Anthralin: Chemical Instability and Glucose-6-phosphate Dehydrogenase Inhibition

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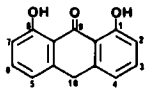
Abstract □ The chemical stability of the antipsoriatic drug, anthralin (1,8-dihydroxy-9-anthrone), in solution has been studied using high-performance liquid chromatographic analysis. The time course for decomposition in solution has been correlated with that of the inhibition of glucose-6-phosphate dehydrogenase, one of the most widely documented biochemical properties associated with anthralin. Solutions of anthralin in aqueous buffer (37°, pH 7.5, under light protection) decomposed completely within 4 hr giving the 10,10′-dimer (40%), no detectable 1,8-dihydroxy-9,10-anthraquinone, and a greatly increased potency of inhibition of glucose-6-phosphate dehydrogenase. This increased inhibitory potency could not be explained by formation of the dimer which, like anthralin and its quinone, were shown to be only weak inhibitors of the enzyme. In acetone solution exposed to light and air, anthralin decomposed completely within 4 days, in part *via* the dimer as intermediate. The final solution had the characteristic color of anthralin-brown, contained the quinone (20%), and like decomposed aqueous solutions of anthralin, completely inhibited glucose-6-phosphate dehydrogenase. The results show that neither anthralin, nor either of its two identified decomposition products, is the potent toxic species against glucose-6-phosphate dehydrogenase.

Keyphrases □ Anthralin—chemical instability and glucose-6-phosphate dehydrogenase inhibition □ High-performance liquid chromatography—chemical instability and glucose-6-phosphate dehydrogenase inhibition of anthralin □ Decomposition—anthralin, chemical instability and glucose-6-phosphate dehydrogenase inhibition

Anthralin (1,8-dihydroxy-9-anthrone, I) has been used successfully for over 60 years in the topical treatment of psoriasis (1–3). However, this treatment suffers from two main disadvantages: staining and irritation of the skin (4). It is well known that anthralin is unstable in solution and, depending on the conditions, can present a complex mixture of products and colors which have as their endpoint anthralin-brown (4, 5).

In view of this chemical instability, the *in vitro* biological properties which have been attributed to anthralin itself might, in fact, be related to some of its decomposition products. Furthermore, the therapeutic action and side effects following topical application of anthralin might result from the decomposition of this molecule into other chemical species having in turn antipsoriatic, irritating, and staining properties. Several attempts have been made to study the behavior of some known breakdown products in test systems which are claimed to give some indication of antipsoriatic or potentially toxic properties (6–8). Despite interest in the subject, little is known about which compounds play a major role in the action of this drug.



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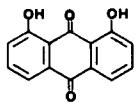

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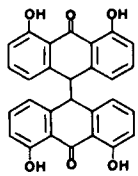

III

In a reinvestigation of anthralin, the first objective was to evaluate the stability of the molecule, particularly in a physiological buffer, using high-performance liquid chromatography (HPLC), and to relate the findings to one of the most widely documented biochemical properties attributed to anthralin itself: the inhibition of glucose-6-phosphate dehydrogenase (9, 10, and references therein)[1].

EXPERIMENTAL

Chemicals—The following chemicals were analytical grade reagents[2]: 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid, iso-octane, diisopropyl ether, methanol, ethanol, 1-butanol, 1,3-butanediol, acetone, acetonitrile, 2-butanone, dimethylformamide, 1-propanol, 2-propanol, and acetic acid. 2-Butanol, tetrahydrofuran, and 1,4-dioxane were chromatographic grade[3]. 1,8-Dihydroxy-9-anthrone (anthralin/dithranol, I)[4] was purified by column chromatography (6). 1,8-Dihydroxy-9,10-anthraquinone (quinone, II) and bi(1,8-dihydroxy-9-anthron-10-yl) (dimer, III) were synthesized[5] following published procedures (7).

Enzymological studies were performed using pure yeast glucose-6-phosphate dehydrogenase[6], with glucose-6-phosphate and nicotinamide adenine dinucleotide phosphate as substrate and coenzyme, respectively[7]. All other chemicals were pure grade reagents[8].

High-Performance Liquid Chromatography—Analysis of anthralin and its derivatives was performed on an HPLC system[9] set at 254 nm (reference at 500 nm), and equipped with a reversed-phase 25-cm column[10], following the analytical conditions developed in this laboratory (11). In all experiments, samples (15 μl, either aqueous or organic solutions) were injected directly into the chromatographic system without previous solvent extraction.

