Journal of Steroid Biochemistry, 1975, Vol. 6, pp. 395–400. Pergamon Press. Printed in Great Britain

EFFECT OF CALCIUM ON PREGNENOLONE FORMATION AND CYTOCHROME P-450 IN RAT ADRENAL MITOCHONDRIA

E. R. SIMPSON and J. WATERS

Department of Physics Applied to Medicine, Middlesex Hospital Medical School, Cleveland Street, London, WIP 6DB

and

D. WILLIAMS-SMITH

Department of Physics, Guy's Hospital Medical School, London, England

SUMMARY

The effect of succinate-supported calcium uptake on pregnenolone formation by rat decapsulated and capsular adrenal mitochondria, and on 11β-hydroxylation of added DOC in decapsulated adrenal mitochondria, has been studied. In the absence of additional sources of reducing equivalents, calcium uptake inhibited both these reactions, due to competition with transhydrogenase for high-energy intermediates.

In the presence of succinate, isocitrate and $NADP^+$, calcium stimulated pregnenolone formation but did not affect 11β-hydroxylation of added DOC. Addition of valinomycin also produced a stimulation of pregnenolone formation, although to a lesser extent than calcium.

Both calcium and valinomycin when added after succinate produced a difference spectrum resembling a type I spectral change with cytochrome P-450.

These results have been interpreted to suggest that respiration-driven uptake of cations by adrenal mitochondria results in an increase in the proportion of cytochrome P-450 bound to endogenous substrate, which is consequently available for pregnenolone formation.

INTRODUCTION

The conversion of cholesterol to pregnenolone appears to be the rate-limiting step in adrenal steroidogenesis [1]. Several reports [2–5] have indicated that adrenal mitochondria from rats injected with ACTH or subjected to ether anaesthesia stress have an increased capacity to synthesize pregnenolone from endogenous mitochondrial cholesterol, when compared to controls.

In an attempt to understand the mechanism of this activation, the optical and electron paramagnetic resonance (E.P.R.) spectra of adrenal mitochondrial cytochrome P-450 have been examined. The study of steroid-induced difference spectra [6] and E.P.R. spectra [7] has suggested that adrenal mitochondria contain at least two distinct species of cytochrome P-450, one involved in cholesterol side-chain cleavage ($P\text{-}450_{scc}$) and one involved in 11β hydroxylation ($P\text{-}450_{11\beta}$) [8]. Proof of this concept has been obtained by separation and partial purification of these forms of cytochrome P-450 [9–11]. As yet a distinct species of cytochrome P-450 responsible for 18-hydroxylation remains to be identified. Spectral titrations of adrenal mitochondria prepared from rats subjected to ether anaesthesia stress [3] or ACTH injection [4] indicate an increase in the inverted type I difference spectrum produced by pregnenolone, when comparison is made with controls. Since this spectrum is believed to arise from displacement of endogenous cholesterol [12], these results have been interpreted to mean that the action of ACTH has resulted in an increase in the proportion of mitochondrial cytochrome P-450 bound to cholesterol. On the other hand, there is little change in the type I difference spectrum produced by adding deoxycorticosterone (DOC), which is believed to be the substrate-binding spectrum of cytochrome $P\text{-}450_{11\beta}$. Consistent with this, E.P.R. studies have shown an increase in the amount of high-spin ferric cytochrome P-450 in adrenal mitochondria from ACTH treated or ether stressed rats [4, 13].

Since ferric cytochrome P-450 undergoes a low- to high-spin state transition on binding substrate [14], these results, together with the observations on steroid binding spectra, have been taken to indicate that the acute action of ACTH results in an increased association of adrenal mitochondrial cytochrome $P\text{-}450_{scc}$ with cholesterol.

