The effects of metal ions on the enzymatic reduction of adrenodoxin, on cholesterol side chain cleavage, and on 11β-hydroxylation, all catalyzed by purified enzymes, have been compared. Both monovalent and divalent ions activated adrenodoxin reduction by NADPH and adrenodoxin reductase, confirming previous findings (Lambeth, J. D., Seybert, D. W., and Kamin, H. (1979) J. Biol. Chem. 254, 7255-7264). Increasing ionic strength continuously increased the $V_{\text{max}}$ for cholesterol side chain cleavage when adrenodoxin was both saturating and fully reduced by excess adrenodoxin reductase. No effect of ionic strength on 11β-hydroxylation $V_{\text{max}}$ was observed under these conditions. At lower [adrenodoxin], both activities declined at high ionic strength due to similar increases in $K_m$ for adrenodoxin. The decrease in activity was well described by a simple Michaelis-Menten function of $V_{\text{max}}, K_m$, and [adrenodoxin]. When [adrenodoxin reductase] was insufficient to fully reduce adrenodoxin, inhibition of both side chain cleavage and 11β-hydroxylation by oxidized adrenodoxin was demonstrable. For 11β-hydroxylation, a decrease in ion concentration (80 to 40 mM NaCl; 5 to 1 mM CaCl$_2$) greatly decreased inhibition by oxidized adrenodoxin (full activity with 40% oxidized adrenodoxin), while for side chain cleavage, the inhibition was marked under all conditions. 11β-Hydroxylation was activated to the same maximum activity (70 min$^{-1}$ at 30 °C) by CaCl$_2$ (2 mM) and NaCl (100 mM). In contrast, side chain cleavage activation by CaCl$_2$ reached at most only 15% of that by NaCl. CaCl$_2$ (30 to 100 μM) strongly inhibited side chain cleavage after activation by 100 mM NaCl. This inhibition was associated with decreased electron transfer from reduced adrenodoxin to P-450$_{sec}$, under steady state conditions. Magnesium chloride fully stimulated both monoxygenases.

In the adrenal cortex, three steps of steroidogenesis are catalyzed by mitochondrial cytochromes P-450: cholesterol side chain cleavage, and 11β- and 18-hydroxylation of Δ⁴-3-ketosteroids (1-3). While cytochrome P-450$_{sec}$ is specific for cholesterol side chain cleavage is distinct from cytochrome P-450$_{11β}$ specific for 11β-hydroxylation (2, 3), the latter can also catalyze 18-hydroxylation of 11-deoxycorticosteroids (4, 5). The mitochondrial cytochromes P-450 are dependent on a ferredoxin-type iron sulfur protein and a flavoprotein with one cofactor (FAD) which acts as the ferredoxin reductase (6-8). In adrenal cortex mitochondria, the concentration of P-450 is equal to that of adrenal ferredoxin (adrenodoxin), while the concentration of the flavoprotein adrenodoxin reductase appears to be one-tenth of these enzymes (9, 10). The mitochondrial cytochromes P-450, like all cytochrome P-450 isolated from mammalian tissues, are integral membrane proteins, while in contrast, adrenodoxin and adrenodoxin reductase behave as peripheral membrane proteins (2, 3, 11-13).

Recent studies with purified enzymes indicate that adrenodoxin transfers electrons from adrenodoxin reductase to P-450 by shuttling between these two enzymes and not within a ternary complex of the three proteins (12-14). Metal ions strongly modulate adrenodoxin binding to adrenodoxin reductase and to P-450 and can activate adrenodoxin reduction by adrenodoxin reductase, as well as cholesterol side chain cleavage and 11β-hydroxylation (13, 16-19). The mechanism of ionic activation of adrenodoxin reduction by adrenodoxin reductase has been elucidated by the recent studies of Lambeth et al. (18). In a preliminary communication of the results presented here, we have noted that the ionic activation of the monooxygenase activities of cytochromes P-450$_{sec}$ and P-450$_{11β}$ is the combined result of several different processes and cannot be explained solely by the activation of adrenodoxin reduction (20).

