Biochemical Pharmacology, Vol. 53, pp. 189–197, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00661-2

Mechanism for the Stabilization *in Vivo* of the Aziridine Precursor 2-(4-Acetoxyphenyl)-2-chloro-*N*-methyl-ethylammonium Chloride by Serum Proteins

Ann Louw, Pieter Swart, Schalk S. de Kock and Kirsten J. van der Merwe*
DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF STELLENBOSCH, MATIELAND 7602, REPUBLIC OF SOUTH AFRICA

ABSTRACT. Oral and intraperitoneal administration of 2-(4-acetoxyphenyl)-2-chloro-*N*-methyl-ethylammonium chloride (Compound A), an analogue of phenyl aziridine precursors that occur in the shrub *Salsola tuberculatiformis* Botsch., had a contraceptive effect on female Wistar rats with a concomitant decrease in total body, uterus, and ovary mass and an increase in adrenal mass. Compound A elicited a Type II difference spectrum and inhibited the Type I deoxycorticosterone (DOC) induced difference spectrum of sheep adrenal cytochrome P450c11 in a manner similar to that of S2, a biologically active fraction isolated from *S. tuberculatiformis*. The effects of Compound A on the spectral properties of P450c11 were diminished with time in PBS. Electrospray mass spectrometry (ES-MS) indicated that the rate of cyclization of Compound A to the corresponding aziridine followed a time course similar to the attenuation of cytochrome P450c11 inhibition. It was concluded that the aziridine precursor, Compound A, rather than aziridine itself, was the inhibiting agent of sheep adrenal P450c11. Addition of sheep and rat plasma prevented the attenuation of the effect of Compound A on the spectral properties of cytochrome P450c11. Subsequent ES-MS analysis indicated that Compound A was stabilized in plasma by sex hormone binding globulin and corticosteroid binding globulin. These results suggest a mechanism whereby natural plant products, which are highly reactive and unstable *in vitro*, can be stabilized by binding to plasma proteins, and so remain biologically active *in vivo*. *Copyright © 1996 Elsevier Science Inc.* BIOCHEM PHARMACOL 53;2:189–197, 1997.

KEY WORDS. *Salsola tuberculatiformis* Botsch.; P450c11; adrenal steroidogenesis; aziridines; corticosteroid binding globulin; sex hormone binding globulin; rats; oestrus cycle

The contraceptive properties of plants belonging to the genus *Salsola* (Family: Chenopodiaceae) were first described by Ploss in 1902 [1]. Bushman folklore also mentions the use of the Namibian shrub *Salsola tuberculatiformis* Botsch. for contraceptive purposes [2]. Feeding experiments with the shrub caused contraception in rats [3, 4]. In addition, *S. tuberculatiformis* and extracts from the shrub have also been implicated in a syndrome of prolonged gestation and foetal post-maturity in sheep. This syndrome is characterized further by under-development of the udder and anaemia in pregnant ewes [3, 4].

Investigation into the contraceptive compounds occurring in *S. tuberculatiformis* led to the isolation of an active but labile HPLC fraction (S2)† from the dried plant material [5]. S2 elicits a Type II difference spectrum and inhibits the Type I DOC-induced difference spectrum of sheep adrenal cytochrome P450c11 [4, 5].‡ S2, however, is very labile and decomposes to synephrine (**I** in Fig. 1) in an acidic aqueous medium. Acetylation of S2 yields the acetylated aziridine structure **III** in Fig. 1, while acetylation of synephrine yields synephrine triacetate (**II** in Fig. 1) [6]. Further investigation into the chemical nature of S2 suggested that the active component might be 2-4-hydroxyphenyl)-1-methylaziridine (**V** in Fig. 2) or the corresponding open chain precursor (**IV** in Fig. 2) [6–8]. However, phenolic aziridines are extremely labile and therefore not ideal test compounds [8]. An aziridine precursor, Compound A (**VI** in Fig. 2), which cyclizes to the corresponding acetoxyphenyl methylaziridine (**VII** in Fig. 2) at physiological pH, was synthesized to test the hypothesis that the

* Corresponding author: Dr. Ann Louw, Department of Biochemistry, University of Stellenbosch, Private Bag X1, Matieland 7602, Republic of South Africa. Tel. 27-21-8083038; FAX 27-21-8083022; E-mail: al@maties.sun.ac.za

† *Abbreviations:* CBG, corticosteroid binding globulin; Compound A, 2-(4-acetoxyphenyl)-2-chloro-*N*-methyl-ethylammonium chloride; DOC, deoxycorticosterone; ES-MS, electrospray mass spectrometry; S2, HPLC fraction isolated from *Salsola tuberculatiformis* Botsch.; and SHBG, sex hormone binding globulin.

Received 1 May 1996; accepted 16 July 1996.

‡ Swart P, Swart AC, de Villiers CA and van der Merwe KJ, Manuscript submitted for publication.

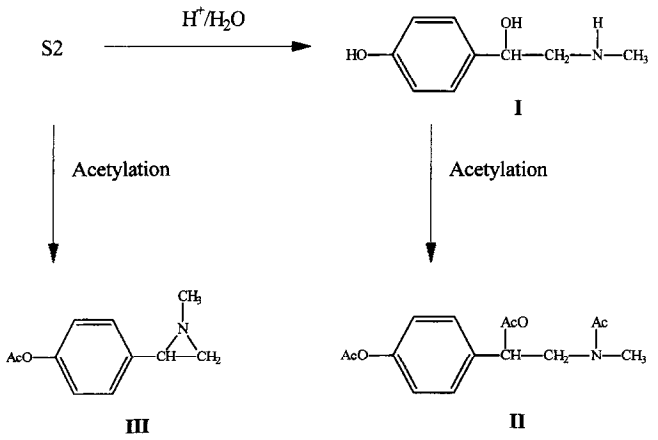

FIG. 1. Reaction scheme for the formation of synephrine (I), synephrine triacetate (II) and acetylated S2 (III).

biologically active component of *S. tuberculatiformis* is a phenylaziridine or its corresponding open chain precursor.

The present study tested the suitability of Compound A as a model for the biological effects of *S. tuberculatiformis* and S2 in certain biological systems. The effects of both oral and intraperitoneal administration of Compound A on rat ovarian cycling and the effect of Compound A on the spectral properties of sheep adrenal cytochrome P450c11 were investigated. In addition, the equilibrium between Compound A and its corresponding aziridine was studied with the aid of ES-MS.