It has been previously suggested that the ability of adrenal mitochondrial cytochrome $P\text{-}450_{scc}$ to form an active enzyme-substrate complex with cholesterol is rate-limiting for pregnenolone formation [15, 3]. Consequently, these results provide an explanation for the enhanced ability to synthesize pregnenolone in adrenal mitochondria from ACTH treated rats.

Steroid biosynthesis in the zona glomerulosa is stimulated by a number of factors such as ACTH, angiotensin, high potassium concentration, and serotonin. In addition to activation of an early pathway, presumably pregnenolone formation, there appears to be in addition, a further activation at a late stage,

namely the last step in aldosterone biosynthesis [16]. As yet, it remains to be established whether the mechanism of activation of pregnenolone formation in this tissue is similar to that discussed above.

In an attempt to explore further the changes described above, the effect of calcium ions added in vitro to adrenal mitochondria was examined. Calcium can stimulate steroid hydroxylation reactions of adrenal cortex mitochondria *in vitro* [17, 2]. This stimulation when NADPH was used as source of reducing equivalents could be partially attributable to calcium-induced permeability of the mitochondria to pyridine nucleotides [18]. However, in a previous communication [19] we reported that millimolar concentrations of calcium could bring about an increase in the inverted type I difference spectrum induced by pregnenolone in rat adrenal mitochondria. More recently [20], E.P.R. studies have shown that these concentrations of calcium bring about an increase specifically in the amount of high-spin ferric cytochrome P-450_{scc} bound to cholesterol within the mitochondria and thus mimic to some extent the *in vivo* effect of ACTH.

The object of this present investigation was to extend these studies to much lower concentrations of calcium, in the micromolar range, and in particular to see if respiration-supported uptake of low concentrations of calcium could activate cholesterol side chain cleavage in mitochondria of rat decapsulated and capsular adrenals.

EXPERIMENTAL

Mitochondria were prepared from decapsulated adrenals and capsular strippings from 150–200 g female rats of the Sprague–Dawley strain. The contamination of the capsules with fasciculata cells was assessed to be routinely between 3–5% in terms of cell number (21). The tissues were homogenized with a Teflon–glass homogenizer in ice-cold 0·25 M sucrose containing 10 mM triethanolamine hydrochloride, 100 μM EDTA and 1% w/v BSA, pH 7·2. The ratio of homogenizing medium to tissue was 1 ml per rat equivalent in the case of decapsulated adrenals and 0·2 ml per rat equivalent in the case of capsular mitochondria.

The homogenates were centrifuged for 10 min at 600 ***g*** in an M.S.E. High speed 18 centrifuge at 4°C. The resulting supernatant was pipetted off and recentrifuged at 10,000 ***g*** for 10 min. After discarding the supernatant, the centrifuge tubes were wiped free of adhering fat and the pellet re-suspended in 0·25 M sucrose and re-centrifuged at 10,000 ***g*** for 10 min. The resulting washed mitochondrial pellet was re-suspended in 0·25 M sucrose to give a protein concentration of 5–8 mg/ml.

Mitochondrial aliquots were incubated in 0·25 M sucrose containing 20 mM KCl, 15 mM triethanolamine hydrochloride and 5 mM $MgCl_2$, pH 7·1 at 27°C in air. The volume of incubations was 1 ml in the case of decapsulate adrenal mitochondria, and 200 μl in the case of capsular mitochondria. The final mitochondrial concentration was around 1 mg/ml. After a 5 min pre-incubation in the presence of 3 μM 2α-cyano-4,4,17α-trimethyl-17β-hydroxy-5-androsten-3-one (cyanoketone), calcium chloride or valinomycin where indicated, pregnenolone formation was initiated by the addition of the source of reducing equivalents. Where added sodium succinate was 2·5 mM, D,L-sodium isocitrate was 5 mM, and $NADP^+$ was 125 μg/ml. After incubating for a further 10 min, 200 μl aliquots were removed for pregnenolone assay into 10 ml ice-cold water.

