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EFFECTS OF ETOMIDATE ON STEROID BIOSYNTHESIS IN SUBCELLULAR FRACTIONS OF BOVINE ADRENALS

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Abstract—The imidazole derivative, etomidate, inhibits the 11β-hydroxylase in cell-free systems and mitochondria isolated from bovine adrenal cortex. Fifty per cent inhibition is achieved at 3.10^{-7} M. The less active hypnotic L-enantiomer is also a less potent inhibitor of the 11-hydroxylation. At a 2 times higher concentration, etomidate affects the cholesterol side chain cleavage. The inhibition of both steroidogenic enzyme systems may be due to binding of the unhindered nitrogen of the imidazole ring of etomidate to the heme iron atom of the adrenal cortex mitochondrial cytochrome P-450 species.

Etomidate† is used for the induction and maintenance of anaesthesia. Recently, it has been reported that long-term infusion of etomidate in patients in intensive care units may be associated with a low plasma cortisol level and failure to respond to β-1-24-corticotropin (Syncathen®) [1–3]. A restoration of normal adrenocortical function is found when etomidate infusion is stopped [1]. High dose etomidate perfusion also affects cortisol plasma levels in dogs [4]. Plasma corticosterone concentrations in rats are lowered by etomidate [5] and the ACTH stimulated cortisol secretion by dispersed guinea pig adrenal cells is found to be sensitive to this imidazole derivative [6].

The present study was designed to examine the effects of etomidate on the cytochrome P-450 species in adrenal mitochondria and microsomes and on the cytochrome P-450-dependent steps in the adrenal steroid biosynthesis. The results obtained indicate that etomidate interacts selectively with the mitochondrial cytochrome P-450 species resulting in an inhibition of the 11β-hydroxylase system (EC 1.14.15.4) and, at somewhat higher doses, of the cholesterol side chain cleavage.

MATERIALS AND METHODS

Materials

Reagents used in the present work were obtained from the following sources: [4-^{14}C]pregnenolone, deoxy-[1α,2α(*n*)-^{3}H]-corticosterone, [4-^{14}C]-cholesterol and 17α-hydroxy[1,2,6,7-^{3}H]-progesterone from Radiochemicals (Amersham); 17α-[1,2-^{3}H]-hydroxy-11-deoxycorticosterone (11-deoxycortisol) from New England Nuclear (Du Pont); NADP, NADPH, D-glucose-6-phosphate, D-glucose-6-phosphate dehydrogenase and ATP from Boehringer (Mannheim); organic solvents (p.a. grade) from Baker Chemicals or Merck (Darmstadt); thin layer plates: silicagel $60F_{254}$, 0.25 mm thickness from Merck (Darmstadt), silicagel G-plates 0.25 mm thickness from Anachem (England). In all experiments etomidate hydrochloride (R 16 660) was used [(+)-ethyl(R)-1-(α-methylbenzyl)imidazole-5-carboxylate hydrochloride]. The laevo-enantiomer of etomidate is R 36 932. Dimethylsulfoxide (DMSO) was used as solvent.

Methods

Isolation of the E-, mitochondrial- and microsomal fractions from the adrenal cortex

Bovine adrenals were obtained from a local slaughterhouse and transported to the laboratory in ice-cold physiological saline. All further operations were carried out at about 4°. Adhering fat and connective tissue were removed and the medullary tissue dissected from the cortex. The cortex was scraped from the capsula membrane and minced with a pair of scissors in Sucrose–Albumin–EDTA (SAE) containing 0.25 M sucrose, 5 mM EDTA and 1.5 g/l bovine albumin Fraction V from Sigma (pH: 7.0). The minced cortex was washed to eliminate blood and homogenized in 5 vol. of SAE using a Potter–Elvehjem homogenizer with Teflon pestle.

The homogenate was centrifuged at 900 *g* for 10 min. Part of the cell-free extract (E-fraction) was stored at −80°, the rest was centrifuged at 9000 *g* for 20 min. The pellet was resuspended in SAE and centrifuged as before. A small part of the 9000 *g*-pellet, containing mitochondria, was suspended in 0.25 M sucrose to determine the ADP/O ratio. The rest was suspended in 0.1 M potassium phosphate buffer (pH 7.4) and stored at −80°.

The combined 9000 *g* supernatants were centrifuged at 105,000 *g* for 60 min. The pellet was resuspended in 1.15% KCl to remove haemoglobin and

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† (+)-Ethyl(R)-1-(α-methylbenzyl)imidazole-5-carboxylate (R 16 659). Etomidate used without a prefix indicates D-etomidate.

centrifuged as before. The pellet containing microsomal membranes was suspended in 0.1 M potassium phosphate buffer and also stored at −80°.

Cytochrome P-450

The cytochrome P-450 (cyt. P-450) content was determined according to Omura and Sato [7] by measuring the reduced carbon monoxide difference spectrum (extinction coefficient used: 91/cm/mM). The sample and reference cells contained a suspension of the mitochondrial or microsomal fraction in 0.1 M potassium phosphate buffer (pH 7.4). The suspension was diluted in the potassium phosphate buffer to obtain a cyt. P-450 content of 0.1 nmole/ml. The difference spectrum in the presence of etomidate, its laevo enantiomer (R 36 932) or DMSO alone was determined after the suspension in the sample cell was reduced with dithionite and saturated with carbon monoxide.

