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Inhibition of Aldosterone Production in Rat Adrenal Mitochondria by 18-Ethynyl-11-Deoxycorticosterone: A Simple Model for Kinetic Interpretation of Mechanism-Based Inhibitors[1]

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A simple mathematical model for studying mechanism-based inhibitors (MBIs) is presented. The mathematical equations are deduced for an experimental protocol consisting of a first incubation of the enzyme in the presence of MBI followed by a washing protocol to eliminate free MBI. Finally enzyme activity (initial velocity) is measured with specific substrate. The representation of the final equation obtained is a straight line, and the MBI-specific association constant of velocity (k) can be calculated from its slope. The mathematical model was then challenged with the effect of 18-ethynyl-11-deoxycorticosterone (18-EtDOC) as an MBI on aldosterone biosynthesis from 11-deoxycorticosterone (DOC) in rat adrenal mitochondria. The last step of the mitochondrial biosynthesis of aldosterone consists of the conversion of DOC into corticosterone (B) or 18-hydroxy-11-deoxycorticosterone (18-OHDOC), and both steroids can then be transformed into aldosterone. The k ($mM^{-1} \cdot min^{-1}$) values obtained for 18-EtDOC were: 451 ± 36 for DOC to aldosterone; 177 ± 16 for B to aldosterone; 175 ± 15 for 18-OHDOC to aldosterone; and 2.7 ± 0.2 for DOC to B. These results show that this MBI practically does not affect the metabolism of DOC to B in our enzyme preparation and that conversions of B and 18-OHDOC into aldosterone are catalyzed by the same enzyme.

Key words: adrenal gland, aldosterone, 18-ethynyl-11-deoxycorticosterone, mechanism-based inhibitors, suicide inhibitors.

Mechanism based inhibitors (MBIs), also named time-dependent or suicide inhibitors, are useful tools for the investigation of metabolic pathways. The action of MBIs involves reversible formation of one or more enzyme-inhibitor complexes prior to irreversible covalent modification. MBIs are relatively unreactive compounds with structural similarity to the substrate for a particular enzyme that, via its normal mechanism, converts the inhibitor molecule into a form that reacts covalently with the enzyme. In other words, MBIs bind to the active site of an enzyme and react covalently, irreversibly blocking access to that site by the specific substrate (*1–4*). Since the binding of the MBI is covalent and irreversible, the enzyme to which the MBI is bound is permanently inactivated; only *de novo* synthesis of the enzyme is able to restore enzyme activity of the cell. The longer the inhibitor is allowed to act, the greater the inhibition will be. This is the reason for the alternative name of time-dependent inhibitors.

The strategy for design of an MBI lies in the introduction of a reactive group into a molecule of substrate, thereby allowing covalent reaction with the binding site of the enzyme. The reactive group must not change the structure of the substrate so much that the affinity of the enzyme for the substrate is substantially lowered. Also, the group must not be so reactive that it forms a covalent reaction product with other cell proteins. The ideal group would be one that is made reactive by the action of the enzyme when the substrate is bound to the active site, thereby allowing the covalent reaction to take place at the time of metabolism (*5–8*). These characteristics can be found in steroidal synthetics by ethynyl groups (*6, 7*).

In this work, we report a simple model for studying the action and potency of MBIs, and experiments to challenge the model by using 18-ethynyl-11-deoxycorticosterone (18-EtDOC) (*9*) in aldosterone biosynthetic pathway of rat adrenal mitochondria.

Aldosterone, the main natural mineralocorticoid, is produced in adrenal zona glomerulosa from cholesterol. This sterol is succesively converted to pregnenolone, progesterone and 11-deoxycorticosterone, which is finally transform-

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Abbreviations: 18-EtDOC, 18-ethynyl-11-deoxycorticosterone; 18-OHB, 18-hydroxycorticosterone, 18-OHDOC, 18-hydroxy-11-deoxycorticosterone; ALDO, aldosterone; B, corticosterone; CYP11B1, 11β hydroxilase, CYP11B2, 11,18-hydroxylase/aldosterone synthase; DMSO, dimethyl sulfoxide; DOC, 11-deoxycorticosterone; MBI, mechanism-based inhibitor; P450-scc, cytochrome P450 that catalyses the side-chain cleavage of cholesterol; RIA, radioimmuno assay; TCA, trichloro acetic acid.

ed into aldosterone in mitochondria (Fig. 1).

In rat adrenal the last step of this pathway is catalyzed by 11,18-hydroxylase/aldosterone synthase (CYP11B2) (*10*), which is localized in the zona glomerulosa. Thus the cytochrome P450 CYP11B2 possess the capability to 11- or 18-hydroxylate 11-deoxycorticosterone (DOC) to yield corticosterone (B) or 18-hydroxy-11-deoxycorticosterone (18-OH-DOC) respectively. Additionally, CYP11B2 is also able to 18-hydroxylate B and its product 18-hydroxycorticosterone (18-OHB), producing aldosterone (Fig. 1).