Measurements of Glucose-6-phosphate Dehydrogenase Activity—All incubation experiments of glucose-6-phosphate dehydrogenase with anthralin and its derivatives were performed in a Ringer buffer composed of 140 m*M* NaCl, 5.2 m*M* KCl, 2.8 m*M* $CaCl_2$, 1.3 m*M* $MgSO_4$, and 20 m*M* 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid at pH 7.5. In this buffer, the enzymatic activity remained almost constant over a 2-hr period at 37°. Before starting the experiments, the commercial enzyme suspension was diluted 300-fold in cold Ringer buffer and kept at 0°.

Time Course of the Influence of Anthralin, Dimer, and Quinone on

[1] This study was presented in part at the "Anthralin Symposium" Sophia-Antipolis, France, Oct. 1980.
[2] Merck.
[3] Carlo Erba.
[4] Bayer.
[5] Laboratoires de Recherches Fondamentales, L'Oréal, Aulnay-sous-Bois, France.
[6] Enzyme Commission Number 1.1.1. 49, yeast enzyme, grade II, Boehringer.
[7] Boehringer.
[8] Prolabo.
[9] Model 1084 B. Hewlett-Packard.
[10] Merck Lichrosorb RT 250-4/5 μm/RP 18.

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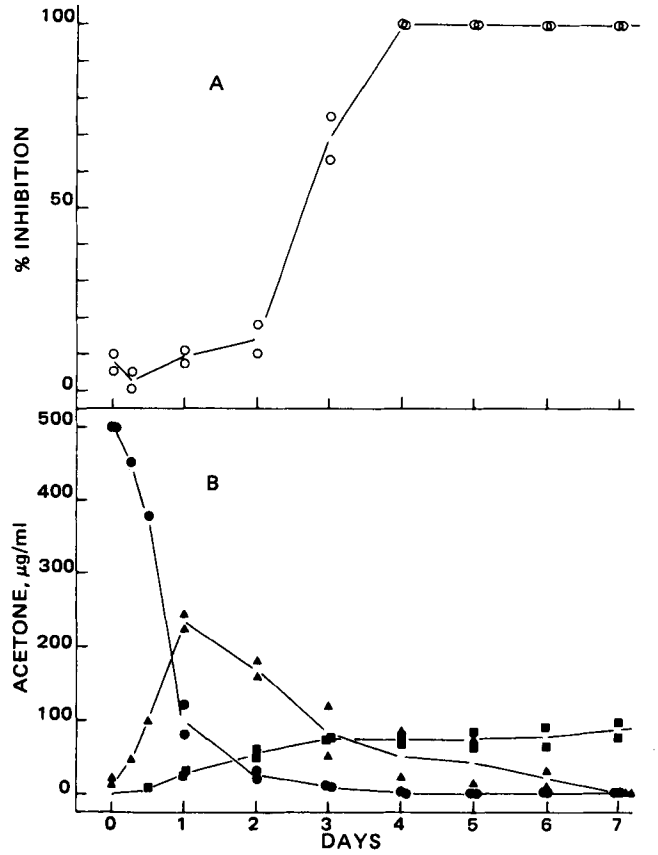

Figure 1—*Time course of the decomposition of anthralin in acetone (initial concentration = 0.5 mg/ml = 2.2 mM, 25°, continuous exposure to artificial light): inhibitory potency against glucose-6-phosphate dehydrogenase (A) and chemical composition of the medium as assayed by HPLC (B). Key: (○) percent of inhibition obtained by incubating the enzyme with 1% of the acetone solution for 5 min; (●) concentration of anthralin; (▲) concentration of dimer; (■) concentration of quinone.*