After addition of an aliquot of [7-H^3]-pregnenolone (18 mC/mmol; the Radiochemical Centre, Amersham) as recovery indicator, steroids were extracted with 2 × 30 ml. dichloromethane. This extract was washed twice with 3 ml water and evaporated to dryness. The residue was taken up in ethanol–water 1:4 (1 × 5 ml; 2 × 0·5 ml) and partitioned against 20 ml cyclohexane. Pregnenolone was found essentially entirely in the cyclohexane layer. This was taken to dryness and the residue taken up in a suitable volume of ethanol for radioimmune assay. The recovery of pregnenolone throughout this procedure was 85–90%.

Radioimmunoassay was performed using antisera raised to a BSA conjugate of pregnenolone prepared as follows: (7-H^3)-pregnenolone-20-(0-carboxymethyl) oxime was prepared according to the method of Erlanger, Borek, Beiser and Lieberman [22]. This was conjugated to BSA using isobutyl chlorocarbonate in tri-n-butylamine. The conjugate was purified by acetone and acid precipitation and lyophilised. On the basis of tritium incorporation, it was estimated that the conjugate contained 14 mole pregnenolone per mole.

Antisera to this conjugate were raised in sheep (kindly provided by the Central Veterinary Laboratory, Newhall, Weybridge, Surrey) by injection of a suspension in Freunds Incomplete Adjuvant at fortnightly intervals. The animals were bled prior to injection and aliquots of the serum were tested for pregnenolone binding. The method used for assay of pregnenolone was based on a published method for assay of corticosterone [23].

Samples were incubated at 30°C for 4 h with a 1:1000 dilution of antiserum in 0·1 M sodium phosphate buffer. Free and bound steroid were separated by adding 0·5 ml 0·0625% w/v charcoal mixture. After standing at −15°C for 10 min the samples were centrifuged and aliquots of the supernatant counted by liquid scintillation spectrometry. A standard curve was run with each experiment within the range 0·3125 to 2·5 ng pregnenolone and quantification of results was achieved using a computer program executed in BASIC [23].

Figure 1 shows a typical standard curve in which the ratio of free/bound steroid is plotted against amount of steroid added. The value of the y-axis intercept in this case was 1·2789, the gradient was 5·4353, and the R squared value 0·9903.

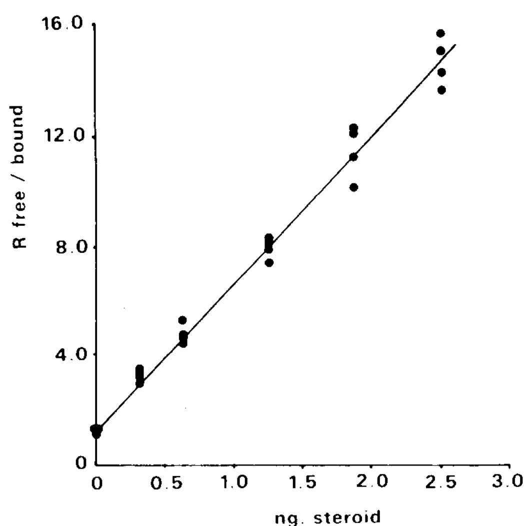

Fig. 1. Typical standard curve for the estimation of pregnenolone.

The cross-reactivity of the antiserum with other steroids is shown in Table 1. Of the steroids tested, only progesterone gave a significant cross reaction. However, this was not a problem in the studies described here, since cyanoketone, a potent inhibitor of 3β-ol dehydrogenase, was added to all the incubations to prevent conversion of pregnenolone to progesterone.

Assay of extracts of incubation buffer in the absence of mitochondria gave a blank value of less than 2% of the amount of pregnenolone detected in a standard assay. The response of the assay was linear over a wide range of concentration of the sample extracted from an incubation. Further purification of incubation extracts on a Sephadex LH20 column developed with chloroform:petroleum ether (60–80° boiling fraction):ethanol 200:200:1 (water saturated) gave values for the pregnenolone concentration which were not significantly different from the unchromatographed extracts.