MATERIALS AND METHODS

Synthesis of Compound A

DL-Synephrine hydrochloride (10 g) was dissolved in glacial acetic acid (27 mL) and cooled to 10° in an ice waterbath. Acetyl chloride (27 mL) was added slowly while stirring thoroughly, and the reaction temperature was kept below 30°. A white precipitate formed from the resulting clear solution within 12 hr. The fine white precipitate, Compound A, was collected by filtration under nitrogen, washed with cold anhydrous ether, followed by cold anhydrous acetone, and kept in a vacuum desiccator over silica gel for 12 hr before storage at −80°. The yield of the reaction was 13.21 g or 83.6% of Compound A. The compound was highly hygroscopic and decomposed after the absorption of moisture or when left open at room temperature. The structure and purity of Compound A were verified by proton nuclear magnetic resonance spectroscopy and fast atom bombardment mass spectrometry, which gave the expected molecular ion at 228,230.

Rat Ovarian Cycling

Nulliparous female Wistar rats with body mass between 190 and 210 g were housed individually in metabolic cages for oral administration experiments and communally in battery cages (five rats/cage) for intraperitoneal administration experiments. The temperature was held between 20 and 22°, and the animals were subjected to a 14:10 light:dark cycle. The rats in metabolic cages were fed daily with 20 g of crushed rat pellets, while those in battery cages received rat pellets *ad lib.* All rats received water *ad lib.* Rats were weighed daily at the same time, their conditions were noted, and a vaginal smear was taken [9]. The vaginal smears were stained on the day they were obtained [10]. The amount of food and water consumed by the rats in metabolic cages was also noted. A period was allowed for acclimatization, and vaginal smears were taken to determine the length and regularity of the oestrus cycle. After at least four consecutive 4-day cycles (before administration period: days 0–15), the rats were divided into experimental groups (six rats/group). The first day of administration (oral or intraperitoneal) of Compound A was designated arbitrarily as day 16 and coincided with oestrus. Administration of Compound A was continued for 16 days (days 16–31). After the administration period, monitoring of the oestrus cycle was continued for an additional 16 days (days 32–47) for one group of rats, while another group was killed on day 31 to obtain the mass of specific organs (uterus, ovaries, adrenals, and liver) and blood.

For oral administration, each rat received 0.16 to 94 μmol of Compound A per day, dissolved by sonication in 5 mL of 96% ethanol. The ethanolic solution of Compound A (5 mL) was mixed with 20 g rat feed, and the treated feed was left for 3–4 hr in a fume cupboard to allow evaporation of the ethanol. The control rats received 20 g rat feed treated with 5 mL of 96% ethanol only. For intraperitoneal administration, each rat received 0.16 to 3.4 μmol of Compound A per day, dissolved in 0.5 mL of sterile filtered PBS (0.1 M phosphate buffer and 0.9% NaCl; pH 7.12). The solution was injected intraperitoneally directly after preparation using a 1-mL tuberculin syringe. Control rats were injected intraperitoneally with 0.5 mL PBS only.

Spectral Studies with Sheep Adrenal Cytochrome P450c11

All spectra were recorded on a Beckman DU 650 spectrophotometer equipped with a six-cell attachment. Lyophi-

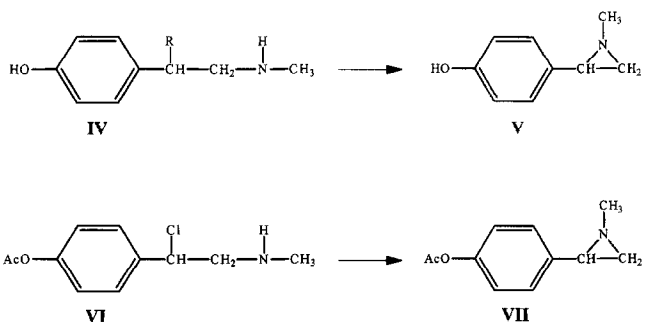

FIG. 2. Chemical structures of aziridine precursors and their corresponding aziridines: 2-(4-hydroxyphenyl)-1-methylaziridine (V) and its open chain precursor (IV); 2-(4-acetoxyphenyl)-1-methylaziridine (VII) and its open chain precursor: Compound A (VI). R = good leaving group.

lized mitochondrial powder from sheep adrenals was prepared as previously described and used as a source of cytochrome P450c11 [5]. Inhibition of DOC-induced difference spectra and induction of difference spectra by test compounds were performed using the method of Swart *et al.* [5]. Compound A was prepared either in PBS, in sheep or rat plasma, or in heated sheep or rat plasma, and immediately added to the test system. The blank for DOC-induced difference spectra contained all components except DOC to eliminate contributions by endogenous DOC in serum and other P450–substrate complexes that may be present in the mitochondrial powder preparation.

Preparation of Plasma

Sheep blood was collected from the abattoir in containers with 2.5 mL potassium oxalate/50 mL of blood as anticoagulant. Rat blood was collected from control rats in tubes containing EDTA and heparin. The blood was centrifuged at 3000 *g* for 15 min at room temperature and the plasma decanted into 1-mL Eppendorf tubes and stored at −20°. Heated plasma was obtained by heating plasma at 60° for 1 hr in a rapidly circulating waterbath, centrifuging for 15 min at 3000 *g*, and decanting the heated plasma. Heating the plasma destroys the low capacity but high affinity steroid binding proteins, CBG and SHBG. Albumin, which has a low affinity but high binding capacity for steroids, is not affected by the same treatment [11].

ES-MS Studies

All ES-MS studies were carried out on a VG-Biotech mass spectrometer. Compound A (M^+ = 228,230), at a concentration of 17 mM, was prepared in either PBS or sheep plasma, diluted 225 times with 50:50 water:acetonitrile containing 1% formic acid, and assayed immediately. The injection volume was 10 μL and the mobile phase was 50:50 water:acetonitrile containing 1% formic acid at a flow rate of 10 μL/min. The rate of aziridine (M^+ = 192) and synephrine (M^+ = 168) formation was monitored at 5-min intervals for 30 or 60 min by positive mode ES-MS analysis. A focus:skimmer value of 16/21 was needed to prevent decomposition, the cone voltage was set at 20, and the source temperature was kept at 65°. The signal of an injection of diluted buffer or plasma was subtracted from the spectra obtained.

To test the effect of albumin on the stabilization of Compound A, BSA at a concentration of 36 mg/mL was added to PBS. Sheep plasma was preincubated for 60 min at 37° with cortisol or testosterone in 40 μL propylene glycol (final concentration in plasma was 4%) at molar ratios of Compound A:cortisol or testosterone of 242:1 and 3:1 to determine the effect of CBG or SHBG on the stabilization of Compound A.

RESULTS

Rat Ovarian Cycling

Daily intraperitoneal administration of Compound A for 16 days prolonged the oestrus cycle in four of the six experimental animals at a dosage of 0.16 μmol Compound A/day and in all six experimental animals at a dosage of 3.4 μmol Compound A/day (Fig. 3). Oral administration of Compound A for the same period showed no prolongation of the oestrus cycle at a dosage of 0.16 μmol Compound A/day. Prolongation in all six of the experimental animals was only achieved at a much higher dosage (94 μmol Compound A/day) when compared with animals receiving Compound A via intraperitoneal administration (Fig. 3). The average number of oestrus days per 16-day period during intraperitoneal administration of 3.2 and 3.4 μmol Compound A/day showed a significant decrease ($P < 0.01$) relative to the control groups, while during oral administration a significant decrease ($P < 0.05$) was seen only at an intake of 94 μmol Compound A/day (Table 1). In all cases, the average number of oestrus days per 16-day period showed a tendency to return to the values observed in the control groups after administration of Compound A was discontinued.

