for fractions obtained when an aliquot of sample no. 1 (Table III) was passed through a 11.5-cm. chromatographic column. Each fraction represents 5 ml. of eluant.

(11) J. F. Nyc, D. M. Maron, J. B. Garst and H. B. Friedgood, *Proc. Soc. Exper. Biol. Med.*, **77**, 466 (1951).

to light in the presence of riboflavin and $MnCl_2$ is essentially the same as that of pure estrone. The chromatographic data (Fig. 4) on estrone exposed to light in the presence of riboflavin alone show a marked deviation from those expected for estrone. Further work with larger rubber chromatographic columns showed that there are at least three chemically unstable products of irradiated estrone.

Discussion

On the basis of the chemical, spectrophotometric, chromatographic and bioassay data presented in this article, it appears that the estrogens, and more specifically estrone, are oxidatively altered on exposure to light when dissolved in a 95% ethanol solution containing either riboflavin or lumichrome. Under these experimental conditions, the photooxidation of estrone is accompanied by a large loss in biological activity. Manganous ions present in a concentration of 10^{-2} mole per liter almost completely inhibit these photoöxidations.

The finding that estrogens are destroyed by light when in solution with riboflavin or lumichrome is of importance in isolation studies on urinary and blood estrogens, since in these fluids steroids are mixed with appreciable quantities of flavins.

LOS ANGELES, CALIFORNIA

[CONTRIBUTION FROM VENABLE CHEMICAL LABORATORY OF THE UNIVERSITY OF NORTH CAROLINA]

Kinetics of the Catalytic Hydrogenation of Certain Schiff Bases[1]

BY ARTHUR ROE AND JOHN A. MONTGOMERY

RECEIVED SEPTEMBER 2, 1952

The rates of hydrogenation of benzalaniline and twenty-four of its derivatives have been studied. It was found that Hammett's equation does not hold for this reaction, although substituents on the ring do definitely affect the rate of hydrogenation. These effects are discussed.

This work was undertaken to determine quantitatively the effects of various ring substituents on the rate of catalytic hydrogenation of the carbon–nitrogen double bond of various Schiff bases. The Schiff bases used are listed in Table I as compounds 1–24; the secondary amines resulting from their reduction are listed in Table I as compounds 1a–24a. Adams platinum catalyst was chosen for this study because of the reproducibility of results and because its activity remains constant for relatively long periods of time. The hydrogenations were carried out at atmospheric pressure, as this procedure gave convenient reaction rates which could be observed easily and accurately by following the change in volume of hydrogen in a gas buret.

Experimental

Materials.—The Schiff bases used were prepared in one of two ways. **Method A.**—The aldehyde and amine were dissolved in benzene or petroleum ether and the solution refluxed in a round-bottom flask attached to a Dean–Stark trap and reflux condenser until the theoretical amount of water collected in the trap. The solvent was then removed and the Schiff base distilled *in vacuo* or recrystallized from a suitable solvent, usually either ethyl alcohol or petroleum ether.

Method B.—The aldehyde and amine were fused on a steam-bath for about a half hour as described by Law.[2] The product was then purified as in method A.

The Adams platinum catalyst was prepared in the usual manner.[3] Three different batches of the catalyst were prepared, but no appreciable difference in the activity of the three batches could be detected. Ethyl alcohol was used as a solvent for the reductions and tank hydrogen was used directly.

Hydrogenation Procedure.—The hydrogenations were carried out in a 500-ml. erlenmeyer flask which could be connected, by means of a three-way stopcock, to either a water aspirator or a 500-ml. gas measuring buret (filled with water and equipped with a leveling bulb). By means of the same stopcock the measuring buret could be filled with hydrogen from a tank. The solution in the erlenmeyer flask was stirred with a magnetic stirrer and variations in the rate of stirring due to line voltage fluctuations were eliminated by the use of a thordarson automatic voltage regulator.

The anil (0.01 mole) was placed in the erlenmeyer flask with 0.02 g. of prereduced Adams catalyst and 100 ml. of ethyl alcohol. The system was evacuated and then filled with hydrogen. The initial reading of the buret was taken and the maguetic stirrer cut on. The reaction temperature was maintained between 24 and 25° by means of a water-bath. There was no appreciable variation in barometric pressure during any run. Benzalaniline was reduced several times during the course of these experiments to check on reproducibility of results; excellent checks were obtained in every case.

Hydrogenation Products.—After each hydrogenation the platinum catalyst was filtered off and the secondary amine formed by the reduction isolated and identified either as the hydrochloride or the free base (except for benzal-*p*-iodoaniline, which decomposed when the alcohol was removed by distillation). The Schiff bases and their reduction products are listed in Table I along with their physical properties and analytical data for new compounds or a literature reference if the compound is known.

Discussion

In agreement with previous work,[4,5] it was established that the hydrogenations were of zero order with respect to the substrate and directly proportional to the amount of catalyst used. This is shown in Tables II, III and IV.

Four typical hydrogenation curves are shown in Fig. 1. These show that a plot of ml. of hydrogen used *versus* time results in a straight line,

(1) Taken in part from the dissertation of John A. Montgomery submitted in June, 1951, in partial fulfillment of the requirements for the Ph.D. degree.

(2) H. D. Law, *J. Chem. Soc.*, **101**, 158 (1916).

(3) R. Adams, V. Voorhees and R. L. Shriner, "Organic Syntheses," 2nd Ed., Coll. Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1940, p. 463.

(4) L. Herandez and F. F. Nord, *J. Colloid Sci.*, **3**, 363 (1948).

(5) H. A. Smith and co-workers, THIS JOURNAL, **67**, 272, 276, 279 (1945); **71**, 81, 413, 415 (1949).