Samples for determination of the reduced level of adrenodoxin were removed from the same incubations in which pregnenolone was determined three minutes after initiation of side-chain cleavage by means of a syringe and immediately placed into quartz E.P.R. tubes and frozen in liquid nitrogen. E.P.R. spectrometry was performed using a Varian E9 spectrometer equipped with a liquid nitrogen cryostat.

Table 1. Cross-reactivity of pregnenolone antiserum with other steroids

Steroid	Cross reaction (%)
Pregnenolone	100
Progesterone	24
Corticosterone	0.27
Aldosterone	0.29
Cholesterol	>0.001
Cyanoketone	0.15

Optical studies were performed using an Aminco DW-2 spectrophotometer operating either in scanning or dual-beam mode.

RESULTS

Figure 2 shows succinate-supported pregnenolone formation in the presence of different concentrations of calcium chloride in decapsulated and capsular mitochondria, and also 11β-hydroxylation of added deoxycorticosterone (DOC) by decapsulated adrenal

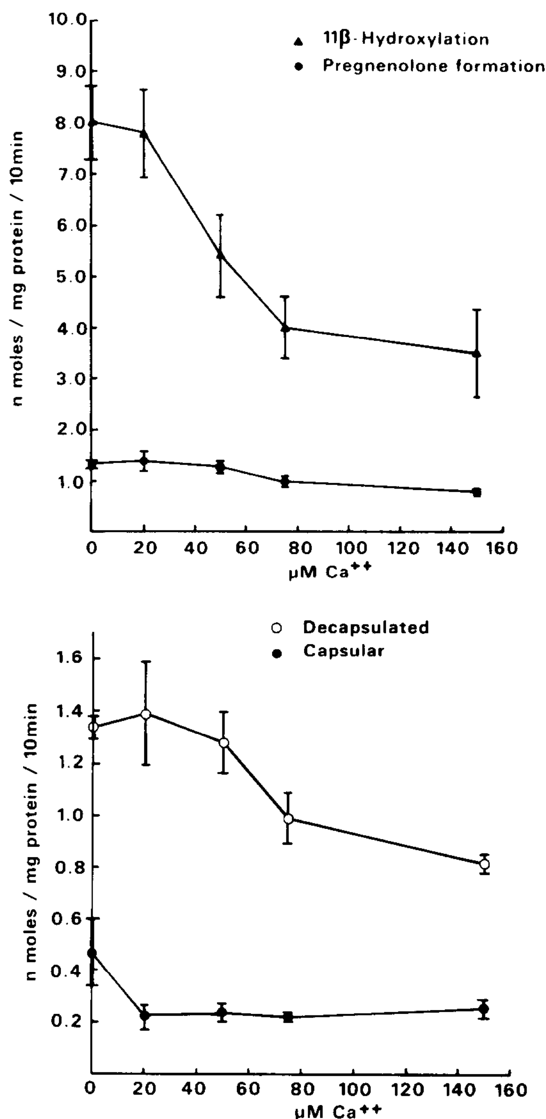

Fig. 2a. Succinate-supported pregnenolone formation (●) and 11β-hydroxylation of added DOC (▲) in rat decapsulated adrenal mitochondria in the presence of different concentrations of calcium. The results are expressed as mean ± S.E.M. for four determinations on two separate preparations of mitochondria.

Fig. 2b. Succinate-supported pregnenolone formation in rat decapsulated (○) and capsular (●) adrenal mitochondria in the presence of different concentrations of calcium. The results are expressed as mean ± S.E.M. for four determinations on two separate preparations of mitochondria.