The body weight gain of rats receiving Compound A

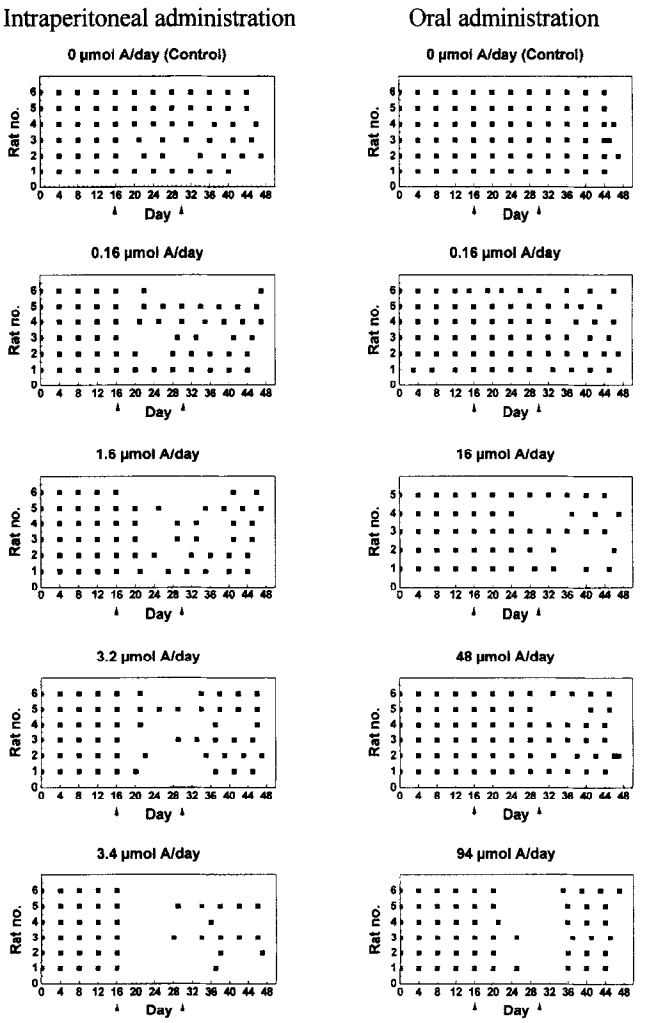

FIG. 3. Effect of intraperitoneal or oral administration of Compound A on the oestrus cycle of female Wistar rats. Key: (■) day of oestrus. The time period between arrows indicates the duration of administration.

TABLE 1. Summary of the effect of oral or intraperitoneal administration of Compound A on the average number of oestrus days per experimental period

Compound A (μmol administered/day)	Number of oestrus days per 16-day experimental period		
	Before administration (days 0–15)	During administration (days 16–31)	After administration (days 32–47)
Oral			
0.00 (N = 6)	4.00 ± 0.00	4.00 ± 0.00	4.00 ± 0.00
0.16 (N = 6)	4.00 ± 0.63	4.00 ± 0.00	3.83 ± 0.41
16.00 (N = 5)	4.00 ± 0.00	3.80 ± 0.45	3.20 ± 0.84*
48.00 (N = 6)	4.00 ± 0.00	4.00 ± 0.00	3.67 ± 0.82
94.00 (N = 6)	4.00 ± 0.00	2.00 ± 0.71*	3.11 ± 0.33†
Intraperitoneal			
0.00 (N = 6)	4.00 ± 0.00	3.83 ± 0.41	3.67 ± 0.52
0.16 (N = 6)	4.00 ± 0.00	3.17 ± 0.98	3.33 ± 1.21
1.60 (N = 6)	4.00 ± 0.00	2.83 ± 0.98	3.17 ± 0.75
3.20 (N = 6)	4.00 ± 0.00	2.33 ± 0.82†	3.33 ± 0.82
3.40 (N = 6)	4.00 ± 0.00	1.33 ± 0.52†	1.83 ± 1.72*

Values are means ± SD; N = the number of rats per experimental group.
,† Statistical significance ($P < 0.05$, †$P < 0.01$ vs control values) was evaluated with Dunnett's Multiple Comparisons test.

intraperitoneally was statistically significantly affected at a dosage of 3.2 μmol Compound A/day ($P < 0.05$) and 3.4 μmol Compound A/day ($P < 0.001$) during the initial period of administration (Fig. 4). The weight gain was the lowest 4 days after administration had commenced (day 20), recovered to that of the control group during the latter period of administration (days 28–31), and matched that of the control group for the period after administration (days 32–47). In the case of oral administration of Compound A, a significant ($P < 0.001$) decrease in body weight gain was observed only when 94 μmol of Compound A was administered per day. The decrease in body weight gain was observed throughout the period of administration with the lowest weight gain being observed on the final day of administration (day 31). After administration had ceased, the weight gain recovered but was still significantly ($P < 0.01$) lower than that of the control on the day that the experiment was terminated (day 47).

The effects of intraperitoneal administration of 3.4 μmol Compound A/day on the relative weights of particular organs in rats are presented in Fig. 5. The relative weights of the uterus and ovaries were decreased (though not significantly), while those of the adrenals and liver were increased significantly ($P < 0.05$) relative to that of the control rats.

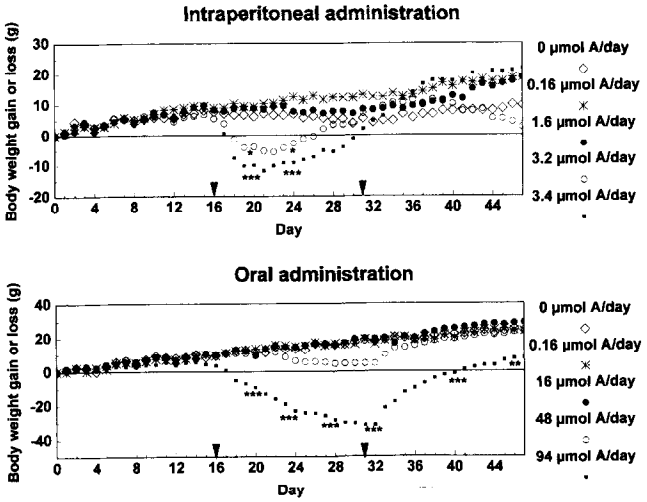

FIG. 4. Average body weight gain or loss (N = 6) of control and Compound A-treated rats. The time period between arrows indicates the duration of administration. Statistical significance (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs control values) was evaluated with Dunnett's Multiple Comparisons test on days 8, 16, 20, 24, 28, 32, 40, and 47.