To trace the spectral transitions of the Soret band of cyt. P-450 associated with the addition of etomidate, the mitochondria were diluted to a cyt. P-450 content of 1 nmole/3 ml. The suspension was divided between the reference and sample cells. A base-line of equal light absorbance was established, etomidate was added to the sample- and DMSO to the reference cell and the resulting difference spectrum (Type II-spectrum) recorded. The Type II spectra and the reduced CO-difference spectra were traced with an Aminco DW-2C double beam dual wavelength spectrophotometer.

Protein content

Protein was determined by the method of Lowry *et al.* [8] or by using the Bio-Rad protein assay (Bio-Rad Laboratories, Cat. No. 500.0006).

ADP/O ratio

The ADP/O ratio of freshly isolated mitochondria was measured as previously described [9].

Steroid metabolism

Cholesterol side-chain cleavage. The cholesterol side-chain cleavage system of bovine adrenal cortex has been studied, following a modification of the method of Simpson and Boyd [10], using intact mitochondria and extracts of sonicated mitochondria. Thawed mitochondria were centrifuged for 20 min at 9000 *g*. The pellet was resuspended in 0.154 M potassium chloride and centrifuged at 9000 *g* for 10 min. After repeating this procedure, the pellet was resuspended in 2 vol. of bi-distilled water and subjected to ultrasonication (Braun Sonic 200) at 0° for 15 min, with intervals for cooling every 5 min. This sonicate was centrifuged at 105,000 *g* for 30 min and the supernatant was used.

Incubations were carried out at 37° for 30 min or 2 hr in 25 ml Erlenmeyer flasks containing 1 ml potassium phosphate buffer (0.1 M, pH 7.4), 25 μmol $MgSO_4$, 2.4 μmol NADP, 20.5 μmol glucose-6-phosphate, 0.5 units of glucose-6-phosphate dehydrogenase, 1.5 ml supernatant (8 mg protein) or 1.5 ml of intact mitochondria (1 mg or 5 mg protein/ml), 0.2 μCi [^{14}C]-cholesterol (SA: 55.7 mCi/mmol) in 50 μl acetone. The final volume was 2.5 ml. During the incubation period, the mixtures were gassed with air. Incubations were stopped by addition of 10 ml ethylacetate and the steroids and cholesterol extracted 3 times 10 min with 50 ml ethylacetate. The combined extracts were evaporated under vacuum until dryness, resuspended in 0.1 ml ethylacetate: chloroform: methanol (1:0.66:0.33) and transferred to a thin layer plate coated with silicagel-G. The solvent system used for the separation was petroleum ether (b.p. 40–60°): diisopropylether: acetic acid (30:70:2). The separation [10] involved two runs per plate; a first run until the solvent front had covered two-thirds of the plate; a second run in the same system was done until the solvent front reached the top of the plate.

The radioactive bands were located by means of contact photography (Kodak® X-omatic Reg. L_2-casette with Kodak® X-omat S X-ray films). The R_f-values of the different fractions were compared with those of standards. After detection, radioactivity was determined by means of liquid scintillation spectrometry [11].

[^{14}C]-*Pregnenolone and* [^{3}H]-*17-OH-progesterone incorporation.* [^{14}C]-Pregnenolone incorporation into steroids of bovine adrenal cortex was studied in E-fractions. The following incubation mixture was used: 8 ml E-fraction (2 mg protein/ml), 16 μmoles NADPH, 50 μmoles ATP and 30 μmoles $MgCl_2.6H_2O$ in a final volume of 10 ml. The reaction was started by the addition of 0.2 μCi [^{14}C]-pregnenolone (SA: 56 mCi/mmole). Incubations were carried out in 100 ml Erlenmeyer flasks at 37° for 30 min in a reciprocating shaker. During the entire period, the incubation mixtures were gassed with air.

[^{3}H]-17-OH-progesterone incorporation was also studied in an E-fraction (protein concentration 2 mg/ml) to which 10 μCi of tritium-labelled 17-OH-progesterone (SA: 55 Ci/mmole) was added. Similar experiments were done using adrenocortical microsomes (protein concentration 1 mg/ml).

After incubation the reactions were stopped by adding 50 ml ethylacetate. Steroids were extracted 3 times 10 min with 50 ml ethylacetate. The extracts were combined evaporated under vacuum. The residue was resuspended by adding 25 ml $CH_3OH:H_2O$ (9:1) and 25 ml *n*-hexane. The methanol–water layer was evaporated under reduced pressure until dryness. The steroids were redissolved in 5 ml ethylacetate, transferred to a glass tube, evaporated under a stream of nitrogen and redissolved in 0.1 ml ethylacetate. The steroids thus obtained were separated with thin layer chromatography (TLC) together with reference compounds. A two-dimensional TLC-system was used. The solvent system for the first dimension was benzene: ethylacetate (1:1). For the second dimension 1 vol. of isooctane: isopropylether: acetic acid (2:1:1) and 1 volume of benzene: ethylacetate (4:3) was used as soivent system. To get a better separation between cortisol and corticosterone, in some experiments a 2nd TLC-system with chloroform: acetic acid (8:2) as solvent was used.