Table 2. Steroid production and level of reduced adrenodoxin in decapsulated adrenal mitochondria

Additions	Pregnenolone Formation nmoles/mg. protein/10 min	Level of Reduced Adrenodoxin percentage of dithionite-reduced
none	0.06	9
succinate	1.83	92
succinate + Ca^{++}	0.48	32
succinate + valinomycin	0.49	27
succinate + FCCP	0.29	18
succinate + isocitrate + $NADP^+$	2.09	100

Conditions: succinate—2·5 mM; isocitrate—5 mM; $NADP^+$—125 μg/ml; Ca^{2+}—350 μM; valinomycin—0·25 μM; FCCP—4 μM.

mitochondria. Under these conditions, calcium inhibited both these reactions. This is to be expected because the supply of reducing equivalents for hydroxylation reactions by succinate in rat adrenal mitochondria is believed to be to a large extent mediated *via* energy-linked transhydrogenase and calcium uptake competes with this reaction for high energy intermediates. The validity of this interpretation was indicated by experiments in which the level of reduced adrenodoxin, a component of the electron transport system for mitochondrial hydroxylation reactions, was measured by E.P.R. spectrometry (Table 2). These experiments showed that the presence of calcium severely inhibited the reduction of adrenodoxin by succinate. Similar results were obtained with valinomycin and FCCP.

In order to prevent this occurring, incubations were conducted in the presence of isocitrate in addition to succinate. Isocitrate provides reducing equivalents for hydroxylation reactions *via* $NADP^+$-linked isocitrate dehydrogenase, and consequently reduction is not dependent on energy supply. Under these conditions addition of calcium resulted in a stimulation of pregnenolone formation.

Figure 3 shows pregnenolone formation in the presence of succinate, isocitrate and $NADP^+$ (added to counter possible leakage of pyridine nucleotides from the mitochondria at higher calcium concentrations) as a function of calcium concentration in the range 120–880 μM for mitochondria from capsular and

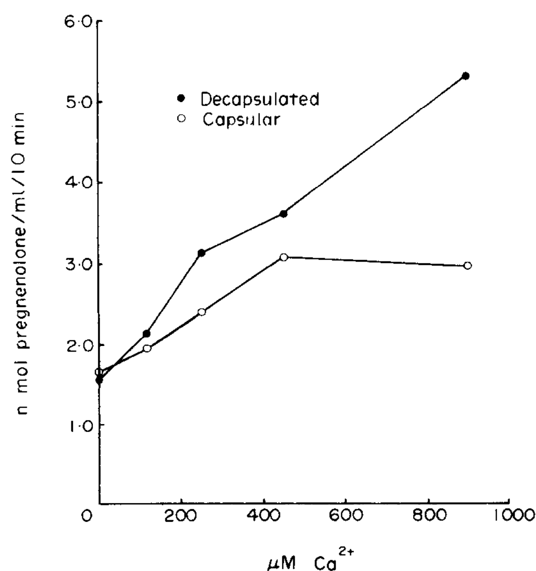

Fig. 3. Pregnenolone formation supported by succinate + isocitrate + $NADP^+$ in the presence of different concentrations of calcium in rat decapsulated (●) and capsular (○) adrenal mitochondria.