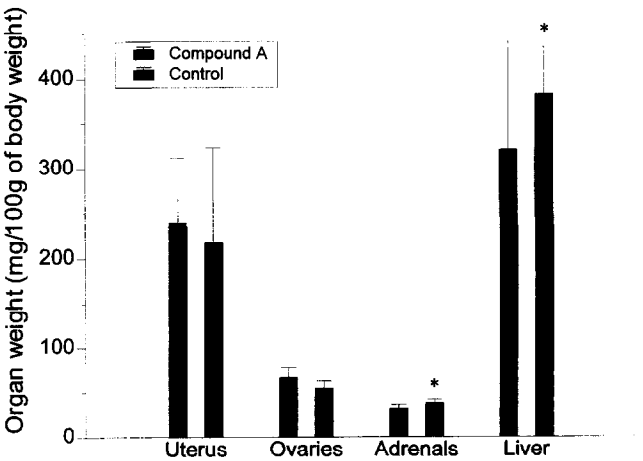

FIG. 5. Effect of intraperitoneal administration of Compound A (3.4 μmol/day) on relative organ weights in female Wistar rats. Statistical significance (*$P < 0.05$ vs control values; N = 6; values are means ± SD) was evaluated with the two-tailed Student's *t*-test. Values for liver were divided by 10.

Spectral Studies with Sheep Adrenal Cytochrome P450c11

INDUCTION OF DIFFERENCE SPECTRA BY TEST COMPOUNDS. DOC, the natural substrate for cytochrome P450c11, elicited a Type I difference spectrum when incubated with mitochondrial powder prepared from sheep adrenals (Fig. 6). The Type I difference spectrum had an absorbance maximum at 390 nm and an absorbance minimum at 420 nm. Compounds that are not substrates for the cytochrome P450c11 enzyme may also bind to the enzyme and elicit a difference spectrum [12]. Compound A induced a Type II difference spectrum with an absorbance maximum at 440 nm and an absorbance minimum at 410 nm (Fig. 6). The Type II difference spectrum of Compound A was attenuated with time in buffer, while the DOC-induced Type I difference spectrum did not change (Fig. 6).

INHIBITION OF DOC-INDUCED DIFFERENCE SPECTRA. Inhibition of DOC-induced difference spectra by Compound A in PBS, sheep plasma, or rat plasma is presented in Figs. 7 and 8. Compound A, prepared in PBS, inhibited the Type I DOC-induced difference spectrum in a concentration-dependent manner. The inhibition however, was attenuated with time. Two hundred micromolar Compound A prepared in PBS showed 52% inhibition at 5 min and no inhibition at 30 min. Native sheep plasma appeared to stabilize the inhibitory effect of Compound A with a higher percent inhibition being shown at 5 min than in PBS (80 vs 52%) and little decrease in percent inhibition being observed after 30 min (Fig. 8: curve 3). In contrast to native sheep plasma, heated sheep plasma showed attenuation of the inhibitory influence of Compound A with time in a manner similar to that of Compound A in PBS. In native rat plasma, the percent inhibition was greater than 100%, which indicated the presence of a peak instead of a trough at 420 nm (see Figs. 7 and 9). Curve 5 in Fig. 8 suggests that native rat plasma stabilized Compound A to a greater degree than native sheep plasma and that heating of rat plasma (Fig. 8: curve 6) abolished some of the stabilizing effect of rat plasma but not all. The inhibitory effect of Compound A in heated rat plasma (Fig. 8: curve 6) was

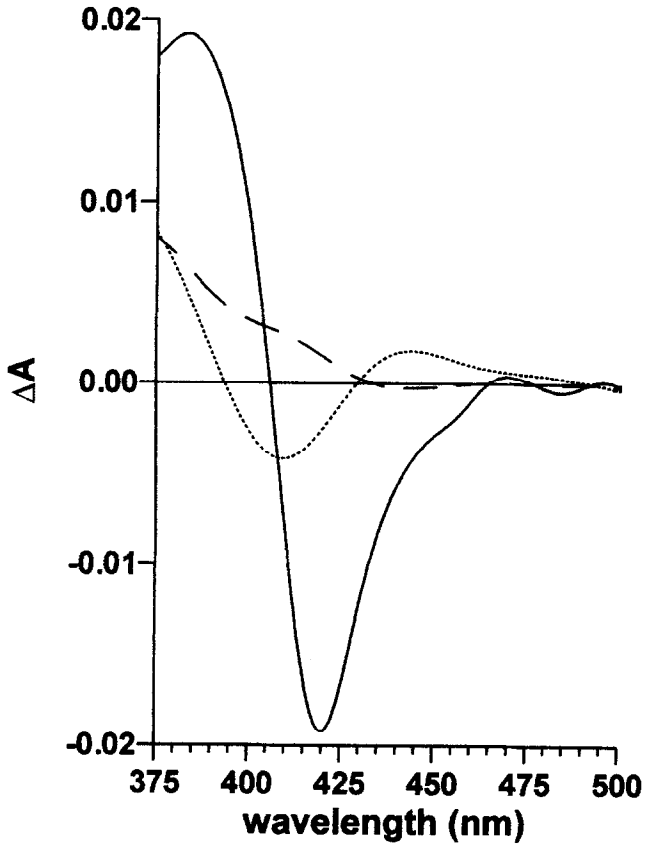

FIG. 6. Type I difference spectrum of a sheep adrenal cytochrome P450 (0.71 μM) preparation induced by 5 μM DOC (—) and Type II difference spectra of an adrenal cytochrome P450 preparation induced by 200 μM Compound A prepared in PBS, 5 min (---) and 60 min (– –) after addition of test compound to the experimental system. Each spectrum represents the average of six spectra taken.

