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Stability of estrogens to oxygen during exposure on silica gel*

In a recent paper it was inferred that estradiol is decomposed by atmospheric oxygen[1] during exposure on thin-layer chromatographic (TLC) sheets coated with silica gel. The evidence presented to support the contention was that the addition of reducing agents was able to prevent the formation of artifacts. No attempt was made to expose estrogens to oxygen in order to test the hypothesis which was at variance with conclusions drawn by other investigators in an earlier report[2]. To ascertain which view is correct, appropriate experiments involving exposure, TLC development, elution and crystallization to constant specific activity were carried out which unequivocally prove that exposure of estrogens on silica gel to oxygen does not result in the formation of artifacts. The experimental basis for the latter conclusion is described herein.

Materials

Chromatogram sheets, silica gel, without fluorescent indicator, Eastman No. 6061; disposable microsampling pipets (10 μl) for applying specimens to TLC sheets, Corning No. 7099S; [2,4,6,7-$^3H_4$]estradiol, 100 Ci/mmole (Amersham/Searle) was repurified by gradient elution chromatography[3] as necessary and stored in the benzene–ethyl acetate eluant; flow-meter, Emil Greiner No. G9144B; oxygen, USP, National Cylinder Gas; plexiglas chambers, top-loading, specially constructed 25 × 25 × 5 cm height, nearly air-tight, with suitable ports for maintaining a controlled internal environment. Solvents, chemicals, and other materials were as described in an earlier publication[4].

Methods

General. Ultraviolet analyses were done with a Beckman Model DB spectrophotometer. Determination of radioactivity by liquid scintillation counting was done with a Nuclear-Chicago Model 725 spectrometer with an efficiency of 34% for tritium using as the scintillation medium toluene with 5 g "PPO" and 0.1 g dimethyl-POPOP per liter. Vials of low potassium content were re-used after cleaning[5] and were sealed with disposable polyethylene caps (Owens, Ill., No. 5212-1, formerly No. OS-3K, Pittston, Pa.) as described earlier[4]. TLC was done in unlined Thomas-Kolb tanks (A. H. Thomas, Philadelphia, Pa.) using glass plates to partially support the flexible TLC sheets. A space was left between the sheets and the plates at the bottom to prevent solvent from moving up unevenly behind the sheets by capillary action. Radiochromatogram scanning, elution from chromatograms and other procedures were done as described earlier[4].

General procedure for exposure of [2,4,6,7-$^3H_4$]estradiol to oxygen and related procedures. A 10 μl specimen of stock [2,4,6,7-$^3H_4$]estradiol solution containing 490 pg (4×10^7 d.p.m./ml) was applied to each of six TLC sheets (4 × 20 cm) along the middle half of a line drawn 2.5 cm from one end of the sheet. The applications were done rapidly in a nitrogen purged plexiglas hood designed for this purpose. Non-radioactive estradiol (10 μg) was applied similarly to three other plates. All of the sheets were placed in TLC developing tanks to which previously had been added 200 ml

of a mixture of 25 % ethyl acetate in benzene. The solvent front was allowed to migrate 2–3 cm up the plate in order to move the estradiol out of the origin and thereby distribute it more uniformly on the silica gel. Two of the specimens containing the [^{3}H]estrogen and one standard containing 10 μg of non-radioactive estradiol were removed from one tank, allowed to air-dry and immediately returned to the tank. The remaining specimens containing the [^{3}H]estrogens were placed in a plexiglas chamber of approximately 3 l capacity and a stream of oxygen was directed into the chamber at a rate of 500 ml/min.

When the solvent front reached a "line" scored in the silica gel 2.5 cm from the top of the sheets, the sheets were removed from the developing tank, air-dried and the [^{3}H]estrogens located by radiochromatogram scanning. The appropriate region, corresponding in mobility with that of the peak on the scan and with the nonradioactive estradiol on the adjacent sheet (located by exposing the sheet to vapors of iodine) was eluted with methanol and the eluate was stored in the refrigerator.

Two of the four specimens maintained in the plexiglas chamber under oxygen were removed after 4 h and the other two were removed after 20 h. Each set was developed and scanned exactly as described for the zero time exposure and the [^{3}H]estradiol at the appropriate radioactive regions of the sheets which were exposed to oxygen for 20 h were eluted with methanol. Each of the scans showed a single peak of radioactivity with only a minor trace of activity at or near the origin. There were no significantly distinguishing features among the scans of the unexposed specimens and those exposed to oxygen for 4 or 20 h.

Crystallization to constant specific activity. Crystallization was done by classical techniques involving slow growth of large crystals in order to avoid entrapment of impurities[6]. In the experience of the author, slow growth of crystals results in more efficient elimination of impurities than rapid growth, recent unconvincing arguments to the contrary notwithstanding[7].