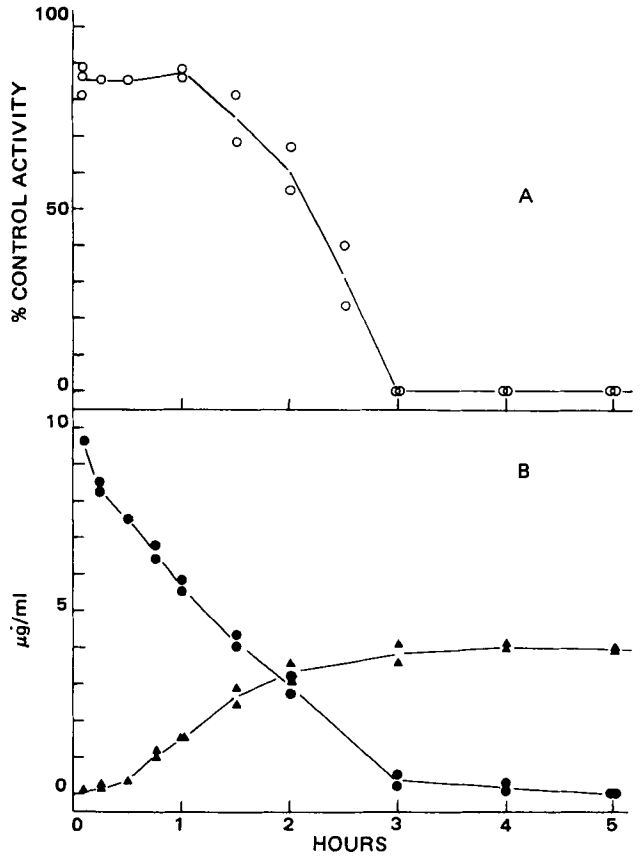

Figure 2—*Activity of glucose-6-phosphate dehydrogenase as a function of time of incubation with a solution of anthralin (A) (10 μg/ml in 1% acetone–Ringer buffer, pH 7.5, 37°, light protection), and the concurrent chemical composition of the medium, as determined by HPLC (B). Key: (○) percent of control enzymatic activity (the enzyme and anthralin were added at time zero); (●) concentration of anthralin; (▲) concentration of dimer.*

the Activity of Glucose-6-phosphate Dehydrogenase—The incubation medium (final volume = 3 ml) consisted of 2.92 ml of Ringer buffer, 50 μl of enzyme solution, and 30 μl of a 1 mg/ml solution of anthralin, dimer, or quinone in acetone to give a final concentration of 10 μg/ml in the incubation medium. Two test solutions and one control solution (to which acetone alone had been added) were kept for each specified time period at 37° in a water bath under light protection. The first test solution was then immediately analyzed by HPLC. To the second test solution and its control was added 50 μl of 10 m*M* nicotinamide adenine dinucleotide phosphate (final concentration 0.17 m*M* = 3 K_m under the present conditions)[11] and 50 μl of 18.6 m*M* glucose-6-phosphate (final concentration 0.31 m*M* = 3 K_m)[11], and the appearance of reduced nicotinamide adenine dinucleotide phosphate was immediately measured by its optical density at 340 nm at 37°[12].

At time zero of incubation, the control activity in the incubation medium (expressed as the variation of optical density per minute) was 0.21–0.23 ΔOD/min. Due to the presence of 1% acetone, this control activity decreased with increasing incubation time (to 50% of initial activity after 5 hr of incubation). To eliminate this unspecific solvent effect, each determination of the activity in the presence of anthralin or derivatives was corrected using the control activity in the presence of acetone alone at the same incubation time.

Time Course of Decomposition in Ringer Buffer and Inhibition of Glucose-6-phosphate Dehydrogenase—Duplicate solutions (30 μl of 1 mg anthralin/ml of acetone added to 2.92 ml of Ringer buffer) were kept for each selected time period at 37° in a water bath under light protection. To one solution, 50 μl of enzyme solution was then added and the solution (final volume = 3 ml, theoretical anthralin concentration 10 μg/ml = 44.2 μ*M*) maintained under light protection at 37° for an additional 5-min period. Enzymatic activity was measured as described earlier. To the second solution, 50 μl of enzyme solution was added and the mixture immediately analyzed by HPLC as described previously.

Time Course of Decomposition in Acetone and Inhibition of Glucose-6-phosphate Dehydrogenase—Anthralin was dissolved in acetone to a final concentration of 0.5 mg/ml. The solution was continuously exposed to air and artificial light[13] at 25° for a period of up to 1 week. At selected times the chemical composition of the medium was analyzed by HPLC, and its inhibitory potency against the enzyme was assessed by adding 30 μl of the acetone solution to a mixture of 2.92 ml of Ringer buffer and 50 μl of enzyme solution. The medium (final volume = 3 ml, theoretical anthralin concentration 5 μg/ml = 22.1 μ*M*) was maintained in a water bath at 37° for 5 min, and the enzymatic activity determined as described earlier.

RESULTS AND DISCUSSION

Selection of a Ringer Buffer–Solvent System—Despite the presence of the polar groups at C-1, C-8, and C-9, the anthralin molecule is quite lipophilic in nature. Consequently, an aqueous solution of anthralin (suitable for enzymological studies) must be prepared by adding a small volume of a concentrated solution of anthralin in an organic solvent to the aqueous medium. The criteria for a suitable organic solvent were solubility and relative stability of anthralin, water miscibility, and compatibility with the enzyme. Acetone was selected as the most suitable solvent (Table I). Stock solutions of anthralin (0.5 mg/ml or 1 mg/ml) in acetone could be used within 3 hr following the preparation.