On the other hand, the cytochrome P450 CYP11B1 (*10*) is localized in adrenal zona fasciculata and does not produce aldosterone but converts DOC into B and 18-OHDOC (Fig. 1). The ratio CYP11B1/CYP11B2 in adrenal cortex is largely greater than 1, provoking that enzyme activity measurements in rat adrenal capsules (taken as zona glomerulosa) that are contaminated with adrenal cores reflect the presence of CYP11B2 (aldosterone biosynthesis) and also CYP11B1 activity (B and 18-OHDOC productions).

Mathematical Model—1. The reaction: The irreversible association between the enzyme E and the inhibitor I may be represented as:

$$E + I \xrightarrow{k} EI$$

The reaction rate may be expressed as:

$$\frac{d[EI]}{dt} = k[E][I] \quad (1)$$

$[EI]$, $[E]$, and $[I]$ represent the concentrations of enzyme-inhibitor complex, enzyme, and inhibitor, respectively, at t minutes of reaction, and k is the specific constant of velocity.

The mass balances on E and I are:

$$Eo = [E] + [EI] \quad (2)$$

$$Io = [I] + [EI] \quad (3)$$

Eo and Io are the initial total concentrations of E and I. Substituting Eqs. 2 and 3 into Eq. 1, rearranging the differential equation, and integrating over the time of the exposure to inhibitor, we get:

$$\int_0^{[EI]} \frac{d[EI]}{(Eo - [EI])(Io - [EI])} = k\int_0^t dt \quad (4)$$

which solves to:

$$\frac{1}{Eo - Io} \ln \frac{1 - \frac{[EI]}{Eo}}{1 - \frac{[EI]}{Io}} = kt \quad (5)$$

The use of Eq. 5 requires the measurement of $[EI]$, which is often measured by radioactive counting or other direct means. Here it may be impossible to measure it directly, and this will be addressed in the next section.

2. Measurement of [EI]: The following derivation is based on Michaellian enzymes for simplicity.

After incubating the enzyme with the inhibitor for a time t, reaching the enzyme-inhibitor complex concentration $[EI]$, we wash the enzyme free of unreacted inhibitor and measure the initial velocity of the enzymatic reaction.

The general reaction scheme for Michaelis-Menten enzyme kinetics is:

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \quad (6)$$

The initial velocity (V) is given by:

$$V = \frac{V_{max}}{1 + \frac{K_M}{S}} \quad (7)$$

$V_{max} = k_3 Eo$ and $K_M = [E][S]/[ES]$, under steady-state conditions, and k_1, k_2, and k_3 are specific constants of velocity according to scheme (*6*).

The mass balance in the enzymatic reaction is $Eo = [E] + [ES]$. After incubation with an MBI, the term $[EI]$ is added to the above balance to give:

$$Eo = [E] + [ES] + [EI]$$

$[EI]$ is now constant since the inhibitor I has been removed, and, in the EI complex, I is irreversibly bound to the enzyme.

Initial velocity after treatment with the MBI (Vw) is:

$$Vw = \frac{dP}{dt} = k_3[ES] = k_3(Eo - [E] - [EI])$$

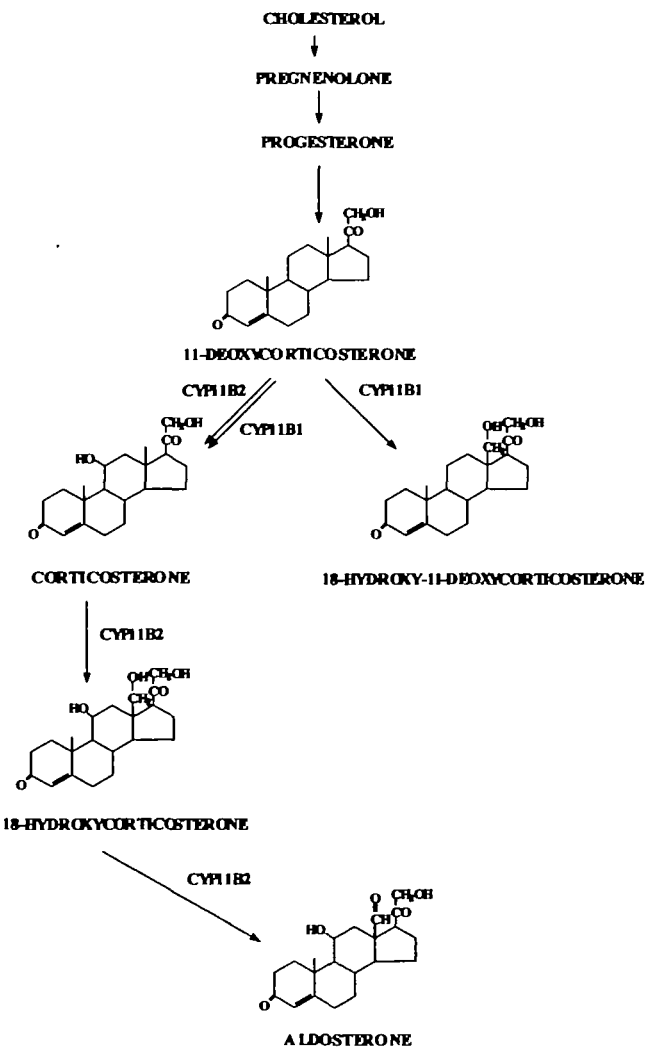


Fig. 1. **Biosynthetic scheme for the production of aldosterone in rat adrenal cortex.** CYP11B2 is only present in zona glomerulosa. CYP11B1 is only present in zona fasciculata. Aldosterone is only produced in zona glomerulosa.

