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The Regulation of Corticosteroid Hydroxylations

1. An Effect of Inorganic Ions in Regulating Iron-Sulphur Protein-Dependent Electron Transport

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Abstract

The enzymes effecting the hydroxylation of steroids in bovine adrenal cortex mitochondria were isolated and their interaction and functioning studied. The results indicated that the sensitivity to ionic strength of the functioning of the hydroxylases studied may be the result of an effect on the transfer of electrons by the iron-sulphur protein, adrenodoxin, and adrenodoxin reductase to cytochrome P450.

Introduction

Among the quantitatively most important derivatives of cholesterol in the vertebrate are the bile acids and the steroid hormones. The metabolic processes leading to production of both these classes of compounds are essentially steroid hydroxylations requiring molecular oxygen and a supply of reducing equivalents resulting from the redox cycle of the cofactor NADP (Nicotinamide-adenine dinucleotide phosphate). The typical steroid hydroxylases relating to bile acid production are found to be located in the microsomal fraction of the hepatic cells while those concerned with the elaboration of the hormones are located in both the microsomes and mitochondria of cells of the endocrine tissues (the adrenal cortex, ovary, testis and placenta).

Most steroid hydroxylations examined leading to bile acid or hormone production have been shown to be cytochrome P450 dependent (Cytochrome P450 is considered to be the site of oxygen activation for a

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number of mixed-function oxidases). In each case too a flavoprotein is considered to be an immediate acceptor of reducing equivalents from NADPH. An important characteristic of the mitochondrial hydroxylations of endocrine glands is the requirement of an iron-sulphur protein to transfer reducing equivalents from the flavoprotein to cytochrome P450. The protein isolated from the adrenal cortex is called adrenodoxin [1] while similar proteins have been identified from the testis [2], ovary [3] and placenta [4]. Evidence has not been found of the participation of similar iron-sulphur proteins in steroid hydroxylations in hepatic tissue [5] or adrenocortical microsomes [6, 7]. Table I summarizes some of the basic differences between those types of steroid hydroxylations most intensively studied to date.

TABLE I. Comparison of some characteristics of three types of steroid hydroxylations[a]

	Class A	Class B	Class C
(1) Tissue:	non-endocrine e.g. liver	endocrine e.g. adrenal cortex	endocrine e.g. adrenal cortex
(2) Location in cell:	microsomal	mitochondrial	microsomal
(3) Type of product:	non-hormonal e.g. bile acids	hormonal and concerned with metabolic regulation (these hydroxylations on the major pathway of mineralocorticoid production)	hormonal
(4) *In vitro* assay:	not very sensitive to ionic concentration	highly sensitive to raised ionic levels (e.g. see Fig. 7)	–
(5) Iron-sulphur protein:	not identified in tissue and not required for enzymic reaction e.g. 7α-hydroxylations [1, 5]	present and required for all hydroxylations viz. cholesterol sidechain cleavage and 11β- and 18-hydroxylations [1, 9]	not identified and not required in enzyme system e.g. 21-hydroxylations [6, 7]

[a] Little is known of hepatic mitochondrial steroid hydroxylations.

The present work describes factors affecting the *in vitro* activity of cytochrome P450 reductase of bovine adrenal cortex mitochondria and a novel mechanism by which the rate of synthesis of steroid hormones, and in particular the mineralocorticoids, can be influenced and determined by the general metabolic state.

Materials and Methods

The purification of adrenodoxin, adrenodoxin reductase and cytochrome P450 was performed by methods similar to those described elsewhere [8,

9]. The activity of adrenodoxin reductase and cytochrome P450 reductase (i.e. adrenodoxin *plus* adrenodoxin reductase) in reducing 2,6-dichlorophenolindophenol and cytochrome c respectively were measured by modifications [8] of previously described methods [9]. The cholesterol sidechain cleavage reaction was measured by determining the percentage conversion of added [4-^{14}C] cholesterol to [4-^{14}C] pregnenolone and [4-^{14}C] progesterone in the presence of NADPH in an aerobic incubation for 5 or 10 min at 37° C[8]. The 11β-hydroxylation of 11-deoxycorticosterone (DOC) was investigated by incubations under similar conditions and the product assayed by the method of Mattingly *et al.* [10, 11]. [4-^{14}C] cholesterol was obtained from the Radiochemical Centre, Amersham and "cleaned" by thin-layer chromatography immediately before use [8]. All other chemicals and reagents were of analytical grade.