The chemical decomposition of anthralin in acetone (0.5 mg/ml) was investigated over a 1-week period (Fig. 1B)[14]. At 25°, continuous exposure

[11] Under the present conditions (*i.e.*, Ringer buffer at pH 7.5 and 37°), the apparent Michaelis constants of glucose-6-phosphate and nicotinamide adenine dinucleotide phosphate were determined and found to be 0.1 m*M* and 0.05 m*M*, respectively.

[12] Model UV 25 spectrophotometer, Beckman.

[13] Mazda lamp, 75 watts.

[14] In all figures presented in this paper, each point is representative of one experiment.

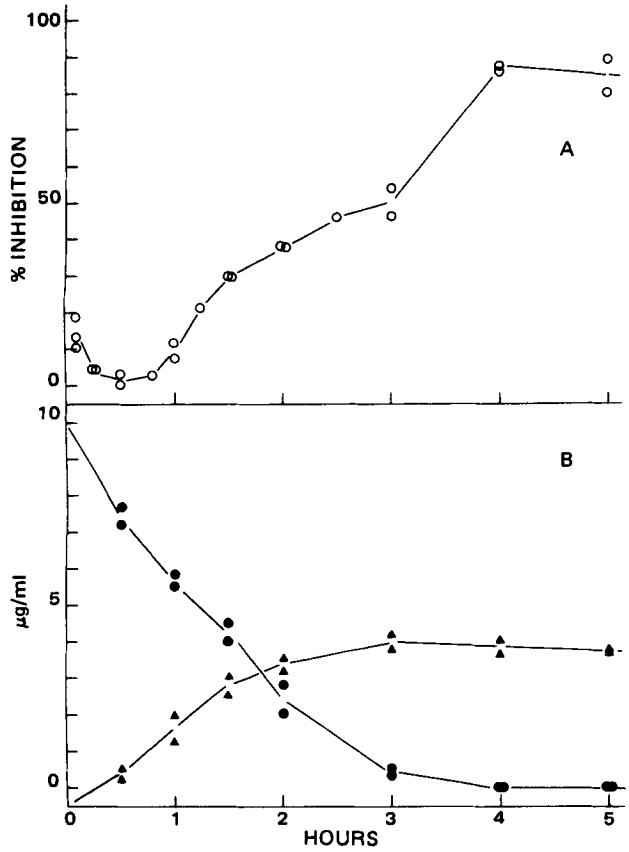

Figure 3—*Time course of the decomposition of anthralin in aqueous buffer (initial concentration = 10 µg/ml in 1% acetone–Ringer buffer, pH 7.5, 37°, light protection): inhibitory potency against glucose-6-phosphate dehydrogenase (A) and chemical composition of the medium as assayed by HPLC (B). Key: (○) percent of inhibition obtained by incubating the enzyme with the medium for 5 min; (●) concentration of anthralin; (▲) concentration of dimer.*

of the solution to light led to the decomposition of anthralin, in part *via* the dimer, and gave a final solution containing 20% quinone and having the characteristic color of anthralin-brown. For the studies in aqueous buffer, a 1% acetone–Ringer buffer system, in which the enzyme compatibility was even better than at 2% (Table I), was finally selected.

Time Dependence of Decomposition of Anthralin with Enzyme—The time dependence of the decomposition of anthralin in Ringer buffer and of the inhibition of glucose-6-phosphate dehydrogenase with enzyme present in the medium is as follows: Anthralin is unstable in aqueous solution at pH 7.5, irrespective of the organic solvent vehicle. When kept in 1% acetone–Ringer buffer at 37° under light protection, anthralin completely decomposed within 4 hr (Fig. 2B), undergoing, in part, oxidation to the dimer (40%), but not to detectable quantities of its quinone. The presence of glucose-6-phosphate dehydrogenase did not influence this process, since very similar results were obtained either in the presence (Fig. 2B) or in the absence (Fig. 3B) of enzyme in the Ringer buffer solution.