After separation, radioactive spots were localized using contact photography. Other fractions were located by spraying with 50% H_2SO_4 and heating at 150°. Radioactivity was determined as above.

Corticosterone and cortisol synthesis from [^{3}H]-*11-deoxycorticosterone and* [^{3}H]-*11-deoxycortisol.* The [^{3}H]-11-deoxycortisol (SA: 51 Ci/mmol) incorporation into cortisol was studied using adrenocortical mitochondria (with succinate as substrate these mitochondria gave an ADP/O ratio of about 1.8). The incubation and extraction procedure were the same as those described for prenenolone. The chloroform:acetic acid (8:2) TLC system was used to separate 11-deoxycortisol from cortisol.

Cortisol synthesis was also studied by using the extract of sonicated mitochondria, as described for the cholesterol side chain cleavage.

The [^{3}H]-11-deoxycorticosterone (SA: 47.8 Ci/mmole) incorporation into corticosterone was studied in a mitochondrial fraction. Incubation, extraction and separation were done as described for cortisol synthesis.

Calculations

Calculations are made by means of "a programming language"-(APL) programme. The data were plotted by means of GI, a full screen APL graphic software from IBM.

RESULTS

Effects of etomidate and L-*etomidate on the adrenal cyt. P-450 species*

The carbon monoxide-difference spectrum obtained with bovine adrenocortical mitochondria showed a Soret maximum at 450 nm. Addition of etomidate to such a mitochondrial suspension resulted in a dose-dependent decrease of the absorption difference between 450 and 490 nm (Fig. 1). A 50% decrease was observed immediately after the addition of $9.4 \pm 0.9 \times 10^{-8}$ M (4 experiments) etomidate. These mitochondrial cyt. P-450 species are much less sensitive to L-etomidate, the less hypnotically active enantiomer. Even at 10^{-5} M only a 40% decrease of the absorption difference between 450 and 490 nm was observed.

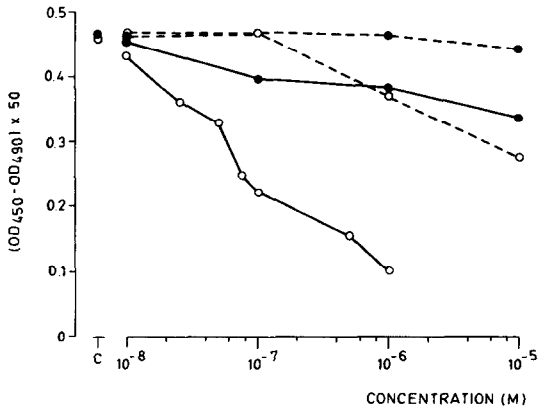


Fig. 1. Effect of D- (solid lines) and L- (broken lines) etomidate on the adrenocortical mitochondrial (open circles) and microsomal (filled circles) cyt. P-450. The $\Delta A_{450-490}$ were determined and plotted against drug concentration. "C" represents the control values. Results are mean values of at least 4 different experiments.

The cytochrome P-450 species in the microsomal fraction are hardly sensitive to either the D- or L-enantiomers (Fig. 1).

To trace the spectral transition of the Soret band of cyt- P-450 associated with the addition of etomidate the mitochondrial suspension containing 0.33 nmoles/ml was divided into the reference and sample cells. After a baseline of equal light absorbance was established, increasing concentrations of etomidate were added and the resulting difference spectrum recorded. As shown in Fig. 2 the imidazole derivative gives with oxidized cyt. P-450 a Type II binding spectrum with a maximum at 426 nm and a minimum at 394 nm. The intensity of the Type II difference spectrum, expressed by the peak to trough value, is dose-dependent. Saturation of the iron binding site seems to be reached at about 5.10^{-7} M of etomidate.

Effects of etomidate on cholesterol side chain cleavage

The intact adrenal cortex mitochondria absorb and/or convert [^{14}C]-cholesterol only slowly. After 2 hr of incubation 25 and 40% of the cholesterol was converted when 1 and 5 mg mitochondrial protein per ml respectively were used.

Most of the radioactivity derived from cholesterol was found in the corticosterone fraction indicating that the mitochondrial fraction is contaminated with microsomes. Fifty per cent inhibition of corticosterone synthesis was reached at about 2×10^{-6} M (Fig. 3). In the presence of 1 mg mitochondrial protein 50% inhibition was obtained at about 10^{-6} M (results not shown).

A much better yield of progestogens (progesterone + pregnenolone) was obtained by using the cyt. P-450 containing mitochondrial extract, prepared from the mitochondria used above. Thirty min (Fig. 4) and 2 hr (Fig. 5) incubation resulted in 50% and more than 60% conversion of cholesterol respectively. After both incubation periods most of the radioactivity is found in the pregnenolone fraction. A 30 or 120-min incubation period in the presence of respectively $6 \pm 3.1 \times 10^{-7}$ M (3 experiments) and $9.6 \pm 7.4 \times 10^{-7}$ M (5 experiments) etomidate yielded 50% inhibition of the cholesterol side chain cleavage.