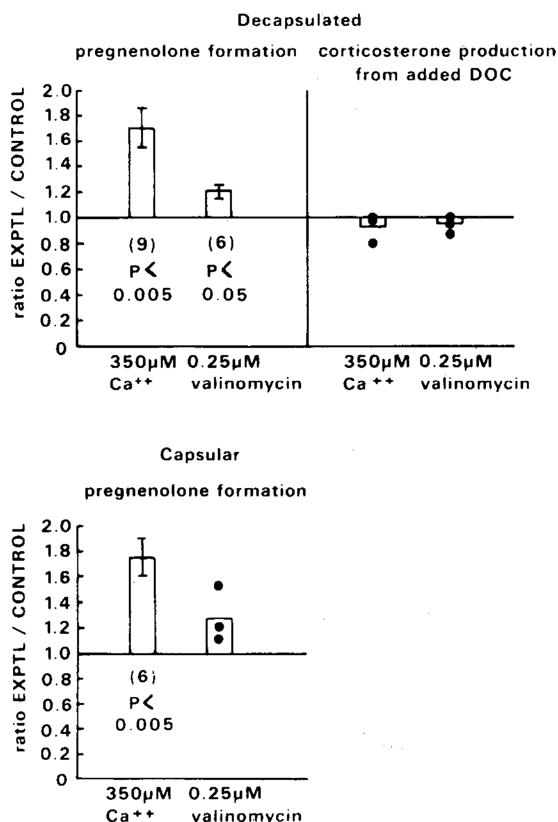

Fig. 4. Effect of calcium and valinomycin on pregnenolone formation and 11β-hydroxylation of DOC in rat decapsulated adrenal mitochondria, and pregnenolone formation in capsular mitochondria. The source of reducing equivalents was succinate + isocitrate + $NADP^+$. Results are expressed as mean $\pm$ S.E.M. for the ratio stimulated/control. The numbers in parenthesis are the numbers of observations.

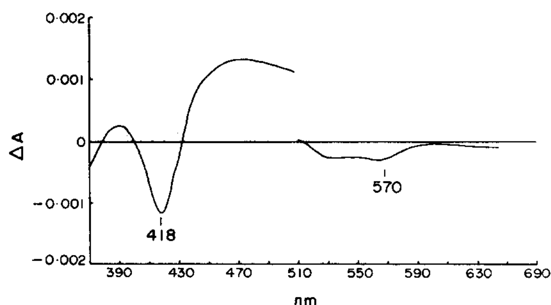

Fig. 5. Difference spectrum induced by calcium in the presence of succinate in rat decapsulated adrenal mitochondria. Succinate was added to both cuvettes to establish a baseline, and then 130 μM calcium chloride added to the sample cuvette to obtain the difference spectrum.

decapsulate adrenals. As can be seen, calcium was capable of stimulating pregnenolone formation 2–4 fold over this concentration range under these conditions.

Data for a number of experiments at a fixed concentration of calcium, namely 350 μM are shown in Fig. 4. The source of reducing equivalents was succinate and isocitrate and $NADP^+$. Also shown in Fig. 4 are experiments in which valinomycin was added. Mitochondria can exhibit energy-linked uptake of potassium ions in the presence of valinomycin, which functions as a potassium-specific channel through the inner mitochondrial membrane. As can be seen from the figure, valinomycin in the presence of 20 mM KCl was capable of eliciting a stimulation of pregnenolone formation, although to a lesser extent than calcium. In addition, the effect of calcium and valinomycin on 11β-hydroxylation of added DOC was also studied in decapsulate adrenal mitochondria. Under these conditions, neither calcium or valinomycin had any effect on 11β-hydroxylation indicating their stimulatory effect was specific for pregnenolone formation from endogenous mitochondrial cholesterol.

Figure 5 shows the difference spectrum induced by calcium in rat decapsulate adrenal mitochondria. Succinate was present in both cuvettes. Although somewhat distorted by the calcium-induced baseline shifts, the resulting spectrum closely resembles a type I difference spectrum, *i.e.*, a substrate-binding spectrum of cytochrome P-450, with a peak at 390 nm, a trough at 420 nm, and a second trough at 570 nm.

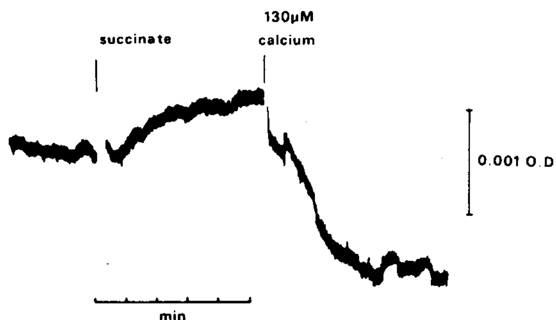

Fig. 6. Kinetics of the spectral change induced by succinate and calcium in rat decapsulated adrenal mitochondria, using the wavelength pair 572–585 nm.