Results and Discussion

Effect of Ionic Strength on Adrenal Cytochrome P450 Reductase

Adrenodoxin and the flavoprotein, adrenodoxin reductase, were freshly prepared from bovine adrenal cortex mitochondria. Figure 1 shows the optical changes observed on titrating adrenodoxin in solution in distilled water with the flavoprotein in solution in distilled water using an Optika spectrophotometer and 1 cm cells divided by a partition to avoid mixing of the two solutions in the cuvette in the reference light

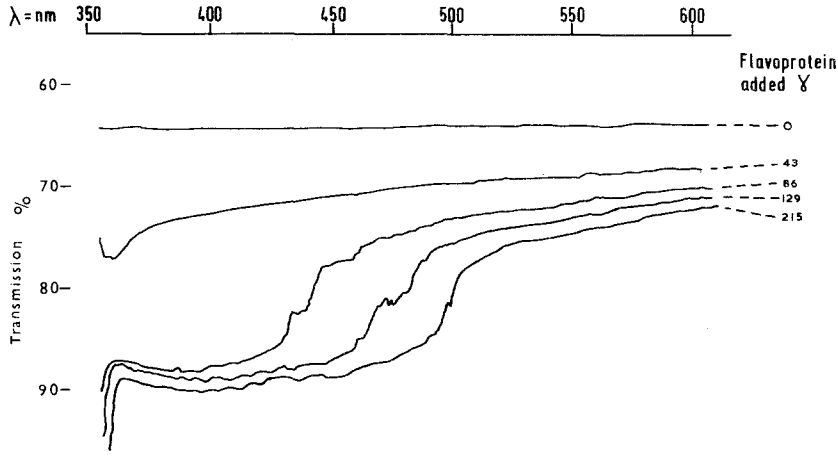

Figure 1. The spectrophotometric titration of adrenodoxin with adrenodoxin reductase in distilled water. 1 ml adrenodoxin solution (0.115 mM or 1.38 mg/ml) was titrated with adrenodoxin reductase solution (8.6 mg protein/ml).

path. The spectra observed give evidence of protein-protein interaction which is not however very similar to that of the related plant iron-sulphur protein, ferredoxin, with the flavoprotein, ferredoxin reductase [12, 13]. However the interaction of the plant proteins were affected by salts [13] and Fig. 2 shows that the same titrations performed in 100 mM-phosphate buffer requires a considerably larger amount of flavoprotein to produce similar spectra to those observed in Fig. 1. This difference in stoichiometry may have been due to the effect of salt or to the buffer affecting the ionisation of the two proteins.

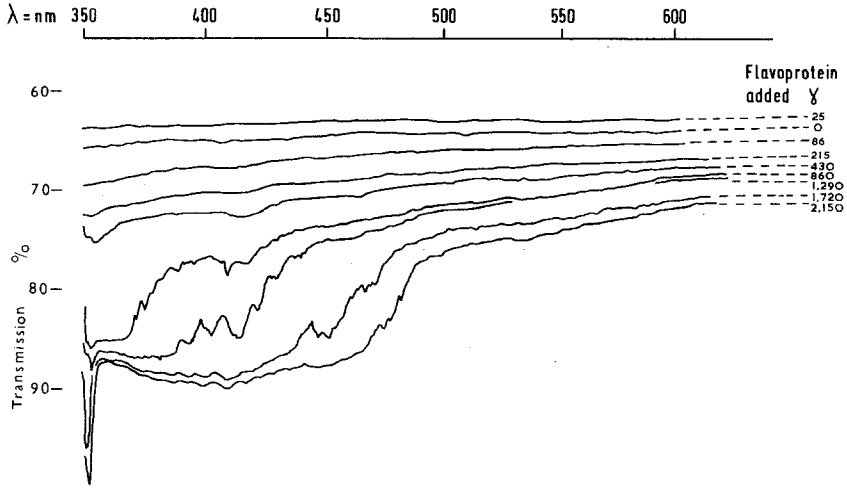

Figure 2. The spectrophotometric titration of adrenodoxin with adrenodoxin reductase in 0.1 M-potassium phosphate buffer, pH 7.4. The protein concentrations were as described for Fig. 1.