Effects of etomidate and of its L-*enantiomer on the utilization of* [^{14}C]-*pregnenolone by an adrenocortical cell-free fraction (E-fraction)*

A 30-min incubation of an E-fraction of an adrenal cortex homogenate resulted in >98% conversion of the added pregnenolone into corticosteroids and androgens. More than 80% of pregnenolone was converted into corticosteroids, especially cortisol and corticosterone (Fig. 6). The addition of increasing concentrations of etomidate resulted in a decreased synthesis of both adrenocortical hormones and in a concomitant accumulation of 11-deoxycortisol and 11-deoxycorticosterone. Interesting is the formation of tetrahydro-11-deoxycorticosterone. Hydrogenation of 11-deoxycortisol has been found in the urine after treating patients with metyrapone, a known inhibitor of the adrenal 11β-hydroxylation [12].

The L-enantiomer of etomidate is a much weaker inhibitor of corticosteroid synthesis, as compared

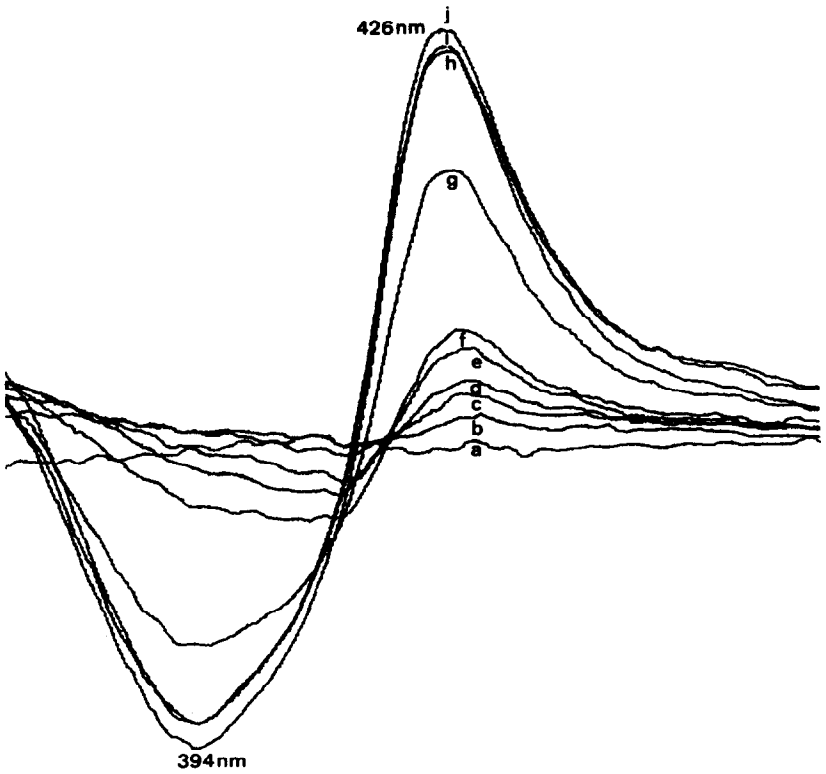


Fig. 2. Spectral transitions of the Soret band of adrenal cortex mitchondrial cyt. P-450. The mitochondria were diluted to 1 nmole cyt. P-450 per 3 ml. The mitochondrial suspensions were divided between the reference and sample cuvettes. After establishment of the base-line (a) DMSO was added to the reference cuvette and (b) 10^{-8}, (c) 2.5×10^{-8}, (d) 5×10^{-8}, (e) 7.5×10^{-8}, (f) 10^{-7}, (g) 2.5×10^{-7}, (h) 5×10^{-7}, (i) 7.5×10^{-7} or (j) 10^{-6} M of etomidate (= D-enantiomer) was added to the sample cuvette. The resulting difference spectra were recorded.

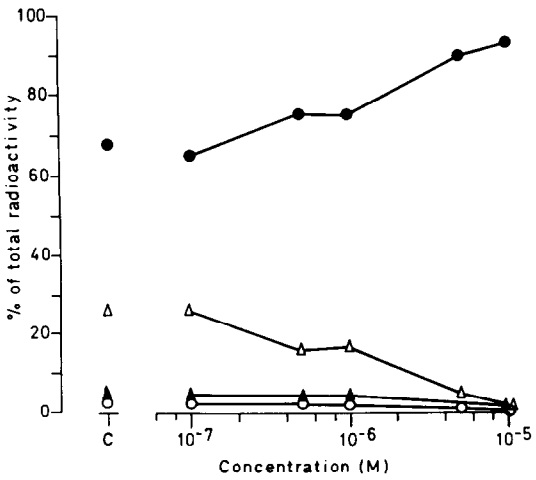


Fig. 3. Effect of etomidate on cholesterol side chain cleavage by a mitochondrial suspension of the bovine adrenal cortex. The mitochondrial fraction was incubated in the presence of [^{14}C]-cholesterol and the radioactivity was determined in the cholesterol (●), pregnenolone- (○), progesterone- (▲) and corticosterone- (△) fraction after 2 hr of incubation at 37°. Results are mean values of 2 experiments. For each experiment mitochondria from another tissue pool were used. "C" represents control value.