In the present studies with purified adrenodoxin, adrenodoxin reductase, P-450$_{sec}$, and P-450$_{11β}$, we have examined the effect of univalent and bivalent metal ions on the reduction of adrenodoxin reductase and on the side chain cleavage and 11β-hydroxylation activities of the cytochromes, all under the same conditions. Our results indicate that ions, particularly Ca$^+$, affect side chain cleavage and 11β-hydroxylation differently. Previously, we have provided evidence that oxidized adrenodoxin (ADX$^*$) inhibits side chain cleavage activity by competing with reduced adrenodoxin (ADX$^-$) for binding to P-450$_{sec}$ (15). The present results indicate that the same effect also operates with P-450$_{11β}$ and that ionic activation of 11β-hydroxylation appears to be associated with a decreased effectiveness of this competition.

**EXPERIMENTAL PROCEDURES**

**Materials**—The phospholipids were purchased from Serdary and Tween 20 and Heps from Sigma Chemical Co. The other materials—testosterone, 11β,21-dihydroxy-4-pregnene-3,20-dione; Heps, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

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were obtained from previously indicated sources (21). [1-2H]-Cholesterol (43 Ci/mmol) was purified as previously described (21).

**Preparation of Phospholipid Vesicles**—Unlabeled cholesterol-containing phospholipid vesicles was prepared according to the procedure of Lambeth et al. (18) with some modifications. Aliquots of nonradioactive cholesterol and [3H]cholesterol solutions in ethanol and [3H]cholesterol solutions in ethanol were added to the vortex tube to give a final concentration of 100 mM NaCl (data not shown and Ref. 19). In the absence of NaCl, side chain cleavage activity was undetectable, while under the same conditions, about 60% of the adrenodoxin molecules were in the reduced form (Fig. 1).

The dependence on KCl in Tween 20 was essentially identical with NaCl (data not shown and Ref. 19). In the absence of NaCl, side chain cleavage activity was undetectable, while under the same conditions, about 60% of the adrenodoxin molecules were in the reduced form (Fig. 1).

The dependence on KC1 in Tween 20 was essentially identical with NaCl (data not shown and Ref. 19). In the absence of NaCl, side chain cleavage activity was undetectable, while under the same conditions, about 60% of the adrenodoxin molecules were in the reduced form (Fig. 1).

**RESULTS**

**Effects of Ions on Cholesterol Side Chain Cleavage and Adrenodoxin Reduction**—Cholesterol side chain cleavage reconstituted with purified adrenodoxin, adrenodoxin reductase, and P-450c17 showed a similar bell-shaped dependence on NaCl both in Tween 20 and phospholipid vesicles (Fig. 1). The dependence on KCl in Tween 20 was essentially identical with NaCl (data not shown and Ref. 19). In the absence of NaCl, side chain cleavage activity was undetectable, while under the same conditions, about 60% of the adrenodoxin molecules were in the reduced form (Fig. 1).

As [NaCl] was increased, the steady state levels of ADX and side chain cleavage activity increased nearly in parallel until all adrenodoxin molecules became reduced at 50 to 60 mM NaCl. Side chain cleavage activity continued to increase from 50 to 100 mM NaCl, then decreased as [NaCl] was increased further, whereas all adrenodoxin molecules remained reduced even at 300 mM NaCl (Fig. 1).

We have recently noted that ADX inhibits side chain...
cleavage probably by competing with ADX for binding to P-450b,c (15) and that the \( V_{\text{max}} \) for side chain cleavage at saturation with adrenodoxin increases linearly with ionic strength from 50 to 200 mM NaCl (13). Therefore, the steep increase of side chain cleavage observed from 0 to 50 mM NaCl in Fig. 1 could be ascribed to a decrease in [ADX'] and/or an increase in the rate of side chain cleavage independent of changes in the steady state [ADX']. In order to distinguish between these possibilities, the experiments in Fig. 2 were conducted with a concentration of adrenodoxin reductase (4 to 5 times higher than in Fig. 1) which was sufficient to reduce all adrenodoxin molecules even at 0 mM NaCl. Under these conditions, side chain cleavage activity increased sharply with increased NaCl. However, from 0 to about 50 mM NaCl with saturating [ADX'], the turnover numbers in Fig. 2 were significantly higher than those in Fig. 1, presumably due to the inhibitory effect of ADX' under the conditions used in Fig. 1. We have recently observed that the \( K_a \) for adrenodoxin in cholesterol side chain cleavage increases exponentially with ionic strength [NaCl] for adrenodoxin in cholesterol cleavage. These simulations are based on the Michaelis-Menten Equation 1 below:

\[
V = \frac{V_{\text{max}} [\text{ADX}_{\text{free}}]}{K_a + [\text{ADX}_{\text{free}}]}
\]  

in which \( V_{\text{max}} \) and \( K_a \) represent values at each ionic strength and adrenodoxin concentration is calculated taking into account the \( K_a \) values for complex formation with adrenodoxin reductase and P-450b,c at each ionic strength. A noteworthy point in these simulations is that as [adrenodoxin] is lowered, the peaks of the curves are shifted to lower values of ionic strength and the peaks become narrower. Indeed, both we (data not shown) and Takikawa et al. (19) have observed sharper peaks with lower [adrenodoxin]. Although the generated patterns are similar to those found experimentally (Fig. 1) with 7.5 and 4.0 mM adrenodoxin, we have consistently observed higher turnover numbers at 900 mM NaCl than those predicted by the simulations. An error in the \( K_a \) is the likely source of this discrepancy. An estimated \( K_a \) for adrenodoxin (40 \( \mu \)) at 300 mM NaCl was obtained by extrapolation of the linear plot of log \( K_a \) versus ionic strength (50 to 200 mM NaCl; Ref. 13, Fig. 8). This \( K_a \) could easily exceed the true value by a factor of 2, particularly in view of the observed saturation of the analogous plot of log \( K_d \) versus ionic strength (13).

In contrast to univalent metal ions, Ca\(^{2+}\) was a very ineffective activator of side chain cleavage activity reconstituted in Tween 20 (Fig. 3) and caused no significant activation of side chain cleavage reconstituted with phospholipid vesicles. The low activation could not be ascribed to the presence of ADX' because at 3 to 4 mM CaCl\(_2\) all adrenodoxin molecules were reduced (Fig. 3). The concentration range of MgCl\(_2\) necessary to increase the steady state levels of ADX' was similar to CaCl\(_2\) (Fig. 3). However, in contrast to CaCl\(_2\), increased [MgCl\(_2\)] continued to increase side chain cleavage activity even after all adrenodoxin molecules were reduced (Fig. 3). A replotted MgCl\(_2\) activation of side chain cleavage activity on the basis of ionic strength was nearly superimposable on that for NaCl.

In the presence of 100 mM NaCl, CaCl\(_2\) inhibited the side chain cleavage activity of P-450b,c in both Tween 20 micelles and phospholipid vesicles (Fig. 4). MgCl\(_2\) caused no inhibition under these conditions. The [Ca\(^{2+}\)] \( \geq 50 \) \( \mu \)M in Tween 20 and
30 μM for vesicle-incorporated P-450 (Fig. 4). This inhibition could not be due to Ca²⁺ effects on adrenodoxin reduction because, in the presence of 100 mM NaCl, Ca²⁺ had no significant effect on either the rate of adrenodoxin reduction by adrenodoxin reductase or the steady state levels of ADX'. The effect of Ca²⁺ on electron transfer from adrenodoxin to P-450 was analyzed by examining the effect on the steady state [ADX'] during cholesterol side chain cleavage. In absence of P-450_{sec}, 17 nM adrenodoxin reductase was sufficient to nearly fully reduce adrenodoxin (Fig. 5). Addition of P-450_{sec} resulted in a new steady state in which only 60% of adrenodoxin molecules became reduced (Fig. 5). Addition of Ca²⁺ during side chain cleavage increased the steady state [ADX'] indicating that Ca²⁺ inhibition of side chain cleavage reaction was associated with a decreased rate of oxidation of ADX'. The effect of Ca²⁺ could be reversed by nearly stoichiometric concentrations of EDTA (Fig. 5) which at these concentrations, in absence of Ca²⁺, did not affect the side chain cleavage activity or the steady state [ADX'] during side chain cleavage. However, addition of increasing concentrations of EDTA in the presence of Ca²⁺ resulted in a further decrease of the proportion of ADX' below that observed in absence of Ca²⁺. At present, the reason for this effect is not clear.