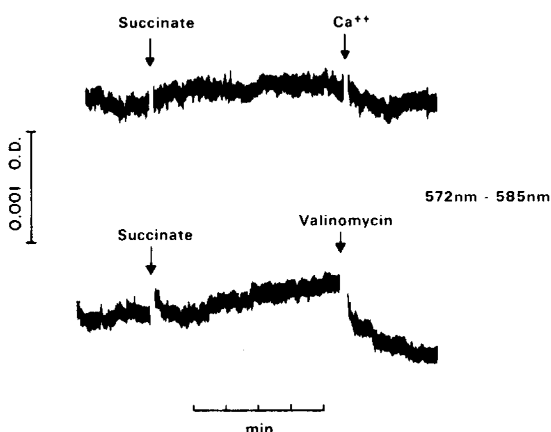

Fig. 7. Kinetics of the spectral change induced by succinate, calcium and valinomycin in rat capsular adrenal mitochondria, using the wavelength pair 572–585 nm.

The kinetics of this spectral change are shown in Figure 6 using the wavelength pair 572–585 nm. Addition of succinate resulted in an inverted type I difference spectrum due to the conversion of cholesterol to pregnenolone. Subsequent addition of calcium resulted in the formation of a type I difference spectrum which was frequently as in this case, greater than the succinate induced spectral change. Similar spectral changes have been observed with valinomycin. Figure 7 shows the spectral changes induced by succinate, calcium and valinomycin in capsular adrenal mitochondria, which respond in a similar fashion to decapsulated adrenal mitochondria.

Addition of succinate and valinomycin to rat liver mitochondria failed to produce any spectral change whatsoever between the wavelengths 572–585 nm, although characteristic spectral changes were observed between the wavelengths 562–585 nm, indicating reduction and oxidation of cytochrome b, as were similar spectral changes in adrenal mitochondria. Since rat liver mitochondria contain no detectable cytochrome P-450, this provides further evidence that the spectral changes observed in adrenal mitochondria do represent a type I difference spectrum of cytochrome P-450.

DISCUSSION

The results presented here indicate that the energy-linked uptake of calcium and potassium ions (in the presence of valinomycin) by decapsulated and capsular adrenal mitochondria results in an increased synthesis of pregnenolone from endogenous precursor. The spectral changes produced by these ions in the presence of succinate suggest an increase in the proportion of cytochrome P-450 bound to substrate within the mitochondria. Since the only endogenous substrate present in the mitochondria in significant amounts is cholesterol, then it is highly likely that these ions are causing an increased association of cytochrome P-450 with endogenous mitochondrial cholesterol. Such increased interaction is likely to be the basis of the enhanced pregnenolone formation

seen under these conditions, whereas 11β-hydroxylation is unaffected.

Final proof of this assertion awaits E.P.R. measurements of adrenal mitochondria incubated with calcium and valinomycin under the conditions used here. However, it has already been established [20] that high concentrations of calcium, namely 5 mM, bring about a large increase in the g = 8·1 signal in decapsulated adrenal mitochondria, indicating that high concentrations of calcium do cause an increase in the binding of cytochrome P-450_{scc} to endogenous substrate.

Thus, uptake of calcium by these mitochondria mimics rather closely the effects on mitochondria of ACTH administered *in vivo*, in terms of pregnenolone formation and spectral changes in cytochrome P-450. The fact that valinomycin-induced potassium uptake produces similar effects suggests the phenomenon is not specific for calcium. Energy-linked uptake of calcium and potassium have similar effects on mitochondria in two ways—both alter the electrochemical potential of the mitochondrial inner membrane, and both produce conformational changes in the mitochondria. Either of these processes could be the underlying mechanism of the stimulation of cholesterol side-chain cleavage since either might reasonably be expected to affect the interaction of a membrane bound enzyme with a membrane bound substrate.