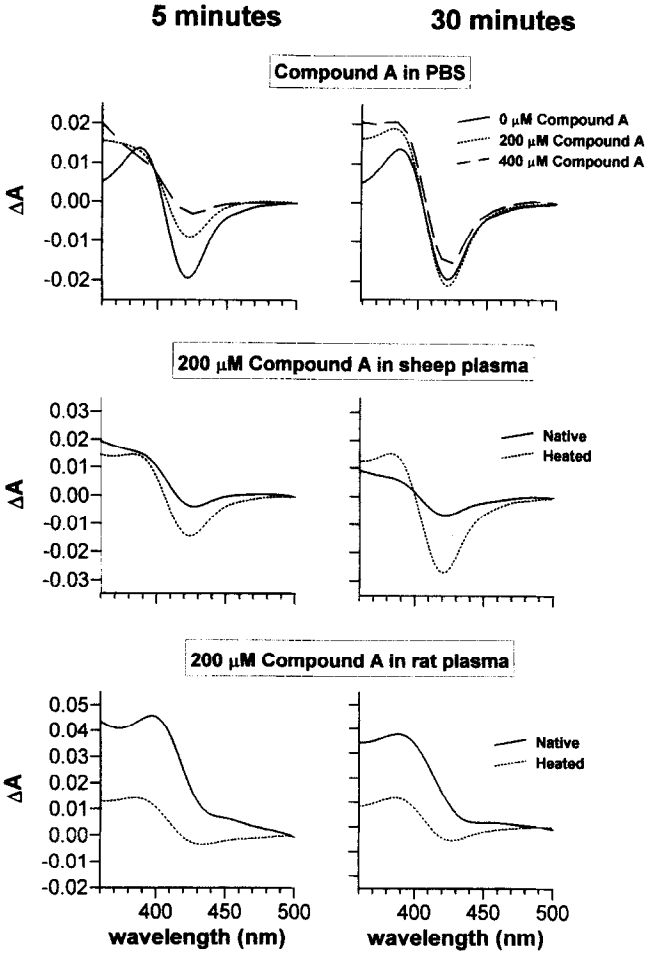

FIG. 7. Inhibition of DOC-induced (5 μM) difference spectra of an adrenal cytochrome P450 (0.88 to 0.99 μM) preparation by Compound A prepared in PBS, native and heated sheep plasma, or native and heated rat plasma. Spectra were taken 5 and 30 min after the addition of Compound A to the test system and are the average of six spectra taken.

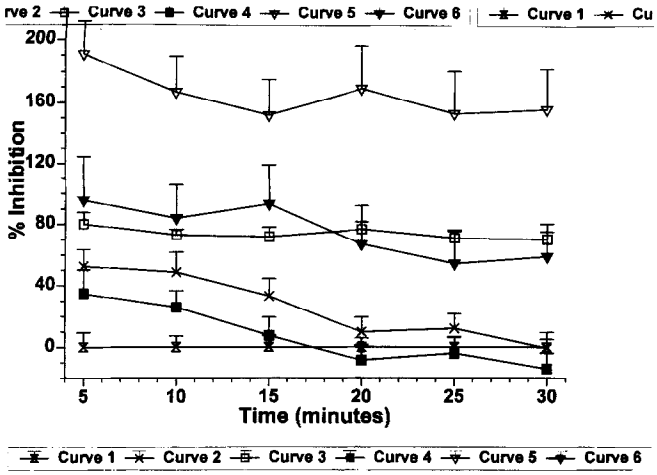

FIG. 8. Time study of percent inhibition of DOC-induced (5 μM) difference spectra of an adrenal cytochrome P450 preparation by Compound A. Values are means ± SD of six points. Curve 1: 0 μM Compound A in PBS; Curve 2: 200 μM Compound A in PBS; Curve 3: 200 μM Compound A in native sheep plasma; Curve 4: 200 μM Compound A in heated sheep plasma; Curve 5: 200 μM Compound A in native rat plasma; Curve 6: 200 μM Compound A in heated rat plasma. The decrease in the trough of the Type I difference spectra was used as the criterion of inhibition and was measured as the absorbance difference at 500 nm minus the absorbance difference at 420 nm per μM P450. Percent inhibition was calculated as follows:

$$\% \text{ Inhibition} = 100 - \frac{(\text{Abs. 500 mm} - \text{Abs. 420 nm sample})/\mu\text{M P450}}{(\text{Abs. 500 mm} - \text{Abs. 420 nm DOC alone})/\mu\text{M P450}} \times 100$$

similar to that obtained for Compound A in native sheep plasma (Fig. 8: curve 3).

The inhibition of the DOC-induced difference spectra by 200 μM Compound A prepared in native rat plasma appeared to differ from the previous spectra and prompted further investigation. In Fig. 9, the inhibition of the DOC-induced spectrum by different concentrations (5, 100, and 200 μM) of Compound A showed an increase and shift in the absorbance maximum to higher wavelengths and a concomitant decrease and shift in the absorbance minimum as the concentration of Compound A was increased. A high concentration of Compound A, however, did not abolish the Type I DOC-induced spectrum but rather appeared to induce an additive spectrum. This suggested that the Type II interaction occurred independently of the Type I interaction, and that the bound Type I compound was not displaced by addition of the Type II compound. This is illustrated by the inset in Fig. 9, where subtraction of the Type I DOC-induced spectrum from the spectrum induced by addition of both DOC and Compound A resulted in a Type II difference spectrum that was similar to the Type II spectrum induced by Compound A alone. Yoshida and Kumaoka [12] found similar results when Type I and II compounds were incubated simultaneously with hepatic microsomes. Thus, the difference spectrum of 5 μM DOC plus 200 μM Compound A in native rat plasma in Fig. 7 was an additive spectrum of both the Type I (DOC-induced) and Type II (Compound A-induced) spectra and suggested that in native rat plasma the effective concentration of Compound A binding to the cytochrome was higher than in native sheep plasma.

ES-MS Studies

The cyclization of Compound A (M^+ = 228,230) to the corresponding aziridine (M^+ = 192) was monitored by ES-MS over a period of time. The cyclization of Compound A prepared in PBS was essentially complete after 60 min, with only about 10% of the total ion count being attributed to Compound A while the rest was the corresponding aziridine (Figs. 10 and 11). The aziridine decomposed to synephrine, and at 72 hr 8% of the total ion count could be attributed to synephrine (results not shown). In contrast to the scenario in PBS, the cyclization of Compound A prepared in sheep plasma proceeded at a slower rate, with 75% of the total ion count being attributed to Compound A at