**Effects of Ions on 11β-Hydroxylation and Adrenodoxin Reduction**—In contrast to P-450_{sec}, the catalytic activity of P-450_{11β} was activated to the same maximum activity by both monovalent (Na⁺, K⁺) and divalent (Ca²⁺, Mg²⁺) metal ions (Figs. 6 to 8). The bivalent ions stimulated at much lower ionic strength than monovalent ions. The activation of the hydroxylase activity was associated with an increase in the proportion of ADX' at steady state (Figs. 6 and 7). However, under these conditions, 11β-hydroxylation reached maximal levels at 40 mM NaCl or 2 mM CaCl₂ while 30 to 40% of adrenodoxin was oxidized (Figs. 6 and 7). As ion concentrations were further increased, all adrenodoxin molecules became reduced and remained reduced even at the highest I examined (300 mM NaCl). Full reduction of adrenodoxin required higher ion concentrations than those required for side chain cleavage (Figs. 1 and 3), probably because of the higher rate of ADX' oxidation by P-450_{11β} and the lower rate of adrenodoxin reduction at 30 °C.

As shown in Fig. 8, the $K_m$ for adrenodoxin increased exponentially with $I$ in a manner similar to that observed with P-450_{sec} (13). However, in contrast to P-450_{sec}, the $V_{max}$ at saturation with adrenodoxin remained essentially constant with ionic strength up to 200 mM KCl (Fig. 8). Thus, the decline in 11β-hydroxylase activity seen at high $I$ in the presence of constant [ADX'] (Fig. 6) was also due to the increase in the $K_m$ for ADX. The $V_{max}$ observed in 5 mM CaCl₂...
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was not different from that observed with up to 150 mM KCl (Fig. 9). The small decline in activity seen at 10 mM CaCl₂ (Fig. 7) cannot be interpreted as a significant effect, because, at these concentrations, CaCl₂ causes much aggregation and turbidity in the reaction mixture.

Previously, we noted that ADX⁺ inhibits side chain cleavage activity probably by competing with ADX⁻ for binding to P-450ᵢ₉ (15). Therefore, the observation that 11β-hydroxylation reaches Vₘₚ in the presence of substantial [ADX⁺] (Figs. 6 and 7) was surprising. Thus, we examined whether ADX⁺ inhibition of monooxygenase activity may be modulated by ions. We varied the steady state [ADX⁺] in the presence of constant total [adrenodoxin] by varying [adrenodoxin reductase] (Figs. 10 and 11). At 80 mM NaCl or 5 mM CaCl₂ percent ADX⁻ and activity correlated directly. However, at lower ion concentrations (40 mM NaCl or 1 mM CaCl₂; chosen on the basis of the results in Figs. 6 and 7), activity increased more rapidly than the percentage of ADX⁻. In particular, increasing the steady state level of ADX⁺ up to 30% did not diminish activity at the lower ion concentrations (Figs. 10 and 11).

**DISCUSSION**

Recent evidence indicates that adrenodoxin transports electrons from adrenodoxin reductase to P-450ᵢ₉ or P-450₁₈ by shuttling between these two enzymes and not within a ternary complex (12-15). As a consequence of this shuttle mechanism, cholesterol side chain cleavage activity shows a Michaelis-Menten dependence on free ADX⁻ (15). Electron transfer from ADX⁻ to P-450ᵢ₉ during cholesterol side chain cleavage appears to be inhibited competitively by ADX⁺ (15). The present results indicate that these principles also hold true for P-450₁₈. Thus, the adrenodoxin dependence of the activities of

**Fig. 7 (left).** Effect of CaCl₂ on adrenodoxin reduction and 11β-hydroxylase activity. The assay conditions were as in Fig. 6.