Acknowledgements—The authors wish to thank Prof. J. F. Tait and Mrs. S. A. S. Tait for helpful discussions. This work was supported by Programme Grant No. G-969/204/C from the Medical Research Council.

REFERENCES

1. Stone D. and Hechter O.: *Archs Biochem. Biophys.* **51** (1954) 457–469.
2. Koritz S. B. and Kumar A. M.: *J. biol. Chem.* **245** (1970) 152–159.
3. Simpson E. R., Jefcoate C. R., Brownie A. C. and Boyd G. S.: *Eur. J. Biochem.* **28** (1972) 443–450.
4. Brownie A. C., Alfano J., Jefcoate C. R., Orme-Johnson W. H., Beinert H. and Simpson E. R.: *Ann. N.Y. Acad. Sci.* **212** (1973) 344–360.
5. Johnson L. R., Ruhmann-Weinhold A. and Nelson D. H.: *Ann. N.Y. Acad. Sci.* **212** (1973) 307–318.
6. Whysner J. A., Ramseyer J., Kazmi G. M. and Harding B. W.: *Biochem. biophys. Res. Commun.* **36** (1969) 795–801.
7. Jefcoate C. R., Simpson E. R., Boyd G. S., Brownie A. C. and Orme-Johnson W. H.: *Ann. N.Y. Acad. Sci.* **212** (1973) 243–261.
8. Jefcoate C. R., Simpson E. R. and Boyd G. S.: *Eur. J. Biochem.* **42** (1974) 539–551.
9. Jefcoate C. R., Hume R. and Boyd G. S.: *FEBS Lett.* **9**, (1970) 41–44.
10. Mitani F. and Horie S.: *J. Biochem. Tokyo* **65** (1969) 269.
11. Shikita M. and Hall P. F.: *J. biol. Chem.* **248** (1973) 5598–5604.
12. Cheng S. C. and Harding B. W.: *J. biol. Chem.* **248** (1973) 7263–7271.
13. Brownie A. C., Simpson E. R., Jefcoate C. R., Boyd G. S., Orme-Johnson W. H. and Beinert H.: *Biochem. biophys. Res. Commun.* **46** (1972) 483–490.
14. Tsai R. L., Yu C. A., Gunsalus I. C., Peisach J., Blumberg W., Orme-Johnson W. H. and Beinert H.: *Proc. natn. Acad. Sci. U.S.A.* **66** (1970) 1157–1163.
15. Simpson E. R., Jefcoate C. R. and Boyd G. S.: *FEBS Lett.* **15** (1971) 53–58.
16. Williams G. H., McDonnell L. M., Tait S. A. S. and Tait J. F.: *Endocrinology* **91** (1972) 948–960.
17. Peron F. G. and McCarthy J. L.: In *Functions of the Adrenal Cortex* (Edited by K. McKerns). Appleton-Century-Crofts, New York Vol. 1 (1968) p. 261.
18. Vinogradov A., Scarpa A. and Chance B.: *Arch Biochem. Biophys.* **152** (1972) 646–654.
19. Simpson E. R., Jefcoate C. R., McCarthy J. L. and Boyd G. S.: *Eur. J. Biochem.* **45** (1974) 181–188.
20. Simpson E. R. and Williams-Smith D.: unpublished observations.
21. Tait J. F., Tait S. A. S., Gould R. P. and Mee M. S. R.: *Proc. R. Soc. Lond. B.* **185** (1974) 375–407.
22. Erlanger B. F., Borek F., Beiser S. M. and Leiberman S.: *J. biol. Chem.* **234** (1959) 1090.
23. Tait S. A. S., Tait J. F. and Bradley J. E. S.: *Aust. J. exptl. Biol. Med. Sci.* **50** (1972) 833–846.