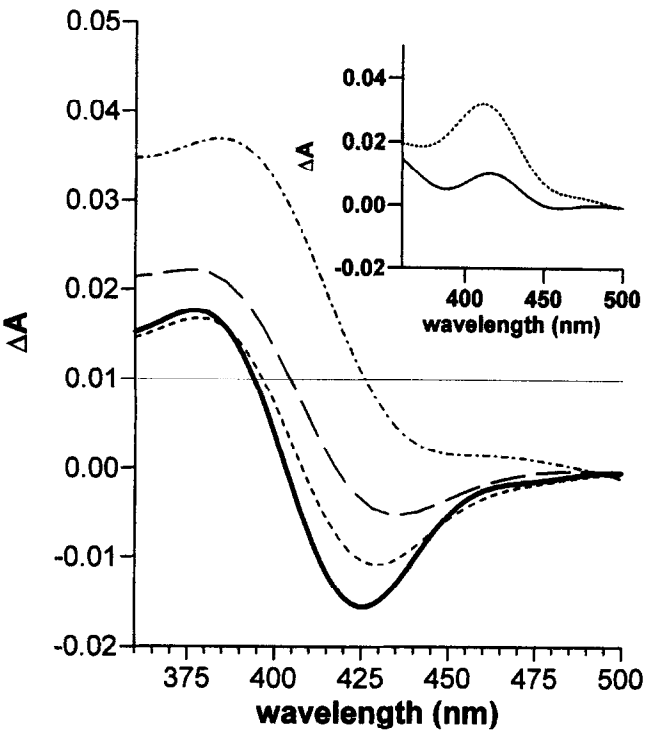

FIG. 9. Inhibition of DOC-induced (5 μM) difference spectra of an adrenal cytochrome P450 (0.78 μM) preparation by Compound A prepared in native rat plasma 30 min after addition of Compound A to the test system. Spectra are the average of six spectra taken. (—) 0 μM Compound A; (···) 5 μM Compound A; (– –) 100 μM Compound A; and (–·–) 200 μM Compound A. Inset: (···) subtraction of the Type I DOC-induced spectrum from the spectrum induced by addition of both DOC (5 μM) and Compound A (200 μM); and (—) the Type II spectrum induced by Compound A (200 μM) alone.

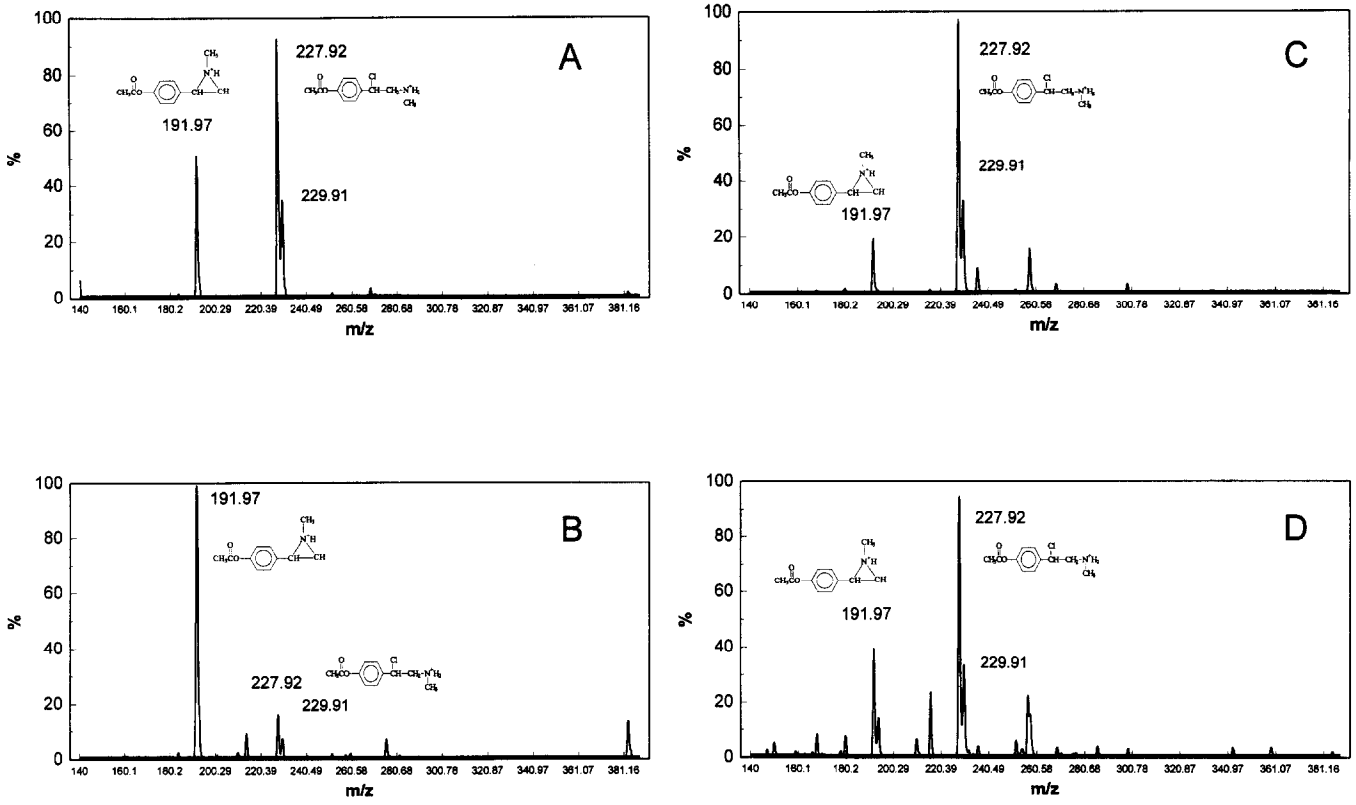

FIG. 10. ES-MS spectra of 17 mM Compound A prepared in PBS or native sheep plasma. Spectra were taken as described in Materials and Methods. Key: (A) After 5 min in PBS, (B) after 60 min in PBS, (C) after 5 min in native sheep plasma, and (D) after 60 min in native sheep plasma. Compound A (M^+ = 228,230); aziridine (M^+ = 192).

60 min. The remaining 25% was the corresponding aziridine (Figs. 10 and 11).

Sheep plasma contains several steroid binding proteins that may be responsible for the stabilization of Compound A. The most important include albumin, CBG, and SHBG. Addition of BSA to PBS (Fig. 11, curve 7) did not stabilize Compound A, which indicated that albumin did not contribute significantly to the stabilization of Compound A. However, addition of both cortisol (Fig. 11, curve 4) and testosterone (Fig. 11, curve 6) to sheep plasma in a molar ratio of 1:3 relative to Compound A decreased the percentage of Compound A present, and suggested that both CBG (cortisol binds primarily to CBG) and SHBG (testosterone binds primarily to SHBG) could be involved in the stabilization of Compound A. The addition of 4% propylene glycol alone to sheep plasma had no effect on the degree of cyclization.

The time scale of cyclization of Compound A to aziridine suggested that it may be Compound A rather than the aziridine which is the active species involved in inhibiting the DOC-induced difference spectra of adrenal cytochrome P450c11. In Fig. 12 it can be seen clearly that in PBS as well as in sheep plasma the percent of Compound A present correlated with the percent inhibition of the DOC-induced difference spectra (r = 0.96 in PBS and r = 0.81 in sheep plasma).