$$= k_3(Eo - \frac{K_M[ES]}{[S]} - [EI])$$

$$= k_3Eo - \frac{k_3[ES]K_M}{[S]} - k_3[EI]$$

$$= V_{max} - V_wK_M/[S] - k_3[I]$$

$$Vw = \frac{V_{max} - k_3[EI]}{1 + \frac{K_M}{[S]}} \quad (8)$$

Equation 8 represents the initial velocity of the Michaelis-Menten enzyme after treatment with the MBI.

The K_M is unaltered by pretreatment with MBI. The initial velocity, Vw, is decreased by an amount k_3 $[EI]/(1+(K_M/[S])$. The denominators of Eq. 7 and Eq. 8 are identical. Note that in any comparison between pretreated and non-pretreated enzyme, the substrate concentration [S] must be the same in both situations, or it will not be possible to cancel the denominators.

Let us now make the ratio of Vw/V; this is the initial velocity of the MBI-treated enzyme to the initial velocity of the untreated enzyme. A plot of this quantity *vs.* t will allow an estimate of the potency of the MBI.

The ratio of Eq. 8 to Eq. 7 is:

$$\frac{Vw}{V} = \frac{V_{max} - k_3[EI]}{V_{max}}$$

And this simplifies to:

$$R = \frac{Vw}{V} = 1 - \frac{[EI]}{Eo} \quad (9)$$

Here R is the quantity Vw/V. If we substitute this quantity into Eq. 5, we get:

$$\frac{1}{Eo - Io} \ln\left(\frac{R}{1 - \frac{[EI]}{Io}}\right) = kt$$

We will make the assumption that $Io \gg Eo$. As above, $Eo - Io \approx Io$ and $[EI]/Io \approx 0$. With these approximations, we obtain:

$$\ln\frac{Vw}{V} = -kIot \quad (10)$$

This equation shows a relationship between Vw/V (R) and t. Note that in the original equations the relationship was between $[EI]$ and t. As an alternative, we may consider a constant incubation time t, varing Io.

The plot of Eq. 10 is a straight line with a y-intercept of zero and a negative slope. The value of k may be obtained from the slope.

By changing t or Io we obtain a family of straight lines starting at the origin, differing only in slope. It is important to note that Eq. 10 is independent of substrate concentration (as long as the same concentration is used for control and experiment) and thus valid for both saturating and non-saturating substrate concentrations.

If the velocity of the enzymatic reaction is measured over too long a period, nonlinearity may arise in the curve generated. There are at least two reasons for this.

The first reason is that the complex EI can be metabolized (especially when using organelles or cells), so that the concentration of EI may not be constant over the time of the experiment. The second reason is that new enzyme can be synthetized (when using cells), thereby increasing Eo. Both of these difficulties can be expected to be minimized by use of purified enzyme preparations.

Both reasons cause the left side of Eq. 10 to be lower than theorically predicted, and this occurs as a function of time. So when plotting t on the x-axis, we would expect a positive deviation from linearity as t increases.

The half-maximal velocity can be obtained by setting equation 10 equal to $Vw = V/2$, giving:

$$t_{1/2} = \frac{-\ln 0.5}{kIo} = \frac{0.693}{kIo} \quad (11)$$

Equation 11 may also be used to calculate k, by measuring $t_{1/2}$ at various inhibitor concentrations, and plotting $t_{1/2}$ against the reciprocal of Io.

MATERIALS AND METHODS

Chemicals—Radioinert steroids, buffer components and BSA (fraction V) were purchased from Sigma Chemical. Organic solvents were of analytical grade. Merrel Dow Lab kindly provided 18-ethynyl-11-deoxycorticosterone (18-EtDOC).

Preparation and Incubation of Rat Adrenal Mitochondria—Male CHBB-Thom rats (150–200 g) were sacrificed by decapitation and adrenals were immediately removed, placed in ice-cold saline, trimmed free of fat and surrounding tissue, and finally decapsulated. Adrenal capsules were homogenized in Krebs-Ringer-bicarbonate-glucose buffer (*11*) (pH 7.2), and mitochondria were separated by centrifugation as reported elsewhere (*12*). The final mitochondrial pellet was reconstituted in the same buffer and aliquots (0.6 mg mitochondrial protein) were used.

Mitocondrial preparation was preincubated in the presence of vehicle (DMSO) or 18-EtDOC (as MBI). After this pretreatment, samples were submitted to a washing protocol. For this purpose, samples were centrifuged as before to obtain a mitochondrial pellet, resuspended in the same buffer without MBI and centrifuged again. The pellet was reconstituted in the same buffer and finally incubated with radioinert steroids as substrates. Final incubation was started by the addition of 0.5 mM NADPH and stopped by placing tubes into an ice-water bath for 15 min. Incubation supernatants were submitted to steroid determination. In some experiments washing procedure was omitted (see "RESULTS").