The corresponding time course of inhibition of glucose-6-phosphate dehydrogenase by anthralin (at its upper limit of solubility of 10 µg/ml) showed an initial slight inhibition (~15%) (Fig. 2A) which was rapidly established and lasted for ~1 hr, followed by an increasing inhibition, which was complete after 3 hr of incubation. Therefore, it was intriguing to speculate why enzyme inhibition should be initially maintained and then increased to reach its maximum over a 3-hr period, since HPLC analysis showed that anthralin was 25% degraded even after 30 min. The maintenance of the level of enzyme inhibition during the first phase could indicate that anthralin interacted slightly but irreversibly with the enzyme.

The second, slowly rising phase could be explained by the breakdown of anthralin into highly toxic species, which were responsible for the total destruction of enzymatic activity. Since the dimer was formed, it could have been suspected as being the highly toxic anthralin derivative. A solution of pure dimer (10 µg/ml = 22.1 µ*M*) was prepared in 1% acetone–Ringer buffer, at pH 7.5 and kept at 37° under light protection in the presence of the enzyme. At selected intervals, the composition of the medium was determined and the enzymatic activity was measured. The dimer was essentially stable over 2 hr (Fig. 4B) and caused only a modest, stable inhibition (10–20%) of glucose-6-phosphate dehydrogenase (Fig. 4A). Similar results were obtained with pure quinone (10 µg/ml, not shown).

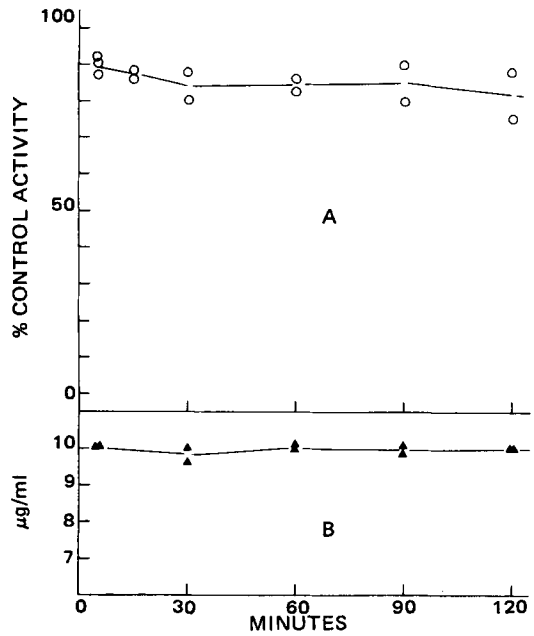

Figure 4—*Activity of glucose-6-phosphate dehydrogenase as a function of time of incubation with a solution of dimer (A) (10 µg/ml in 1% acetone–Ringer buffer, pH 7.5, 37°, light protection), and the concurrent chemical composition of the medium, as assayed by HPLC (B). Key: (○) percent of control enzymatic activity (the enzyme and dimer were added at time zero); (▲) concentration of dimer.*

These results demonstrate that the dramatic inhibition of glucose-6-phosphate dehydrogenase by anthralin solutions cannot be explained by the formation of dimer (40%), and suggest that one (or more) other unidentified breakdown products must be the toxic species. These breakdown products could not be detected under the present analytical conditions.

Time Dependence of Decomposition of Anthralin without Enzyme—The time dependence of the decomposition of anthralin in Ringer buffer, without enzyme and the inhibitory potency of the medium against glucose-6-phosphate dehydrogenase is as follows: Solutions of anthralin

Table I—Stability Properties of Anthralin in Pure Solvents and Compatibility of Solvent with Glucose-6-phosphate Dehydrogenase

Solvent	Stability[a]	Enzyme Compatibility[b] (percent solvent)
Methanol	– –	
1-Propanol	– –	ND[c]
1,3-Butanediol	– –	
Dimethylformamide	– –	
Ethanol	–	2
2-Propanol	–	1
1-Butanol	–	0
Tetrahydrofuran	–	0
2-Butanol	+	0
1,4-Dioxane	+	1
Acetonitrile	+	1
Acetone	++	2
2-Butanone	++	1
Acetic acid	++	0

[a] After 3 hr of exposure to day light at room temperature, the percentage of initial anthralin (0.5 mg/ml solvent) which has decomposed is >15% (– –), between 5 and 15% (–), between 1 and 5% (+), <1% (++). [b] Enzyme compatibility with solvent is expressed as the maximum percentage of solvent in the incubation medium, for which <10% of initial enzymatic activity is lost after 30 min of incubation at 37°. [c] Not done.

in 1% acetone–Ringer buffer (10 μg/ml) were left for appropriate periods at 37° under light protection in the absence of enzyme. The enzyme inhibitory potency of the aged solutions was assayed by adding glucose-6-phosphate dehydrogenase for an additional incubation period of 5 min.