DISCUSSION

The study of the effect of Compound A on the ovarian cycling in female rats was performed primarily to assess the effect on reproductive performance in relation to previous results [3, 4]. Female Wistar rats exhibited a prolonged oestrus cycle, characterized by extended vaginal dioestrus, when Compound A was administered intraperitoneally at a dosage of 3.2 and 3.4 μmol Compound A/day and orally at a dosage of 94 μmol Compound A/day for 16 days. Oestrus cycles returned to that of the control group upon discontinuation of Compound A. The effect on the oestrus cycle was accompanied by a decrease in body weight gain, a decrease in the relative weights of ovaries and uterus, and an increase in the relative weights of the adrenals and liver. A similar reversible prolongation of the oestrous cycle in female rats was observed by Basson *et al.* [3] during feeding experiments with S. *tuberculatiformis*. They also observed a decrease in body weight, a decrease in the weights of the uterus and ovaries, and an increase in the weight of the adrenals. The weight of the liver also decreased but not significantly. In feeding experiments with metyrapone (a known inhibitor of adrenal cytochrome P450c11) van der Merwe *et al.* [4] found oestrus cycle blocking activity accompanied by severe anorexia. Several other authors have shown that adrenal corticosteroid biosynthesis inhibitors

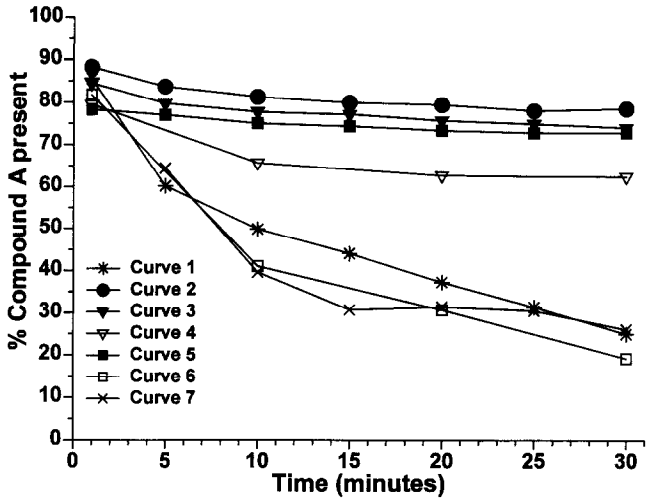

FIG. 11. Degree of cyclization of Compound A to aziridine over time as measured by ES-MS. Values are means of six assays. The results are expressed as percent Compound A present and was calculated as follows:

$$\% \text{ Compound A present} = \frac{\text{Total ion count for peaks } M^+ = 228{,}230}{\text{Total ion count for peaks } M^+ = 228{,}230 \text{ and } 192}$$

Curve 1 = Compound A in PBS; curve 2 = Compound A in native sheep plasma; curve 3 = Compound A in native sheep plasma preincubated with cortisol (242 mol Compound A:1 mol cortisol); curve 4 = Compound A in native sheep plasma preincubated with cortisol (3 mol Compound A:1 mol cortisol); curve 5 = Compound A in native sheep plasma preincubated with testosterone (242 mol Compound A:1 mol testosterone); curve 6 = Compound A in native sheep plasma preincubated with testosterone (3 mol Compound A:1 mol testosterone); and curve 7 = Compound A in PBS plus BSA (36 mg/mL).

(like metyrapone, aminoglutethimide, and amphenone B) cause growth inhibition, adrenal and hepatic hypertrophy, and uterine hypotrophy [13–16]. Our results indicate that Compound A had effects similar to that of *S. tuberculatiformis* on the reproductive function in female rats and that these effects are probably mediated by a disturbance in the corticosteroid profile of the animals.

Compound A interacted with mitochondrial powder prepared from sheep adrenals to induce a Type II difference spectrum, which indicates the presence of an amino group that reacts with the heme iron of the cytochrome [12]. S2, prepared from *S. tuberculatiformis*, also induced a Type II difference spectrum [5]. In addition, Compound A inhibited the Type I DOC-induced difference spectrum of mitochondrial powder prepared from sheep adrenals in a manner similar to that observed by Swart *et al.* [5] with S2. Our results indicate that the resultant spectrum was a sum of a Type I DOC-induced spectrum and a Type II Compound A-induced spectrum, which suggests that the two substrates bind independently and that Compound A does not displace DOC upon binding. This is consistent with the results obtained by Yoshida and Kumaoka [12] when Type I and Type II compounds were added simultaneously. These results strongly suggest that Compound A and S2 interact with cytochrome P450 prepared from sheep adrenals in a similar manner.

The time studies that monitored the effect of Compound A prepared in PBS on the Type I DOC-induced difference spectrum over a period of 30 min clearly showed that Compound A inhibited the Type I spectrum but that this inhibition was diminished with time. ES-MS indicated that the rate of cyclization of Compound A to the corresponding aziridine in PBS correlated with the attenuation of inhibition found in the cytochrome P450c11 system if Compound A was prepared in PBS. It is, therefore, the aziridine precursor, Compound A, rather than the aziridine, that is the inhibiting agent in the cytochrome P450c11 system.

Spectrophotometric and ES-MS time studies with sheep plasma showed that the plasma stabilized the inhibitory effect of Compound A in the P450c11 system by retarding the cyclization of Compound A to the corresponding aziridine. The correlation between the amount of Compound A present and the percent inhibition of the DOC-induced spectra was high. Heating of the sheep plasma, which destroys CBG and SHBG but not albumin, resulted in an inhibition pattern similar to that observed for PBS, i.e. no stabilization of Compound A [17]. The suggestion that CBG and SHBG may be involved in the stabilization of Compound A in sheep plasma was confirmed by ES-MS studies, where sheep plasma preincubated with cortisol (which binds primarily to CBG) or testosterone (which binds primarily to SHBG) resulted in a higher degree of cyclization of Compound A than in sheep plasma without cortisol or testosterone. Albumin, on the other hand, did not appear to have a significant effect on the stabilization of Compound A. These results correlate with the fact that

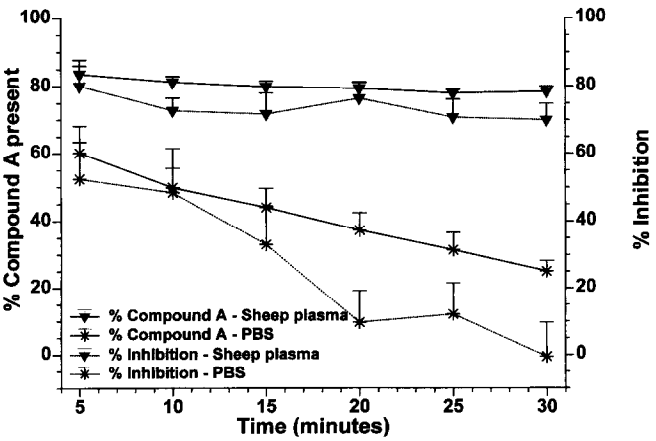

FIG. 12. Correlation between the percent inhibition of DOC-induced difference spectra by Compound A (see legend of Fig. 8) and percent Compound A present as measured by ES-MS (see legend of Fig. 11). Values are means ± SD of six assays. The correlation coefficient (r) between percent inhibition and percent Compound A was 0.81 in sheep plasma and 0.96 in PBS.

although albumin has a high capacity to bind steroids in plasma it has a low affinity for steroids [18].