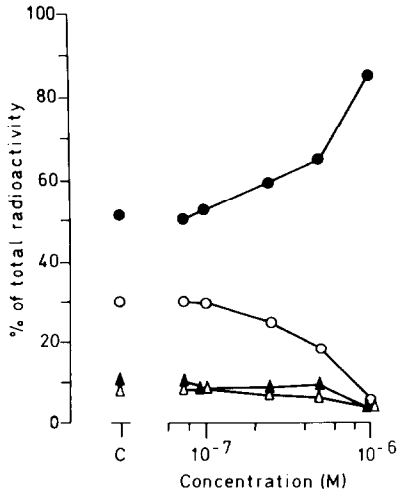


Fig. 4. Effect of etomidate on cholesterol side chain cleavage by a mitochondrial extract: incubation time, 30 min; temperature, 37°. Results are mean values of 3 experiments. Further details are given in the legend to Fig. 3.

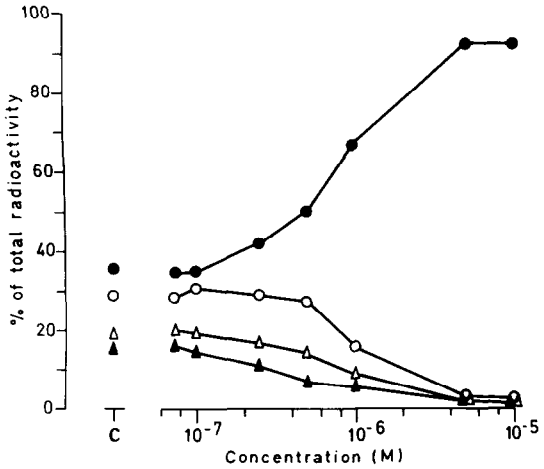


Fig. 5. Effect of etomidate on cholesterol side chain cleavage by a mitochondrial extract; incubation time, 2 hr. Results are mean values of at least 5 experiments. Further details are given in the legend to Fig. 3.

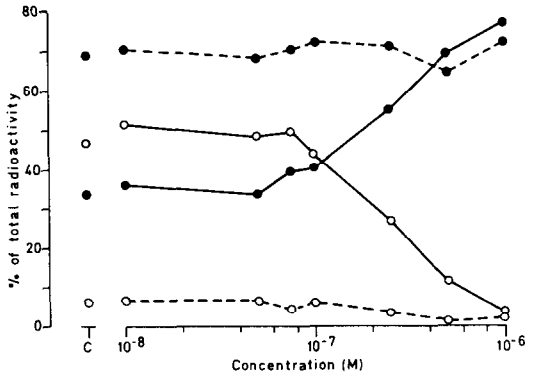


Fig. 7. Effect of etomidate on the incorporation of radioactivity derived from [^{3}H]-17-OH-progesterone into cortisol (○) and 11-deoxycortisol (●) by an E-fraction (solid line) and a microsomal fraction (broken line) of bovine adrenal cortex. Results are mean values of 3 experiments. Further details are given in the legend to Fig. 6.

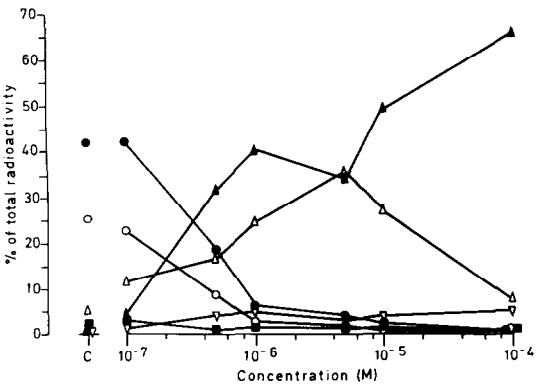
with etomidate (i.e. the D-enantiomer). Fifty and twenty per cent inhibition of respectively cortisol and corticosterone synthesis are observed at 10^{-6} M L-etomidate (results not shown). At this concentration almost 90% inhibition was obtained with etomidate.

Inhibitory effects of etomidate on the utilization of [^{3}H]-17-OH-*progesterone by adrenocortical E- and microsomal fractions*

Using an E-fraction of bovine adrenals and [^{3}H]-17-OH-progesterone as the precursor 60% of the [^{3}H]-label is found in cortisol and 27% in 11-deoxycortisol. In the presence of etomidate the 11-hydroxylation of 11-deoxycortisol is inhibited (Fig. 7). Fifty per cent inhibition was obtained at about $2.3 \pm 0.2 \times 10^{-7}$ M (3 experiments). The microsomal hydroxylation at the 21-position of 17α-hydroxyprogesterone is much less sensitive to etomidate. In fact concentrations up to 10^{-6} M did not affect the synthesis of 11-deoxycortisol out of 17-OH-progesterone (Fig. 7).