**Fig. 8 (right).** Effect of KCl on adrenodoxin dependence of 11β-hydroxylase activity. The adrenodoxin on the abscissa refer to [adrenodoxin]. The assays were carried out in 10 mM Hepes (pH 7.2) with 50 μM deoxycorticosterone, 0.24 μM adrenodoxin reductase, and 0.2 μM P-450₁₈. The concentrations of KCl are shown next to each plot. The numbers on the lower left of the figure represent the apparent Kᵣₚ for adrenodoxin at each concentration of KCl.

**Fig. 9.** Adrenodoxin dependence of 11β-hydroxylase activity in the presence of 5 mM CaCl₂. The assays were carried out in 10 mM Hepes (pH 7.2) with 50 μM deoxycorticosterone, 0.24 μM adrenodoxin reductase, and 0.165 μM P-450₁₈. In the inset, the same data are replotted in double reciprocal form.

**Fig. 10.** Effect of 40 mM and 80 mM NaCl on adrenodoxin reductase dependence of adrenodoxin reduction and 11β-hydroxylase activity. The assays were carried out in 10 mM Hepes (pH 7.2) with 50 μM deoxycorticosterone, 7.6 μM adrenodoxin, and 0.15 μM P-450₁₈.

**Fig. 11.** Effect of 1 mM and 5 mM CaCl₂ on adrenodoxin reductase dependence of adrenodoxin reduction and 11β-hydroxylase activity. The assay conditions were as in Fig. 10.
both cytochromes, at a fixed level of steroid substrate, should be described by the familiar Michaelis-Menten equation that takes into account competitive inhibition:

\[
V = \frac{V_{\text{max}} \cdot [\text{ADX}_{\text{free}}]}{K_m \left(1 + \frac{[\text{ADX}]_{\text{free}}}{K_i}\right) + [\text{ADX}]_{\text{free}}}
\]  

(2)

in which the definition of \(\text{ADX}_{\text{free}}\) includes the \(K_d\) values for the formation of separate complexes by adrenodoxin reductase and P-450 (15).

Previous studies have shown that the \(K_d\) for cholesterol binding to P-450_{26} and the \(K_m\) for cholesterol in side chain cleavage are almost independent of ionic effects (13). However, monovalent and divalent ions can affect most if not all of the variables in Equation 2 for both cytochromes including the \(K_d\) values that determine \(\text{ADX}_{\text{free}}\). Ions activate adrenodoxin reduction by adrenodoxin reductase, resulting in an increase in \([\text{ADX}']\) and a decrease in \([\text{ADX}''\) (15). The \(K_m\) for ADX' in both cholesterol side chain cleavage (13) and 11\(\beta\)-hydroxylation (Fig. 8), and the \(K_v\) values for complex formation with adrenodoxin reductase (18) and P-450_{26} (13) increase with ionic strength. In the special case where adrenodoxin is maintained fully reduced (Fig. 2), Equation 2 simplifies to Equation 1 which provides a good description of the effect of ionic strength on side chain cleavage activity (Fig. 2).

The effectiveness of ADX' to inhibit monooxygenase activity also appears to be modulated by ions at least for P-450_{18} (Figs. 6, 7, 10, and 11). The high correlation between per cent of ADX' and per cent maximal activity at 80 mM NaCl and 5 mM CaCl\(_2\) with near saturating adrenodoxin indicates that at these ion concentrations, the \(K_m\) for ADX' is approximately equal to the \(K_m\) for ADX' as previously noted for P-450_{26} for an ionic strength equivalent to 80 mM NaCl (15). However, at lower concentrations of both ions (40 mM NaCl, 1 mM CaCl\(_2\)), the observation of maximal rates of 11\(\beta\)-hydroxylation in the presence of substantial [ADX'] (Figs. 6, 7, 10, and 11) suggests that at these ion concentrations, ADX' does not effectively inhibit 11\(\beta\)-hydroxylase activity (i.e. in equation 2, \(K_i > K_m\)).

For P-450_{26}, a comparison of the results in Figs. 1 and 2 in the range of 0 to 30 mM NaCl, where there were substantial [ADX'] in Fig. 1 and no ADX' in Fig. 2, indicates that ADX' inhibits side chain cleavage also at this low range of ionic strength.