Rat plasma stabilized Compound A in the P450c11 test system to a greater degree than sheep plasma, which suggests the presence of an effectively higher concentration of Compound A in rat plasma. Even after heating some stabilizing effect was retained.

The discrepancy between the stabilizing effect of native sheep and rat plasma on Compound A in the cytochrome P450 studies may be explained by the fact that although the binding affinities of both rat and sheep plasma for cortisol are similar, the binding capacity of rat plasma (40 μg/100 mL) exceeds that of sheep plasma (8.8 μg/100 mL) [17–19]. The fact that a degree of stabilization was still observed in heated rat plasma, but not in heated sheep plasma, may once again be explained by species differences. In this regard, Seal and Doe [19] found that, although heating to 60° for 15 min inactivated 80% of the binding capacity in rat plasma while in sheep plasma only 60% of the cortisol binding was inactivated, the higher initial capacity of rat plasma before heating resulted in a cortisol binding capacity after heating that equalled that of sheep plasma before heating.

In light of the results obtained with ES-MS and the cytochrome P450 studies, the differences in the contraceptive effect observed between orally and intraperitoneally administered Compound A could be ascribed to the fact that orally administered Compound A cyclizes to the corresponding aziridine during the preparation and absorption of the compound into the blood. Thus, during oral administration the effective plasma concentration of Compound A, the reactive species, will be lower than that of the intraperitoneally administered compound.

To summarize, the results suggest a mechanism whereby natural products, such as those that occur in *S. tuberculatiformis* or in S2, the HPLC fraction isolated from the plant, which are highly reactive and labile *in vitro*, may be biologically active *in vivo* due to stabilization by binding to plasma proteins. Furthermore, both CBG and SHBG may be involved in the stabilization of Compound A in plasma, and Compound A may be involved in the inhibition of adrenal cytochrome P450c11, and thus cortisol or corticosterone biosynthesis. The biological effects of Compound A in both rats and sheep may simply be the interaction with a specific target organ such as the adrenals, in which case binding to plasma proteins acts primarily as a transport mechanism. Alternatively, the binding of Compound A to CBG and SHBG, with possible concurrent displacement of the endogenous steroid ligand, may contribute towards the biological effect or even be the biological effect itself. It has been shown, for example, that some of the extra-adrenal effects of metyrapone may be attributed to the displacement of cortisol from albumin [20].

The authors wish to thank Allister Simons for his expert technical assistance during the rat studies.

References

1. Ploss H, *Das Weib in d. Natur- u. Völkerkunde* **1:** 670, 1902, as cited by Brondegaard VJ, Contraceptive plant drugs. *Planta Med* **23:** 167–172, 1973.
2. Maritz K, Eienskappe van die pil in gannabos. *Dagbreek en Landstem* **33:** 1, 1969.
3. Basson PA, Morgenthal JC, Bilbrough RB, Marais JL, Kruger SP and van der Merwe JLdeB, "Grootlamsiekte," a specific syndrome of prolonged gestation in sheep caused by a shrub, *Salsola tuberculata* (Fenzl ex Moq) Schinz var. *tomentosa* C. A. Smith ex Aellen. *Onderstepoort J Vet Res* **36:** 59–103, 1969.
4. van der Merwe KJ, Hofmeyr JHS, Swart P, Parkin DP, Rossouw J, Hartshorne J, van Rensburg SJ, Morgenthal JC and Basson PA, Natural products affecting the gestation period of sheep and their mode of action. *S Afr J Sci* **72:** 184, 1976.
5. Swart P, van der Merwe KJ, Swart AC, Todres PC and Hofmeyr J-HS, Inhibition of cytochrome $P450_{11\beta}$ by some naturally occurring acetophenones and plant extraxts from the shrub *Salsola tuberculatiformis*. *Planta Med* **59:** 139–143, 1993.
6. van der Merwe KJ, de Kock SS, Swart P and Fourie L, The application of mass spectrometry in the study of labile natural products. *Biochem Soc Trans* **19:** 432s, 1991.
7. van der Merwe KJ, de Kock SS, Swart P and Fourie L, The electron impact and fast atom bombardment mass spectrometry of aziridines and their 2-chloroethylamine precursors. *Biol Mass Spectrom* **21:** 672–674, 1992.
8. Fourie L, van der Merwe KJ, Swart P and de Kock SS, Application of fast atom bombardment mass spectrometry for the analysis of biologically active compounds. *Anal Chim Acta* **279:** 163–166, 1993.
9. Zarrow MX, Yochim JM and McCarthy JL, *Experimental Endocrinology: A Source Book of Basic Techniques*. Academic Press, New York, 1964.
10. Drury RAB and Wallington EA, *Carleton's Histological Technique*. Oxford University Press, New York, 1967.
11. Hammond GL, Lähteenmäki PLA, Lähteenmäki P and Luukkainen T, Distribution and percentages of non-protein bound contraceptive steroids in human serum. *J Steroid Biochem* **17:** 375–380, 1982.
12. Yoshida Y and Kumaoka H, Studies on the substrate-induced spectral change of cytochrome P-450 in liver microsomes. *J Biochem (Tokyo)* **78:** 455–468, 1975.
13. Gower DB, Modifiers of steroid-hormone metabolism: A review of their chemistry, biochemistry and clinical applications. *J Steroid Biochem* **5:** 501–523, 1974.
14. Karlsson MG, Eriksson UJ and Swenne I, Perinatal hypoadrenalism in the rat does not alter glucose tolerance and insulin secretory response to glucose. *Ups J Med Sci* **95:** 147–155, 1990.
15. Chart JJ, Sheppard H, Allen MJ, Bencze WL and Gaunt R, New amphenone analogs as adrenocortical inhibitors. *Experientia* **XIV:** 151–152, 1958.
16. Oohata N, Kawamura I, Lacey E, Nishigaki F, Matsumoto S, Tsujimoto S, Naoe Y, Manda T and Shimomura K, A new aromatase inhibitor, FR901537: II. Pharmacological and antitumor effects. *J Antibiot (Tokyo)* **48:** 763–767, 1995.
17. Keller N, Sendelbeck LR, Richardson UI, Moore C and Yates FE, Protein binding of corticosteroids in undiluted rat plasma. *Endocrinology* **79:** 884–906, 1966.
18. Paterson JYF and Hills F, The binding of cortisol by ovine plasma proteins. *J Endocrinol* **37:** 261–268, 1967.
19. Seal US and Doe RP, Corticosteroid-binding globulin: Biochemistry, physiology and phylogeny. In: *Steroid Dynamics* (Eds. Pincus G, Nakas T and Tait JF), pp. 63–90. Academic Press, New York, 1966.
20. Kehlet H and Binder Chr, Effect of metyrapone on cortisol binding capacity in plasma. *Acta Endocrinol (Copenh)* **81:** 787–792, 1976.