The instability to salt of the association of adrenodoxin with adrenodoxin reductase is further evidenced from the results seen in Fig. 3. Here pairs of cuvettes were lined up in the "sample" and "reference" light paths of a Unicam SP800 spectrophotometer. Cuvettes containing adrenodoxin and flavoprotein in the "sample" light path had their contents mixed. Equal aliquots of sodium chloride were added to each of the four cuvettes and the contents well stirred. Figure 3 shows that the effect of sodium chloride on the optical absorption of the proteins in association is significantly different from the effect on that of the proteins in isolated solutions.

Similar investigations were made (Fig. 4) on the effects of salts on the interaction of adrenodoxin and adrenal mitochondrial cytochrome P450. It is seen that salts affect the optical absorbancy of this association of proteins too. Significantly it was observed that the effects of equimolar sodium chloride and potassium chloride were not the same.

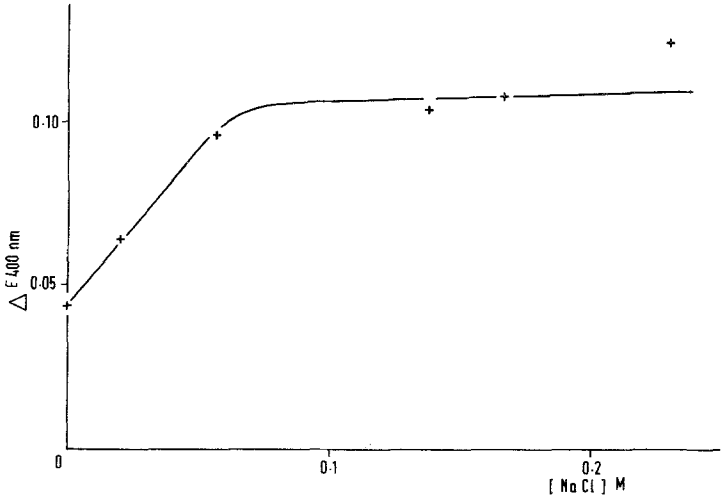

Figure 3. The effect of increasing concentrations of sodium chloride on the light extinction at 400 nm of a mixture of adrenodoxin reductase and adrenodoxin. Two silica cells of 1 cm light path were lined up in the "reference" beam and another two in the "sample" beam of a Unicam SP800 spectrophotometer. 2.5 ml of purified flavoprotein (2.3 mg protein/ml) in 10 mM-Tris-HCl, pH 7.4 was placed in one cell in the "reference" beam and in one cell in the "sample" beam. 2.5 ml adrenodoxin ($E^{415\,nm}$: $E^{280\,nm}$ = 0.8, 0.28 mg protein/ml) in 10 mM-Tris-HCl, pH 7.4 was added to each of the remaining cells. The contents of the two cells in the "sample" beam were mixed and redivided equally between the two cells. Equal aliquots of 1 M-sodium chloride (in the same buffer) were added to each of the four cells, the contents of which were then well stirred. The contents of the two cells in the "sample" beam were mixed and redivided equally into the same cells. The resulting extinction at 400 nm was read and corrected for dilution. Each point shown represents the change in light extinction at 400 nm caused by addition of sodium chloride to the association of adrenodoxin and flavoprotein *minus* any effect of sodium chloride on the separate proteins.

As has been noted in Table I two apparent differences between adrenal mitochondrial and hepatic microsomal steroid hydroxylases are that the former unlike the latter depend on the activity of an iron-sulphur protein and are inhibited *in vitro* by high ionic strength. In the light of the observations made on the effects of salt on the interaction of adrenodoxin with its reductase and with cytochrome P450 it was of interest to speculate whether the presence of the iron-sulphur protein as a component of mitochondrial cytochrome P450 reductase was related to the inhibition by ionic strength of these same steroid hydroxylases. The suggestion has also been made that changes in salt concentration can regulate photosynthetic electron transport pathways by their effect on the interaction of plant ferredoxin and ferredoxin reductase [13]. To further investigate the adrenal enzymes the effect of