Effects of etomidate on the 11-hydroxylation reaction in adrenocortical mitochondria and in a cytochrome P-450 containing mitochondrial extract

When adrenal cortex mitochondria are incubated in the presence of [^{3}H]-11-deoxycortisol or [^{3}H]-11-deoxycorticosterone, almost 90 and 95% of the radioactivity is found in cortisol and corticosterone respectively. Fifty per cent inhibition of the 11-hydroxylase dependent cortisol synthesis was observed after 30 min of incubation with $3.05 \pm 0.9 \times 10^{-7}$ M (4 experiments) of etomidate (Fig. 8). At $5 \pm 0.8 \times 10^{-7}$ M (4 experiments) a simi-



Fio. 6. Effect of etomidate on the incorporation of radioactivity derived from [^{14}C]-pregnenolone into cortisol (○), corticosterone (●), 11-deoxycortisol (△), 11-deoxycorticosterone (▲), aldosterone (■) and tetrahydro-11-deoxycorticosterone (▽) by an E-fraction of bovine adrenal cortex. Incubation time: 30 min, temperature: 37°. $2.8 \pm 0.8 \times 10^{-7}$ M yielded 50% inhibition of cortisol synthesis. Results are mean values of 4 experiments. Three different tissue pools were used.

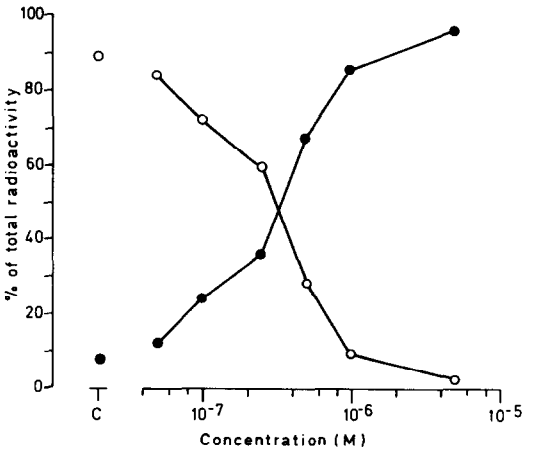


Fig. 8. Effect of etomidate on cortisol (○) synthesis from [^{3}H]-11-deoxycortisol (●) by bovine adrenal cortex mitochondria: Incubation time, 30 min; temperature; 37°. Results are mean values of 4 experiments.

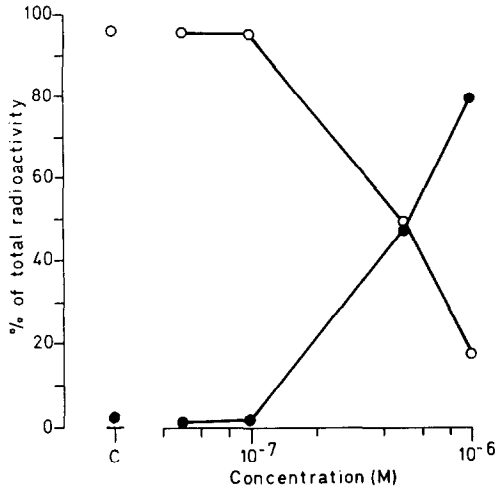


Fig. 9. Effect of etomidate on corticosterone (○) synthesis from [^{3}H]-11-deoxycorticosterone (●) by bovine adrenal cortex mitochondria: incubation time, 30 min; temperature, 37°. Results are mean values of 4 experiments.

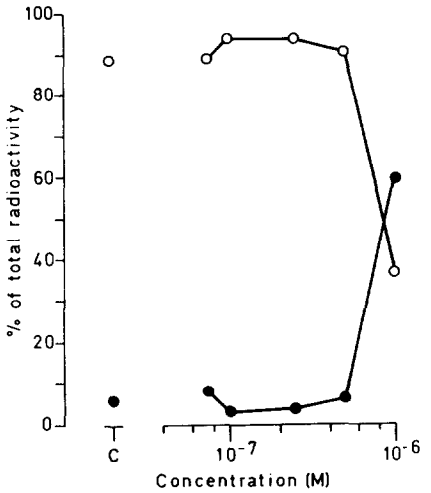


Fig. 11. Effect of etomidate on cortisol (○) synthesis from [^{3}H]-11-deoxycortisol (●) by an extract of bovine adrenal cortex mitochondria. Incubation time: 2 hr. Results are mean values of 3 experiments. Further details are given in the legend to Fig. 10.

lar inhibition of the 11-hydroxylation of 11-deoxycorticosterone was found (Fig. 9).

Using a cytochrome P-450 containing (specific activity 0.19 nmoles/mg protein) extract of adrenal cortex mitochondria about 60% of the added 11-deoxycortisol was hydroxylated at C-11. Thirty minutes of incubation in the presence of $1.3 \pm 1.2 \times 10^{-6}$ M (4 experiments) resulted in 50% inhibition of the cortisol synthesis (Fig. 10). This is about a 5 times higher concentration than that needed to inhibit the hydroxylation reaction in intact mitochondria (Fig. 8). Even after 2 hr of contact 50% inhibition was achieved only at $8.5 \pm 1.2 \times 10^{-7}$ M (3 experiments) (Fig. 11). It should be noted that the decreased cortisol synthesis coincides with a decreased labelling of 11-deoxycortisol derivative(s) of which we were unable so far to identify the chemical structure.