Steroid Determination—Aldosterone was determined by direct RIA in incubation supernatants (*13*). Corticosterone was measured after extracting incubation supernatants twice with 1 ml of hexane (*14*) (see Fig. 2), in order to remove remnant 11-deoxycorticosterone.

Miscellaneous—Stock solution of the MBI 18-EtDOC was 5 mM in DMSO. Radioinert steroids as substrates and cortisol as competitive inhibitor (see "RESULTS") were added to the incubation mixture dissolved in the same buffer described above. Proteins were measured by the method of Bradford (*15*).

The protocol used for testing of MBI was as follows: Identical amounts of enzyme source (pure enzyme, subcellular fraction, cells, *etc.*) were incubated with MBI. The enzyme preparation was then washed out of inhibitor, and the initial velocity (Vw) was measured by incubation with specific substrate in the absence of inhibitor. In parallel, initial

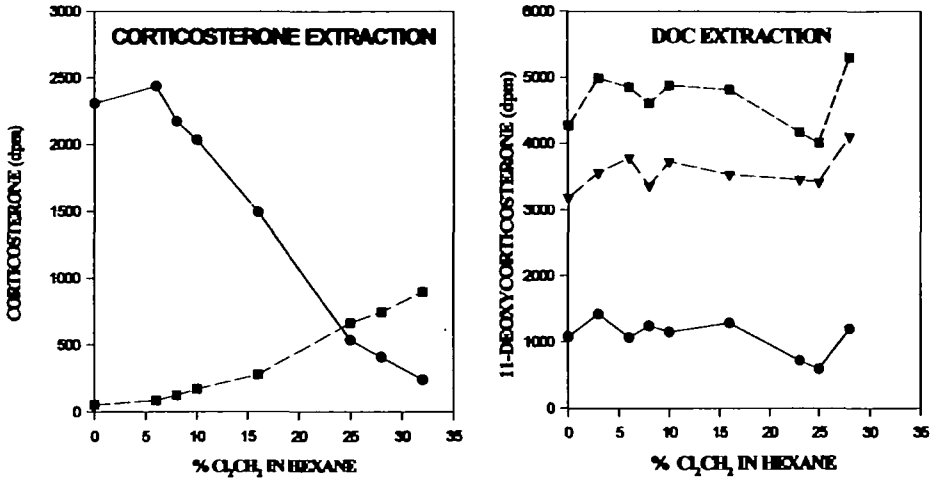


Fig. 2. **Extraction of 11-deoxycorticosterone from aqueous phase.** A solution of known amounts of tritiated 11-deoxycorticosterone (DOC) and corticosterone (B) in incubation buffer was extracted twice with hexane: methylene chloride at various percentages. After extraction, corticosterone and DOC-associated radioactivities were measured in aqueous and organic phases. Left panel shows tritiated corticosterone in aqueous (●) and organic (■) phases. Right panel shows tritiated DOC in aqueous (●) and organic (▼) phases and total radioactivity (■). Since the purpose of this experiment (see "MATERIAL AND METHODS") was to find experimental conditions to remove DOC from the aqueous phase (incubation supernatant), 100% hexane was chosen in further experiments.

velocities were also obtained from mitochondrial enzyme firstly incubated without inhibitor (V). Finally Vw/V ratios were calculated for different MBI concentrations (Io) or incubation times (t), and k (specific association constant of velocity) was obtained according to Eq. 10 (see "RESULTS").

Preparation of Bovine Adrenal Zona Glomerulosa Cell Cultures—Bovine adrenal glomerulosa cell cultures were obtained as described elsewhere (*16*). Briefly, bovine adrenal glands were obtained from a local abattoir and trimmed clean of fat and adhering tissue under sterile conditions. The cells were dispersed with collagenase, cultured as described (*16*), and plated in 24-well plates (200,000/well; Costar, Cambridge, MA) and incubated at 37°C in a 5% CO_2 air environment. The medium was changed 24 h later, and cells were incubated for 3–5 days with medium before use.

Tritiated Leucine Experiments—Incorporation of tritiated leucine into bovine glomerulosa cells was measured as reported (*17*) with minor modifications. Briefly, glomerulosa cell cultures were incubated with tritiated leucine for different incubation times. They were then washed extensively with ice-cold fresh medium and gently scraped from the plate. Cell suspensions were then homogenized, and cell proteins pelleted with ice-cold 25% TCA. Protein pellet was washed twice, and protein-associated radioactivity was measured by liquid scintillation.

P450scc Activity—Cytochrome P450scc activity was measured as described in detail elsewhere (*18*).

RESULTS

When rat adrenal mitochondria were preincubated with plain buffer, the production of aldosterone from 11-deoxycorticosterone upon further incubation decreased as the preincubation time increased. This fact reflects the previously reported instability of the cytochrome P450 involved in aldosterone biosynthesis (*19*, *20*) (see below). For this reason, protocols designed to study the time-course of the effect of different agents contained specific controls (one for each treatment time).