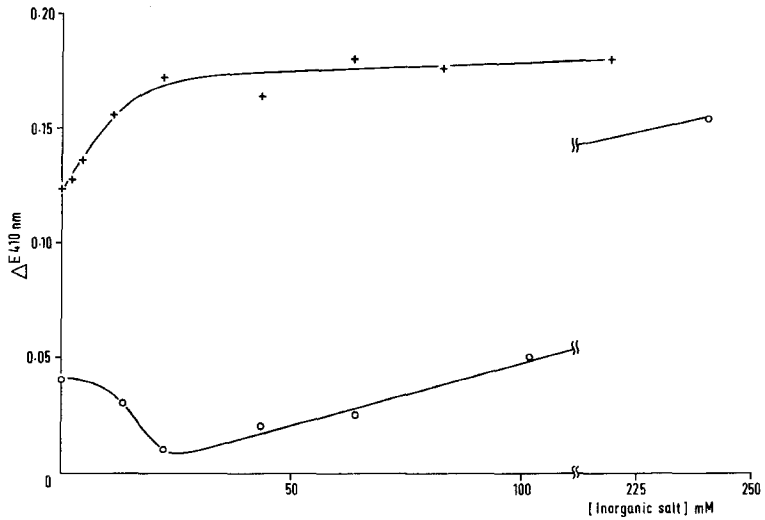

Figure 4. The changes in light extinction at 410 nm of adrenodoxin and cytochrome P450 due to increasing concentrations of sodium chloride or potassium chloride, +——+ sodium chloride, o——o potassium chloride. The determinations were made in a similar manner to that employed in investigating the effect of sodium chloride on the association of adrenodoxin and its reductase (see Fig. 3). For these experiments 2.2 ml solubilized adrenal mitochondrial cytochrome P450 (5.5 mg protein/ml), 2.2 ml adrenodoxin solution ($E^{415\,nm}$: $E^{280\,nm} = 0.8$, 0.18 mg protein/ml) and 1 M-sodium or potassium chloride solutions were used. Each point shown represents the change in light extinction at 410 nm caused by the addition of sodium or potassium chloride to the association of adrenodoxin and cytochrome P450 *minus* any effects on the separate proteins.

ionic strength on the enzymic activity of cytochrome P450 reductase was examined. Adrenal mitochondrial or hepatic microsomal cytochrome P450 reductase activities can be investigated independently of cytochrome P450 by their ability to catalyse the reduction of cytochrome c by NADPH [9, 14]. The activity of hepatic microsomal cytochrome P450 reductase, a single flavoprotein, in reducing cytochrome c is not inhibited by ionic strength [14]. Figure 5 demonstrates however that the rate of NADPH-cytochrome c reduction by adrenal mitochondrial cytochrome P450 reductase is markedly sensitive to the ionic strength of the assay mixture. Figure 6 also shows that the inhibition by the divalent magnesium ion is more marked than that by monovalent cations (the effect of potassium ion is shown). The effect of varying concentrations of Tris-HCl buffer on 11β-hydroxylation of 11-deoxycorticosterone (DOC) by adrenal mitochondrial enzyme extracts (Fig. 7) is similar to that on the NADPH-cytochrome c reduction by the mitochondrial cytochrome P450 reductase (Fig. 5). The pH of the reagent solutions and of the incubation mixtures of the above

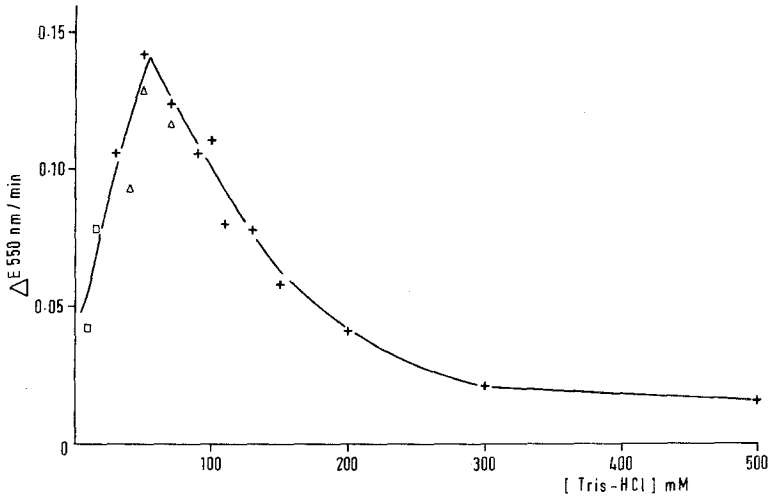

Figure 5. The variation in rate of cytochrome c reduction by adrenodoxin reductase *plus* adrenodoxin at different ionic strengths. The different symbols denote different preparations of Tris-HCl buffer, pH 7.4.