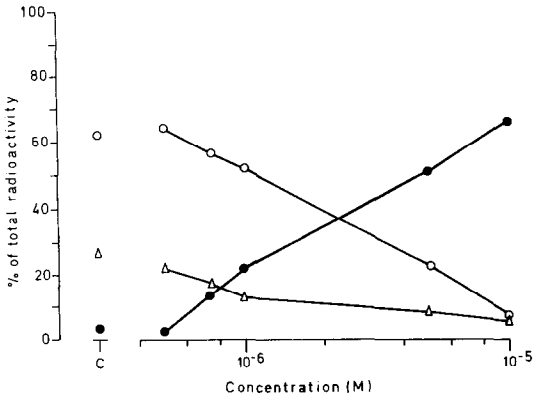


Fig. 10. Effect of etomidate on cortisol (○) synthesis from [^{3}H]-11-deoxycortisol (●) by an extract of bovine adrenal cortex mitochondria. The incorporation of radioactivity in an unidentified 11-deoxycortisol derivative (△) is shown: incubation time, 30 min; temperature, 37°. Results are mean values of 4 experiments.

DISCUSSION

From the experimental evidence presented here it can be deduced that etomidate interferes with cyt. P-450 species in the adrenal cortex mitochondria. Up to 5.10^{-5} M, etomidate influenced only slightly the carbon monoxide-difference spectrum obtained with bovine adrenocortical microsomes. However, by using adrenal cortex mitochondria a 50% decrease in the absorbance increment between 450 and 490 nm was observed immediately after the addition of 9.4×10^{-8} M. Several classes of imidazole derivatives are extremely potent inhibitors of microsomal enzymes of insects and rats. They interact strongly with cytochrome P-450 yielding Type II binding spectra [13, 14] (for review see [15]). According to Schenkman *et al.* [16] the Type II spectral change observed (absorption peak at 426 nm and minimum at 394 nm) suggests that the unhindered nitrogen (N_3) of the imidazole ring binds to the catalytic heme iron atom at the site occupied by the 6th ligand and thus occupies the site of dioxygen binding (Fig. 12). The latter step precedes the formation of an "activated oxygen" complex needed in the hydroxylation of, for example, 11-deoxycortisol or during the oxidation of cholesterol, a first step in the conversion of cholesterol to pregnenolone.

The interaction of etomidate with cyt. P-450 differs from that of metyrapone, another inhibitor of cyt. P-450, in being much more selective. Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) is an inhibitor of two classes of cyt- P-450 enzymes: adrenal steroid 11β-hydroxylase (mitochondria) and phenobarbital-inducible forms of cyt- P-450 (microsomes) [17–19]. At 10^{-4} M metyrapone a 50% decrease in the absorbance increment between 450 and 490 was obtained with liver microsomes from phenobarbital-treated

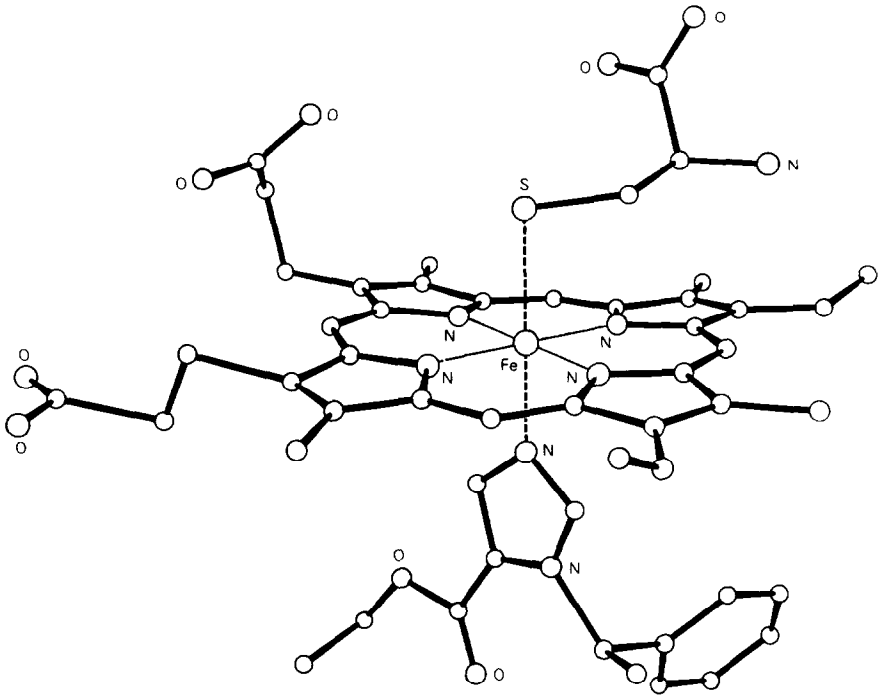


Fig. 12. Proposed perspective drawing of the cyt. P-450-iron protoporphyrin etomidate complex. The fifth ligand is a thiolate anion of cysteine. This figure was modelled using X-ray crystallographic data by H. Moereels (Dept. of Theoretical Medicinal Chemistry, Janssen Pharmaceutica).

rabbits and with bovine adrenal cortex mitochondria (unpublished results). At concentrations up to 10^{-4} M etomidate did not interfere with the phenobarbital- and 3-methylcholanthrene-inducible forms of cyt. P-450 in rabbit liver and the cyt. P-450 from *Saccharomyces cerevisiae*. Only the cyt. P-450 species in piglet testis microsomes were slightly sensitive. A 48% decrease was observed at 10^{-4} M etomidate (unpublished results).