Several grams of estradiol were dried overnight at room temperature in a vacuum desiccator (<1 mm). Two 50 mg specimens were weighed and transferred with acetone to separate 25 ml volumetric flasks. The [^{3}H]estradiol regions from the zero and 20 h exposures to oxygen which had been eluted from the chromatographic sheets were transferred to separate volumetric flasks containing 50 mg of estradiol. The volume was brought to 25 ml with acetone and after shaking the flask to insure complete solubilization 1 ml was removed to a liquid scintillation vial. The remainder was transferred in two batches to tared liquid scintillation vials which were used as the crystallization vessels. The volume was reduced on a hot plate using a gentle stream of nitrogen. Periodically, *n*-hexane was added and the flow of nitrogen was stopped to allow refluxing solvent to wash down crystals deposited on the walls of the vials. A spatula facilitated the latter process as did also the intermittent introduction of a stream of nitrogen to cool the walls. When the volume was down to a level which from experience would yield approximately two-thirds of the estradiol as crystals and one-third in the supernatant the vials were removed from the hot plate and crystallization was allowed to proceed slowly at room temperature without disturbing the vial. When crystallization was complete the supernatant was removed and the crystals were washed sequentially with *n*-hexane–ethyl acetate

washings. The crystals were dried for about 1 h in a vacuum desiccator and an appropriate aliquot based on direct weighing was removed to a separate vial for accurate determination of weight by UV analysis and for radiochemical analysis by liquid scintillation spectrometry. Each crop of crystals was recrystallized until there was no further change in the specific activity.

TABLE I

RECRYSTALLIZATION TO CONSTANT SPECIFIC ACTIVITY

Number of recrystallizations	*Specific activity (c.p.m./mg)*	
	Oxygen (20 h)	*Zero control*
0	1051	954
1	1093	905
2	941	859
3	956	836

In Table I are summarized the results which were obtained. The recovery of intact [^{3}H]estradiol after exposure to oxygen for 20 h was not significantly different from that found with the unexposed control as judged by the relative magnitude of the final specific activities and the rates at which constancy was attained.

Discussion

As more sensitive methodology for the determination of steroidal hormones and other substances of biological interest is developed it becomes necessary to take precautions to avoid potentially destructive effects of impurities in the environment or in procedural materials which come in contact with the determinates. Frequently the effects are of consequence only at far below the microgram range and invariably are an inverse function of the quantities being manipulated[1].

The demonstration that a single factor does or does not cause or contribute to the formation of artifacts can be elusive since the control thereof may preclude maintaining constant all other potential variables. A recent report[8] that the decomposition of dehydroepiandrosterone by air at room temperature while on silica gel could be avoided by keeping the strips at $-15°$ implies that temperature is a significant variable. Although possible to achieve control of temperature without altering exposure to air and light, the most conventional and practical means to reduce the temperature would simultaneously alter the exposure to air as well as light. The variability of the decomposition of estrogens under constant conditions of air and light[2] and the subjective correlation of the extent of the decomposition with the intensity of the effects of eye irritating smog[9] clearly implicated atmospheric contaminants as a major cause of the decomposition[2]. In more recent studies done by the author[10], preliminary results indicate that (1) oxygen, light and atmospheric pollutants all participate in the degradation of estrogens during exposure on silica gel; (2) air-borne particulate matter may be a significant factor in the degradation;

with silica gel than on glass plates coated with Silica Gel G (Merck) presumably because of a partial protective effect exerted by the organic binder which is mixed in with the silica gel on the plastic-backed sheets; and (4) a very gentle stream of oxygen (from a 1/4 in. orifice) directed onto the Silica Gel G (Merck) at the region of the plates where estrogens are located during exposure to ambient conditions in the laboratory, provides nearly complete protection from extensive decomposition found with control specimens similarly exposed without a stream of oxygen. An earlier report[11] of the destruction of a number of steroids by strong ultraviolet light while dissolved in a relatively non-volatile stationary phase on paper chromatograms may involve a mechanism very different from that which is operative in the destruction of estrogens on silica gel, an adsorbant which becomes activated maximally at 300° and declines in activity at higher temperatures[5,12]. The destructive potential of impure methanol and the rapidity with which impurities re-develop[13,14] after simple distillation or after distillation through a Vigreux column illustrate the need for careful attention to the purity of starting materials and the possible need for distillation of some solvents through efficient fractionating columns at a high reflux ratio.

Clearly, on the basis of the data presented in this report oxygen alone is not responsible for the decomposition of estrogens while exposed as a thin film on silica gel as has been inferred[1].

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