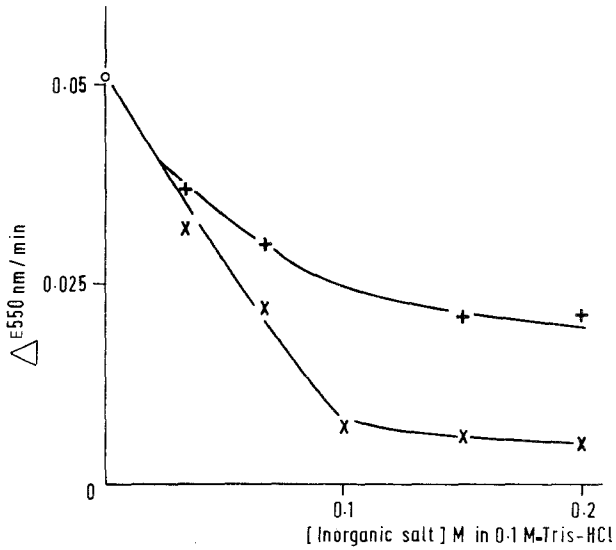

Figure 6. The effect of different concentrations of potassium chloride and magnesium chloride on the rate of cytochrome c reduction by adrenodoxin reductase *plus* adrenodoxin, +——+ in potassium chloride, x——x in magnesium chloride. The assays were performed in 0.1 M-Tris-HCl, pH 7.4. The rates of cytochrome c reduction in the presence of sodium chloride or ammonium chloride were similar to those in the presence of potassium chloride.

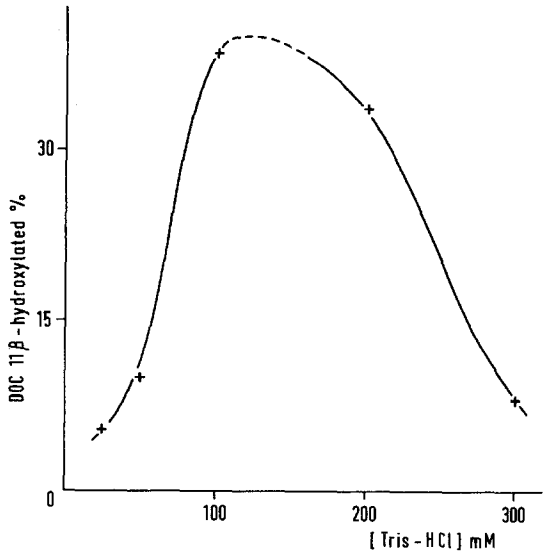

Figure 7. The variation of rate of DOC 11β-hydroxylation at different ionic strengths. The 100,000 g supernatant used was from an adrenocortical mitochondrial enzyme extract [8] made in 100 mM-Tris-HCl, pH 7.4 and then dialysed until the ionic strength reached equilibrium at 10 mM buffer.

experiments were carefully checked to ensure that the above observations were not the effect of changes in pH.

A Possible Relationship of the Effects of Various Monovalent Anions and Cations on Mitochondrial Steroid Hydroxylations

It has been shown (Fig. 4) that sodium and potassium chloride did not have the same effect on the interaction of adrenodoxin with cytochrome P450. A comparison was also made of the effect of various monovalent anions and cations on the steroid hydroxylations by adrenal mitochondrial enzyme. Incubations were carried out in conditions of maximal DOC 11β-hydroxylation. To separate incubations in conditions yielding maximal rates of hydroxylation were added 150 mM concentrations of chlorides of monovalent cations and the relative inhibitory effects on hydroxylations evaluated as follows:

$$\text{Relative inhibition} = \frac{\text{Maximal rate of hydroxylation} - \text{inhibited rate}}{\text{Maximal rate of hydroxylation}} \times 100$$

The inhibitory effects of equimolar amounts of the various chlorides of monovalent cations were found to be not the same. The pH and other variables which may possibly be differently affected by addition of the

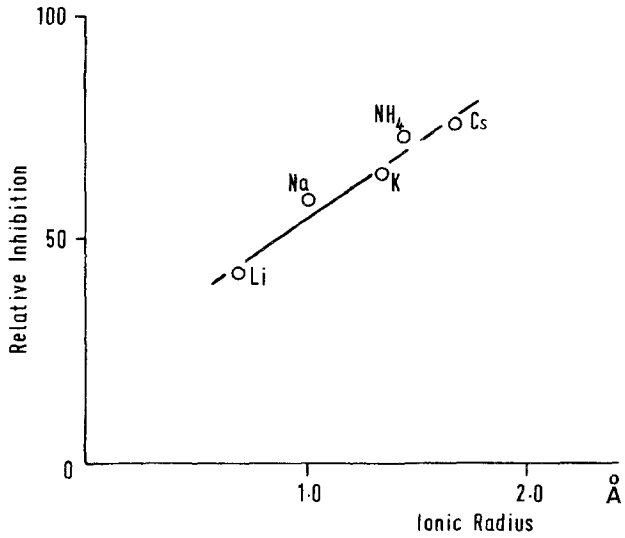

Figure 8. The relationship between the relative inhibitions of DOC 11β-hydroxylation due to salts of monovalent ions and the radius of the cations. For experimental details see text and ref. 8.