In the present studies, one of the major differences observed between P-450_{26} and P-450_{18} is that the \(V_{\text{max}}\) of side chain cleavage, but not 11\(\beta\)-hydroxylation, is increased by increased ionic strength independent of changes in [ADX'] (Figs. 2 and 8). At present, this activation of the rate of side chain cleavage cannot be explained at a molecular level. The studies on cytochrome P-450_{26}, catalyzed-camphor monooxygenation indicate that, for this reaction, the rate-limiting step is that of the second electron transfer from putidaredoxin to P-450_{26} (26). However, we have recently discussed the possibility, based on the high ratio of \(K_m/K_d\) for adrenodoxin, that for P-450_{26}, the dissociation of ADX' from P-450_{26} may be a rate-limiting step in side chain cleavage. Although this remains speculative, it is notable that increasing ionic strength also weakens the binding of ADX' to oxidized P-450 (log \(K_i \approx 1\)). Such an effect would be analogous to the ionic enhancement of adrenodoxin reduction by the reductase (18). A comparison of the data in Figs. 1 and 6 indicates that the rate of 11\(\beta\)-hydroxylation at a fixed [adrenodoxin] declines more rapidly at high \(I\) than does the rate of cholesterol side chain cleavage. This is consistent with activities described by Equation 2 as \(K_m\) values for both activities increase similarly with \(I\) (Fig. 8 and Ref. 13), while only \(V_{\text{max}}\) for side chain cleavage increases to partially offset this effect.

Another major difference between P-450_{26} and P-450_{18} is that while 1 to 2 mM CaCl\(_2\) activates 11\(\beta\)-hydroxylation to the same extent as 40 mM NaCl and KCl, it causes no significant activation of cholesterol side chain cleavage and, in the presence of 100 mM NaCl, inhibits optimally activated side chain cleavage reaction in both detergent micelles and phospholipid vesicles (Fig. 4). The inhibition of side chain cleavage is associated with a decreased rate of electron transfer from ADX' to P-450_{26} (Fig. 5). However, this finding does not mean that Ca\(^{2+}\) is inhibiting side chain cleavage directly at this process. We have previously shown that Ca\(^{2+}\) does not affect the binding of either cholesterol or ADX' to P-450_{26} (13). Lambeth et al. have recently reported that CaCl\(_2\) is more effective than NaCl in the stimulation of cholesterol side chain cleavage activity of phospholipid vesicle reconstituted P-450_{26} (18). However, under the conditions of their experiments with 1 \(\mu\)M adrenodoxin, the turnover of P-450 was 10 to 20 times lower than that obtained in the present experiments, and the maximal activities they observed with CaCl\(_2\) are lower than the maximally inhibited turnover numbers we observe. The side chain cleavage inhibitory effect of Ca\(^{2+}\) is not common to all ion activations in general, since MgCl\(_2\) causes no inhibition and activates side chain cleavage in a manner similar to NaCl on the basis of ionic strength.

At present, the physiological relevance of the opposing effects of low concentrations of Ca\(^{2+}\) on the monooxygenase enzymes is questionable. Extensive evidence indicates that Ca\(^{2+}\) plays a stimulatory role in steroidogenesis (27-32), and to our knowledge, no inhibitory effect of Ca\(^{2+}\) on NADPH-supported steroidogenesis at such low concentrations (30 to 100 \(\mu\)M) has been observed. In studies using isolated mitochondria, Ca\(^{2+}\) has been shown to activate both cholesterol side chain cleavage and 11\(\beta\)-hydroxylation (27, 33, 34) and also to increase the rate of P-450 reduction without an apparent change in the rate of reduction of adrenodoxin (34). Adrenocorticotropic activation of steroidogenesis is accompanied by acute increases in polyphosphoinositides which can avidly bind Ca\(^{2+}\) (35, 36). These phospholipids specifically stimulate mitochondrial cholesterol side chain cleavage (37) and may also affect Ca\(^{2+}\) levels in the immediate environment of the cytochromes P-450_{26} and P-450_{18}.

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