The interaction of etomidate with cyt. P-450 is sterospecific, the D-enantiomer (etomidate) being a more potent inhibitor of the adrenal cortex mitochondrial cyt. P-450 than the L-enantiomer. The latter is also a less active hypnotic. The D-enantiomer is a more potent inhibitor of the conversion of pregnenolone to cortisol and corticosterone too.

To synthesize cortisol and corticosterone from pregnenolone microsomal and mitochondrial cyt. P-450 species are needed. The results presented here suggest that under the present conditions the mitochondrial 11-hydroxylase systems are much more sensitive to etomidate than the microsomal enzyme systems involved in the steroidogenesis. Fifty per cent inhibition of the hydroxylation reactions leading to cortisol and corticosterone was achieved at about 3 and 5×10^{-7} M etomidate respectively. The addition of concentrations $>10^{-5}$ M resulted in an increased accumulation of 11-deoxycorticosterone and a concomitant decrease in 11-deoxycortisol. This is suggestive of an inhibitory effect of high concentrations of etomidate on the 17-hydroxylation, an essential step in the formation of cortisol and not involved in that of corticosterone. The inhibition of the 17-hydroxylation makes more progesterone available for the synthesis of 11-deoxycorticosterone.

Etomidate did not interfere with the utilization of 17-OH-progesterone by a microsomal fraction whereas a 50% inhibitory effect on the 17-OH-progesterone conversion into cortisol was observed at 2.3×10^{-7} M when an E-fraction was used. This conversion depends on the presence of the mitochondrial 11-hydroxylases (sensitive to etomidate) and the microsomal 21-hydroxylases. The fact that we did not find an effect on the conversion of 17-OH-progesterone to 11-deoxycortisol indicates again a much lower sensitivity of adrenocortical microsomes to etomidate as compared with that of adrenocortical mitochondria.

The steroidogenic enzymes residing in the adrenocortical mitochondria are the cholesterol side chain cleavage system, steroid C-11 hydroxylases and C-18 hydroxylases. There is clinical evidence that one 11β-hydroxylase is involved in the 11-hydroxylation of 11-deoxycorticosterone and another in the conversion of 11-deoxycortisol to cortisol [20]. However, other studies indicate that although cyt. P-450$_{11\beta}$ activity is usually characterized with the substrate 11-deoxycorticosterone it can also catalyze the 11β-, 18- and 19-hydroxylation of various steroids [21]. From the results described in the present study, it cannot be deduced that different cyt. P-450 species are involved in the 11-hydroxylation reactions since both the synthesis of cortisol and corticosterone are sensitive to etomidate.

The 11-hydroxylase of intact mitochondria is about 5 times more sensitive to etomidate than the 11-hydroxylation system present in an extract of sonicated mitochondria. On the contrary, no significant

difference in sensitivity was observed when the effects of etomidate on cholesterol side chain cleavage were studied in intact mitochondria or mitochondrial extracts. Fifty per cent inhibition was noticed at respectively 10^{-6} and 9.6×10^{-7} M. Studies of Wang *et al.* [22] suggest that the 11β-hydroxylase is associated with phospholipids while the cholesterol side chain cleavage system (desmolase) is not. In the same context they found that the desmolase is more easily extracted from the mitochondria than the 11β-hydroxylase. This may also indicate that the latter enzyme is more embedded in the hydrophobic part of the mitochondrial inner membrane whereas the desmolase is more oriented towards the hydrophilic part of the membrane. It is thus possible that the extraction procedure, used in this study, resulted in a derangement of the orientation of the membrane components needed for maximum binding of etomidate with cyt. $P\text{-}450_{11\beta}$. Since the desmolase does not seem to depend on the presence of phospholipids, extraction will not disturb the activity, on the contrary more cholesterol is converted in the extracts than in the intact mitochondria.

Using intact mitochondria, the 11β-hydroxylation of 11-deoxycortisol was found to be almost 2 times more sensitive to etomidate than the desmolase. This corresponds to the accumulation of 11-deoxycortisol observed in patients following the administration of etomidate (De Coster, personal communication). If etomidate were a more potent inhibitor of the cholesterol side chain cleavage a decreased 11-deoxycortisol content would have been found.

Sato *et al.* [23] found that an adrenal cyt. P-450 preparation could convert 11-deoxycorticosterone into corticosterone and to 18-OH-corticosterone. Watanuki *et al.* [24] reported that metyrapone inhibits competiviely the 11β- and 18-hydroxylase activities of a highly purified cyt. P-450 from bovine adrenocortical mitochondria. These authors also found that increasing concentrations of antiserum, produced by rabbits to the purified enzyme, produce increasing inhibition of both activities. These findings support the suggestion that the two hydroxylase activities occur in a single system. Studies are under way to determine the inhibitory effects of etomidate on the 18-hydroxylase too.

In conclusion, the results obtained so far suggest that the decrease in cortisol plasma levels found after infusion of etomidate in patients result from interference with the 11β-hydroxylase system. This is, as shown in Fig. 12, probably due to binding of the unhindered nitrogen of the imidazole ring of etomidate to the heme iron atom of the mitochondrial cytochrome P-450 in the adrenal cortex. Experiments are planned to study the kinetics of the inhibition in purified enzyme systems and to get an idea on the interaction of other parts of the etomidate structure with the cytochrome P-450 species.

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