various monovalent cations having been checked and eliminated as likely causes of the effects observed, other possible explanations were investigated. It was found that the relative inhibitions produced by the chlorides of the various cations could be linearly related to the ionic radius of each cation (Fig. 8). Similarly it was found that the relative inhibitions produced by the halides of sodium could also be linearly related to the ionic radius of the anions (Fig 9). (Relative inhibitions evaluated from different experiments may not however be compared since their absolute values are also dependent on other factors such as the duration of the incubation [8].) The explanation of these findings is not certain. However it has been determined by several workers that the dielectric constant around a monovalent ion of either sign changes with the distance from the ion taken as a point charge [15-19] and it has been suggested that the ionic radius could influence this change [15]. It may be that these purely local effects can affect the binding sites for adrenodoxin and NADPH (ref. 8).

Irrespective however of the precise mechanism by which the radius of a monovalent ion exerts its (stimulatory or) inhibitory effect on mitochondrial steroid hydroxylations, the effect itself is of significance. The mitochondrial hydroxylations lead to the synthesis of the mineralocorticoid aldosterone, one of whose functions is the regulation of sodium and potassium balance in the body. The *in vivo* rate of aldosterone synthesis can be altered by factors such as "water-loading" or transuda-

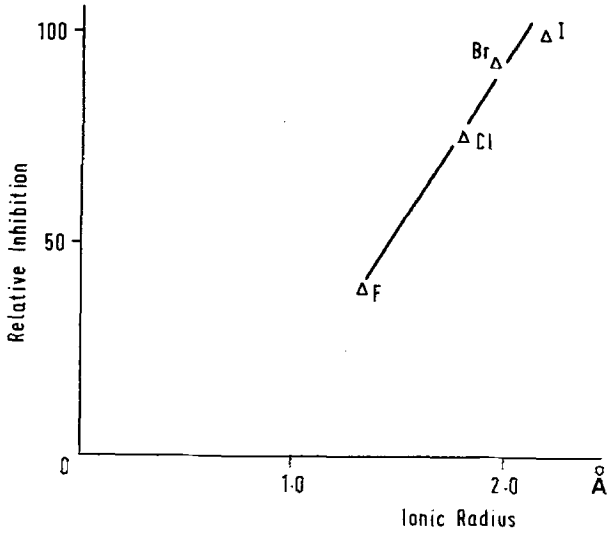

Figure 9. The relationship between the relative inhibitions of DOC 11β-hydroxylation due to salts of monovalent ions and the radius of the anions. For experimental details see text and ref. 8.

tion [20] which affect the composition of the blood plasma and consequently that of the cells of the highly vascularized adrenal cortex [21] or by sodium deprivation, potassium feeding or other measures to affect the Na^+/K^+ balance [20]. The effects of increased Na^+ or decreased K^+ have similar directional influence on aldosterone secretion which is however opposite to that evoked by decreased Na^+ or increased K^+ (see ref. 20). That the stimuli of aldosterone synthesis increase the potassium content of the adrenal cortex tissue *in vivo* [22] and influence steroid production by isolated adrenal cells [23] have been reported. It has also been demonstrated *in vivo* and *in vitro* that lithium and sodium have similar effects on aldosterone secretion which are however opposite to those observed with potassium and other larger monovalent cations [20, 24-28]. In view of the present results on the linear relationship between the effect on steroid hydroxylation and the radius of monovalent ions it is therefore suggested that within the adrenocortical mitochondrion the normal output of aldosterone is assured by an optimal level (or balance) of absolute and relative concentrations of monovalent inorganic ions of smaller radius such as sodium to those of larger radius such as potassium. Fluctuations to either side away from this "normal" or "optimal" level would affect mitochondrial corticosteroid hydroxylations by affecting the rate at which reducing equivalents are transferred from the flavoprotein to cytochrome P450 by adrenodoxin.

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