Figure 3 shows the time-dependent inhibition provoked by the MBI 18-ethynyl-11-deoxycorticosterone (18-EtDOC) on the production of aldosterone from 11-deoxycorticosterone in rat adrenal mitochondria. The inhibitory effect of 18-EtDOC on aldosterone production from 11-deoxycorticosterone was dose-dependent (Table I).

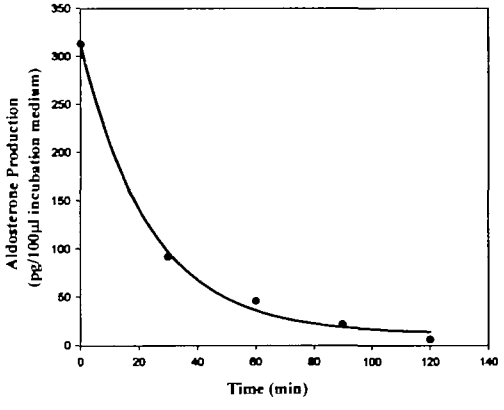


Fig. 3. **Time dependency of 18-ethynyl-11-deoxycorticosterone-mediated inhibition on aldosterone production from 11-deoxycorticosterone (DOC; 10 µM).** Aldosterone production was measured from 1 µM 11-deoxycorticosterone (DOC). Rat adrenal mitochondria were pretreated with 10 µM MBI 18-EtDOC. After washing out the inhibitor, enzyme source was incubated with DOC (1 µM) and 0.5 mM NADPH for different periods at 37°C. After incubation, aldosterone levels were measured by RIA. Values are mean ± SDM of three independent experiments performed in quadruplicate. 18-EtDOC: 18-ethynyl-11-deoxycorticosterone.

18-EtDOC also decreased the production of aldosterone from other substrates such as corticosterone and 18-hydroxy-11-deoxycorticosterone (see below).

Since production of aldosterone from 11-deoxycorticosterone involves the intermediate production of corticosterone (Fig. 1), this last steroid was also measured after each incubation. For this purpose, incubation media were extracted twice with hexane in order to remove remaining substrate (11-deoxycorticosterone) (Fig. 2). Figure 4 shows that corticosterone levels from 11-deoxycorticosterone were unchanged.

To study the reversibility of the effect of 18-EtDOC on the transformation of 11-deoxycorticosterone into aldosterone, further experiments were designed, also using the

competitive (reversible) inhibitor of the same reaction, cortisol (*21*). When the enzyme source was incubated with 11-deoxycorticosterone after treatment with cortisol or 18-EtDOC (without washing), aldosterone production was decreased with respect to controls (Table II). On the other hand, when the transformation of 11-deoxycorticosterone into aldosterone was measured after washing out free cortisol and 18-EtDOC, only the latter was able to provoke inhibition, strongly suggesting irreversibility. It is important to note that control levels were much lower after the preincubation and washing procedure, probably because of CYP-11B2 instability (*22*), sensitivity to mechanical disruption during experimental procedures (*23*), oxidation of CYP-11B2 (*24*), and adrenodoxin leakage from mitochondria (*25*).

NADPH is the well-accepted cofactor for the cytochrome P450-mediated hydroxylation of 11-deoxycorticosterone to yield aldosterone. Preincubation of mitochondria with NADPH decreased the further production of aldosterone from 11-deoxycorticosterone probably due to oxidation of the enzyme. 18-Ethynyl-11-deoxycorticosterone, produced the same inhibitory effect on the reaction with or without preincubation with NADPH (Table III).

The effect of 18-EtDOC was also tested on the activity of cytochrome P450scc, that is, the transformation of cholesterol into pregnenolone. 18-Ethynyl-11-deoxycorticosterone was ineffective in decreasing this enzyme activity (Table IV).

As further evidence of the irreversible binding of 18-EtDOC to aldosteronogenic cytochrome P450, calf adrenal zona glomerulosa cell cultures were pretreated with vehicle or 18-EtDOC for 24 h. After intensive washing, tritiated leucine was added to fresh culture medium. Radioactivity incorporation into cell proteins and aldosterone production were measured at different incubation times. Table V shows that protein synthesis parallels aldosterone secretion, strongly suggesting that *de novo* synthesis of cytochrome P450 aldosterone synthase is required to restore aldosteronogenic activity, and therefore that 18-EtDOC action is irreversible (see Introduction; first paragraph).

Finally, the mathematical model proposed here for the behavior of MBIs and the calculation of inhibition constants (see above) was challenged with our results.

TABLE I. **Dose-dependent effect of 18-EtDOC on aldosterone production from 11-deoxycorticosterone.** Aldosterone production was measured from 1 μM 11-deoxycorticosterone (DOC). Rat adrenal mitochondria were pretreated with different concentrations of the MBI 18-ethynyl-11-deoxycorticosterone (18-EtDOC). After washing out the inhibitor, enzyme source was incubated with DOC (1 μM) and 0.5 mM NADPH at 37°C for 60 min. After incubation, aldosterone levels were measured by RIA. Values are mean ± SDM of three independent experiments performed in quadruplicate.