The results shown in Fig. 3 indicate that the gradual disappearance of anthralin during the first 30 min was responsible for the concomitant decrease in the enzyme inhibitory potency of the solution. The reappearance of a stronger inhibitory activity, however, is in striking contrast with the complete degradation of anthralin which occurred during the following 2–3 hr and, as already noted, could not be attributed to the formation of the dimer.

Similarly, when anthralin was left in acetone (0.5 mg/ml) for several days under continuous exposure to light (Fig. 1), the final dark brown solution, which was totally devoid of anthralin or dimer, showed a strong toxicity against glucose-6-phosphate dehydrogenase (Fig. 1A). Incubation of 1% of the acetone solution with the enzyme in Ringer buffer (5 min) resulted in a total destruction of enzymatic activity, which could not be explained by the presence of quinone.

CONCLUSION

Using a highly sensitive and reproducible HPLC assay, additional evidence has been given for the chemical instability of anthralin in solution, particularly aqueous media. In acetone solution over long periods, anthralin decomposes, in part *via* the dimer, to give a final solution containing 20% quinone. In aqueous media at pH 7.5, 37°, and under light protection, the corresponding dimer (but not quinone) is formed. Near their upper limit of solubility in Ringer buffer, anthralin, dimer, and quinone interact with glucose-6-phosphate dehydrogenase, but this interaction leads to a fairly modest decrease of enzymatic activity. The dramatic changes regarding inhibition of the enzyme cannot be explained by the formation of dimer from anthralin and suggest that other breakdown products, unidentified as yet, must be the most active derivatives against glucose-6-phosphate dehydrogenase. Thus, if neutral aqueous buffers are used for the investigation of the mode of action of anthralin, the chemical instability of this molecule and the possible interference of highly active breakdown products should be kept in mind. Therefore, both *in vitro* and *in vivo* data should be interpreted cautiously.

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Steroidal Oxazoline Derivatives: Synthesis and *In Vitro* Effect on Bovine Pancreatic Ribonuclease Activity

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Abstract □ A series of steroidal oxazoline derivatives, containing different chains attached to the heterocyclic ring, were synthesized and examined for *in vitro* effect on bovine pancreatic ribonuclease activity. The results indicated weak anabolic properties for all products except Compounds III and XII which showed mild catabolic activities.

Keyphrases □ Oxazoline—steroidal derivatives, synthesis and *in vitro* effect on bovine pancreatic ribonuclease activity □ Derivatives—steroidal oxazoline, synthesis and *in vitro* effect on bovine pancreatic ribonuclease activity □ Ribonuclease activity—bovine, pancreatic, steroidal oxazoline derivatives, synthesis and *in vitro* effects

In connection with a program studying modified steroids, a variety of *N,N*-disubstituted aminoethyl ethers of 6-phenyl, benzyl, or thiazolidinyl-17β-estradiol were synthesized and tested for antiestrogenic properties (1). In addition, various androgenic and estrogenic keto-steroids were converted into the corresponding 4-substituted-3-thiosemicarbazone (2) and acylhydrazone (3–5) derivatives, and the products evaluated for anticancer (2, 4) and endocrinological (2, 3, 5) activities.

Extending the studies to steroids containing fused heterocyclic systems, the synthesis of a series of 2′-thio-17-oxoestra-1(10),4-dieno[2,3-*d*]oxazolines (V, VII, IX, and XI) and the corresponding estra-1,5(10)-dieno[4,3-*d*]oxazolines (VI, VIII, X, and XII), possessing methyl or *N,N*-disubstituted aminoethyl moieties in the heterocyclic ring, was undertaken[1]. The *in vitro* effect of the product on the activity of the bovine pancreatic ribonuclease was evaluated as a preliminary measure for their anabolic and catabolic properties (6) (Scheme I).

RESULTS AND DISCUSSION

Chemistry—The 2- (I) and 4-aminoestrones (II), prepared by reduction of the 2- and 4-nitroestrones (7) with sodium dithionite in alkaline medium (8), were treated with carbon disulfide and potassium hydroxide in boiling ethanol to produce 2′-thio-17-oxoestra-1(10),4-dieno[2,3-*d*]oxazoline (III), and 2′-thio-17-oxoestra-1,5(10)-dieno[4,3-

[1] This paper constitutes Part VII of the series on Steroidal Derivatives: Part VI is Ref. 5.

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