[18-EtDOC] (μM)	Aldosterone (ng/500 μl incubation medium)
0	5.8 ± 0.5
0.1	4.5 ± 0.4
1.0	0.47 ± 0.04
10.0	0.15 ± 0.01
50.0	0.0100 ± 0.0001

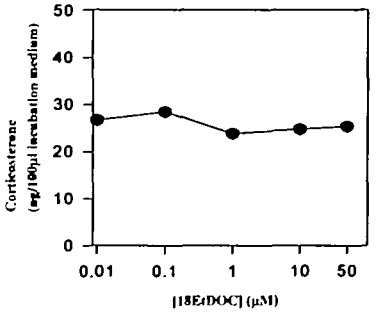


Fig. 4. **Corticosterone (B) production from 11-deoxycorticosterone (DOC).** Rat adrenal mitochondria were pretreated with different concentrations of the MBI 18-EtDOC. After washing out the inhibitor, enzyme source was incubated with DOC (1 μM) and 0.5 mM NADPH for 60 min. After incubation, B levels were measured by RIA. No significant differences were observed between different 18-EtDOC concentrations. Values are mean ± SDM of three independent experiments performed in quadruplicate. 18-EtDOC, 18-ethynyl-11-deoxycorticosterone.

TABLE II. **Effect of washing out the inhibitors.** Aldosterone production was measured from 1 μM 11-deoxycorticosterone (DOC). Rat adrenal mitochondria were pretreated with 10 μM 18-ethynyl-11-deoxycorticosterone (18-EtDOC) as MBI or 10 μM cortisol as competitive inhibitor. Enzyme sources were then incubated with DOC (1 μM) and 0.5 mM NADPH at 37°C for 60 min with (+) or without (−) previous washing out of the inhibitors. After incubation, aldosterone levels were measured by RIA. Values are mean ± SDM of three independent experiments performed in quadruplicate.

Treatment	Washing	Aldosterone (ng/500 μl incubation medium)	% Inhibition
Vehicle	−	24 ± 2	−
Cortisol	−	19 ± 4	19.5
18EtDOC	−	3 ± 1	85.8
Vehicle	+	2.1 ± 0.2	−
Cortisol	+	2.3 ± 0.3	−
18EtDOC	+	1.1 ± 0.1	50.7

TABLE III. **Effect of NADPH during the preincubation on aldosterone production from 11-deoxycorticosterone.** Aldosterone production was measured from 1 μM 11-deoxycorticosterone (DOC). Rat adrenal mitochondria were pretreated with vehicle or 10 μM 18-ethynyl-11-deoxycorticosterone (18-EtDOC) as MBI, with or without 0.5 mM NADPH. After washing out the inhibitor, enzyme source was incubated with DOC (1 μM) and 0.5 mM NADPH at 37°C for 60 min. After incubation, aldosterone levels were measured by RIA. Aldosterone values represent mean ± SDM of three independent experiments performed in quadruplicate.

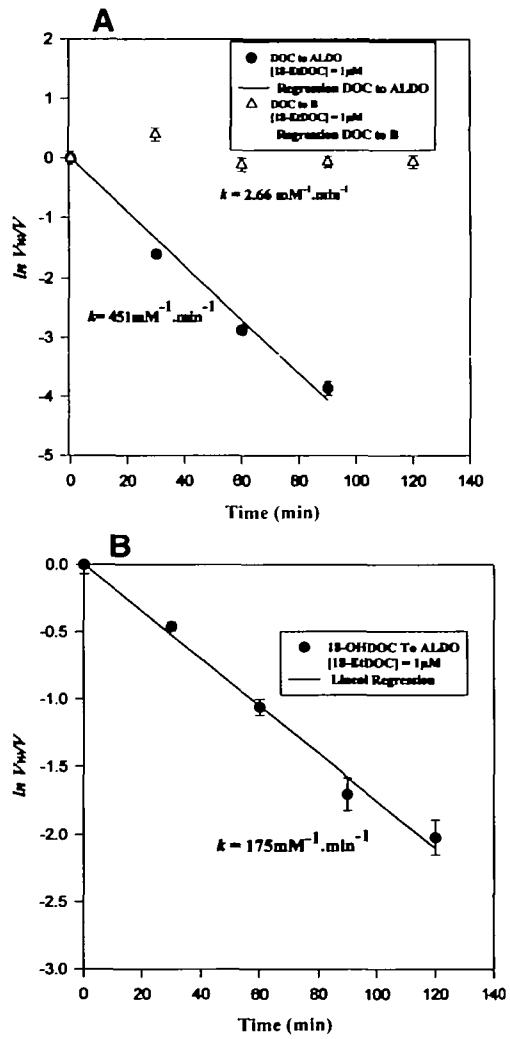
Preincubation	18-EtDOC Treatment	Aldosterone (ng/500 μl incubation medium)	Treated/ control
Vehicle	−	883 ± 129	0.213
Vehicle	+	188 ± 99	
NADPH	−	197 ± 62	0.216
NADPH	+	43 ± 14	

TABLE IV. **Effect of 18-ethynyl-11-deoxycorticosterone (18-EtDOC) on cytochrome P450scc activity.** Tritiated pregnenolone production from [1,2-^{3}H]cholesterol (0.5 μCi) was measured in rat adrenal mitochondria (50 μg protein) after incubation at 37°C for 60 min. Pregnenolone produced was separated by TLC (mobile phase, cyclohexane:ethyl acetate, 3:2), scraped from the plate and submitted to liquid scintillation counting. For details see Ref. *18*. Values are mean ± SDM of three independent experiments performed in quadruplicate.

Treatment	Pregnenolone (dpm/mg protein)
Vehicle	1,628.5 ± 508
18-EtDOC	1,590 ± 402

TABLE V. **Aldosterone production and tritiated leucine incorporation in 18-EtDOC treated bovine adrenal cultured cells.** Bovine adrenal glomerulosa cell cultures were pretreated with 10 μM 18-ethynyl-11-deoxycorticosterone (18-EtDOC) and then incubated with tritiated leucine for different incubation periods. After incubation, cell cultures were extensively washed and homogenized, and the radioactivity associated with the 25% TCA-insoluble fraction was measurement by liquid scintillation. Aldosterone levels were measured in incubation medium by RIA. Values are mean ± SDM of three independent experiments performed in quadruplicate.

Time (h)	Aldosterone (pg/10 μl)	^{3}H-Leucine incorporation (cpm)	% Aldosterone relative to 32.5 h	% ^{3}H-Leucine relative to 32.5 h
1	3.9 ± 0.1	3,498 ± 94	18 ± 1	16.2 ± 0.4
6.6	7.1 ± 0.1	7,171 ± 197	34.3 ± 0.3	33.3 ± 0.9
17.5	10.7 ± 0.4	10,902 ± 175	52 ± 2	50 ± 1
32.5	20 ± 1	21,516 ± 634	100 ± 4	100 ± 2



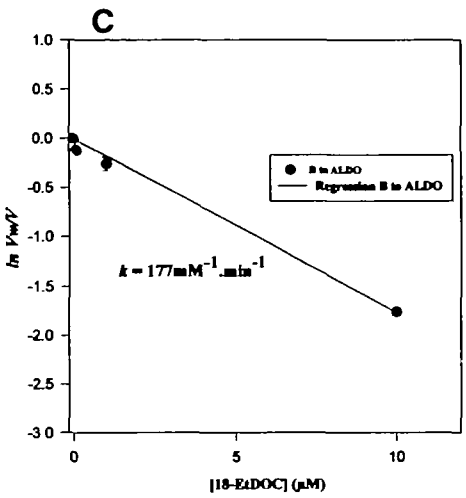


Fig. 5. **Representation of equation 10.** Productions of aldosterone and corticosterone (B) were measured from 11-deoxycoticosterone (DOC) (A); production of aldosterone was measure from 18-hydroxy-11-deoxycorticosterone (18-OHDOC) (B), and from corticosterone (C). When DOC and 18-OHDOC were used as substrates, the experiments were designed with different incubations times at a constant 18-EtDOC concentration (1 μM); and for corticosterone as the substrate, incubation time was maintained constant (45 min) and 18-EtDOC concentration was varied. Similar results were obtained with both experimental designs for each reaction. Rat adrenal mitochondria were pretreated with the MBI 18-EtDOC. After washing out the inhibitor, enzyme source was incubated with the sustrate and 0.5 mM NADPH at 37°C. After incubations, aldosterone and corticosterone levels were measured by RIA. 18-EtDOC, 18-ethynyl-11-deoxycorticosterone. Graphs are representative of at least four independent experiments. Each point of the graph is the mean ± SEM of triplicates.

Figure 5 shows the representation of equation 10 (see above) for the production of aldosterone from 11-deoxycorticosterone, 18-hydroxy-11-deoxycorticosterone, and corticosterone. For the first two substrates, the experiments were performed with different incubations times at a constant 18-EtDOC concentration (1 μM), and for corticosterone as the substrate, the incubation time was maintained constant (45 min) and 18-EtDOC concentration was varied. Thus, both possibilities of experimental design are shown (see above, three successive paragraphs immediately after Eq. 10). However, k values from both experimental approaches for each reaction were practically indistinguishable.

As predicted by the model, a straight line was obtained

TABLE VI. **Apparent inhibition constants.** Rat adrenal mitochondria were pretreated with 10 μM 18-hydroxy-11-deoxycorticosterone (18-EtDOC) (MBI). After washing out the inhibitor, enzyme source was incubated with the corresponding substrate (1 μM) of the reactions shown in Fig. 1 and 0.5 mM NADPH at 37°C for 60 min. After incubation, corticosterone and aldosterone levels were measured by RIA. Values are mean ± SDM of three independent experiments performed in quadruplicate. DOC, 11-deoxycorticosterone; B, corticosterone; ALDO, aldosterone.

Reaction (substrate→product)	k values ($mM^{-1}\cdot min^{-1}$)
DOC→ALDO	451 ± 36
B→ALDO	177 ± 16
18OHDOC→ALDO	175 ± 15

in all cases, and k values were calculated from its slope.

Figure 5 also shows (upper panel) the representation of Eq. 10 as a function of incubation time for the conversion of 11-deoxycorticosterone into corticosterone. In agreement with Fig. 4, a practically horizontal line indicating no inhibitory effect of 18-EtDOC on this reaction was obtained. In effect, the slope of the straight line was close to zero.

Table VI lists k values for the four different reactions shown in Fig. 5.

DISCUSSION

18-EtDOC behaves as an MBI in the adrenal mitochondrial production of aldosterone, since its inhibitory action on aldosterone production from 11-deoxycorticosterone, 18-hydroxy-11-deoxycorticosterone, and corticosterone was time-dependent (Fig. 3) and irreversible (Table II). In effect, the longer 18-EtDOC was allowed to act, the greater was the inhibition (Fig. 3), and the inhibitory action of synthetic was independent of washing (Table II).

In comparison, the reversible inhibitory action of cortisol, a proved competitive inhibitor of aldosterone production from 11-deoxycorticosterone in our rat adrenal preparations (*21*), showed a much lower time-dependency, and it was dramatically lost when cortisol was washed out before the addition of 11-deoxycorticosterone as substrate (Table II).

Additionally, preliminary data from experiments on the action of 18-EtDOC in bovine adrenal cell cultures clearly showed that recovery of aldosterone enzymatic activity after removing 18-EtDOC is associated with *de novo* protein synthesis (Table V).

The inhibitory action of 18-EtDOC on aldosterone biosynthesis from 11-deoxycorticosterone seems to be specific, since the activity of another steroidogenic cytochrome P450, cytochrome P450scc, was unaffected (Table IV).

When enzyme source (rat adrenal mitochondria preparation) was pretreated with NADPH, basal production of aldosterone was dramatically diminished due to redox reactions on cytochrome P450-aldosterone synthase and its environment. However, the inhibitory action of 18-EtDOC was maintained at the same level as in control mitochondria (Table III). This result supports the hypothesis that 18-EtDOC only affects active cytochrome P450-aldosterone synthase. In other words, the synthetic binds to the same enzyme state as the substrate, which primarily discards artifacts or non-specific inhibition.

In the present report we show a new mathematical model for studying the kinetics of MBI. This model was challenged with the effect of 18-EtDOC on aldosterone biosynthesis from 11-deoxycorticosterone in rat adrenal zona glomerulosa mitochondria. According to Eq. 10 of the mathematical model, the specific association constant of velocity (k) for the formation of enzyme-MBI complex can be calculated from the slope of Vw/V *vs.* t or Vw/V *vs.* Io graphs, where Vw is the initial velocity of the enzymatic reaction after treatment with MBI, and V is the initial velocity of the reaction without pretreatment with MBI. Since k values (Table VI) for the transformations of corticosterone and 18-hydroxy-11-deoxycorticosterone to aldosterone are identical, we can hypothetize that both reactions are catalyzed by the same enzyme, which is in agreement with previous reports (*26*).

The k value for the transformation of 11-deoxycorticosterone into corticosterone (not shown in Table VI) was 2.7 ± 0.2 mM^{-1} min^{-1}, which could be taken as a theoretical "zero" since production of corticosterone from 11-deoxycorticosterone was unaffected by 18-EtDOC (Fig. 4).

The conversion of 11-deoxycorticosterone to aldosterone in our enzyme preparation involves not only CYP11B2 but also CYP11B1 activity, because of the contamination of adrenal capsules with zona fasciculata (adrenal core). So the value has to be understood as an apparent k value.

In support of this last assumption, the apparent k value for the transformation of 11-deoxycorticosterone into aldosterone (Table VI) is higher (451 mM^{-1} min^{-1}) than k values for conversions of corticosterone and 18-hydroxycorticosterone to aldosterone. In effect, k values for the conversion of 11-deoxycorticosterone to aldosterone in the presence of CYP11B2 alone are expected to be around 175 mM^{-1} min^{-1} (Table VI).

In summary 18-EtDOC acts as a strong and specific MBI of cytochrome P450 aldosterone synthase. Its potential use in hyperaldosteronism remains to be investigated.

Concluding Remarks—The mathematical model presented here was found to fit the behaviour of MBI, allowing easy calculation of k. This model is very simple and its application is tied to an experimental protocol consisting of preincubation with MBI, followed by washing, and final incubation with specific substrate.

18-EtDOC behaves as an MBI, since: (i) its action was irreversible, (ii) its action was time-dependent, and (iii) its inhibitory potency was independent of substrate concentration.

The k values for the transformation of corticosterone and 18-hydroxy-11-deoxycorticosterone into aldosterone were indistinguishable, suggesting that all these reactions are catalyzed by the same enzyme, CYP11B2. This conclusion is in agreement with other studies (*21*, *26*).

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