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Control of Sterol Metabolism in Rat Adrenal Mitochondria

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Steroidogenesis by adrenal mitochondria from endogenous precursors is stimulated by corticotropin (ACTH) and is sensitive to the protein-synthesis inhibitor cycloheximide. In the present investigation the effect of cycloheximide treatment on the metabolism of a number of analogues of the normal steroidogenic substrate, i.e. cholesterol, by rat adrenal mitochondria was studied. It was observed that the metabolism of analogues such as desmosterol, 26-norcholest-5-en-3β-ol and 5-cholen-3β-ol (that is with non-polar alkyl side chains like cholesterol), was sensitive to cycloheximide treatment. By contrast, the metabolism of those analogues with polar groupings on the side chain, i.e., 20α-, 24-, 25- and 26-hydroxycholesterols was insensitive to pretreatment with cycloheximide. The binding of added sterol to the cytochrome *P*-450 component of the mitochondrial sterol desmolase was studied. Similar studies on the equilibration time on addition of exogenous sterols to achieve maximum rates of pregnenolone production were also made. Both studies show that cholesterol, a non-polar sterol, penetrated slowly through the mitochondrial milieu to reach the cytochrome *P*-450 reaction centre whereas 24- and 26-hydroxycholesterols rapidly attained the enzymic environment. The cycloheximide-sensitive process in sterol metabolism appeared related to the transfer of non-polar sterols such as cholesterol within the mitochondria to a region in close proximity to the enzyme. The importance, and possible mechanism of action, of the cycloheximide-sensitive factor in the control of adrenal steroidogenesis is discussed.

Treatment of rats with cycloheximide blocks the stimulation by corticotropin of pregnenolone synthesis in adrenocortical mitochondria (reviewed by Schulster *et al.*, 1974; Simpson & Mason, 1976). Such studies suggested that cycloheximide inhibited the synthesis of a peptide or of a protein component involved in regulating availability of the substrate to the mitochondrial cholesterol desmolase rather than directly acting on the desmolase. It was attractive to suggest that the cycloheximide-sensitive factor was involved in the transport of extramitochondrial stores of cholesterol (e.g. hydrolysed lipid droplets) into the mitochondria to be available for steroidogenesis (Boyd & Trzeciak, 1973). However, it appears that the cycloheximide-sensitive factor must act, if it acts on transport at all, at the level of intramitochondrial cholesterol transport. This argument depended on the findings that cycloheximide did not prevent the mitochondrial accumulation of cholesterol brought about by corticotropin (Mahaffee *et al.*, 1974; Arthur *et al.*, 1976*a*) as well as e.p.r. evidence on the association of cholesterol with cytochrome *P*-450 (Williams-Smith *et al.*, 1976).

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Jefcoate *et al.* (1974) have implied that the cycloheximide-sensitive process was dependent on the nature of the sterol substrate, since the adrenal metabolism of 25-hydroxycholesterol was insensitive to cycloheximide. Adrenal mitochondrial desmolase can metabolize a wide variety of analogues of cholesterol at similar or even higher rates than the physiological substrate, cholesterol (Arthur *et al.*, 1976*b*). We have now used a number of derivatives of hydroxycholesterol as well as sterols with various lengths of alkyl side chain to elucidate the function of the so-called cycloheximide-sensitive factor on the metabolism of such cholesterol analogues in adrenal mitochondria.

Materials and Methods

Pregnenolone 20–albumin was prepared as described by Erlanger *et al.* (1959). [7α-^{3}H]Pregnenolone (17–20Ci/mol) was supplied by NEN Chemicals G.m.b.H., Dreieichenhain, West Germany. Aminoglutethimide phosphate was a gift from Ciba Laboratories, Horsham, Sussex, U.K. Dr. A. M. Neville, London, generously supplied 2α-cyano-4,4,17α-trimethyl-17β-hydroxyandrost-5-en-3-one (cyanoketone). Corticotropin (zinc hydroxide complex) was obtained from Organon, Morden, Surrey,

U.K. $NADP^+$ and bovine serum albumin (type F, essentially fatty acid-free) were the products of Sigma Chemical Co., St. Louis, MO, U.S.A. Desmosterol was obtained from Organon Laboratories, Newhouse, Strathclyde, U.K.; cholest-5-ene-3β,20α-diol (20α-hydroxycholesterol) was synthesized by the method of Petrow & Stuart-Webb (1956); cholest-5-ene-3β,24-diol (24-hydroxycholesterol), cholest-5-ene-3β,25-diol (25-hydroxycholesterol) and cholest-5-ene-3β,26-diol (26-hydroxycholesterol) were synthesized by Dr. S. A. M. Ali (Ali, 1968). 5-Cholen-3β-ol and 26-norcholest-5-en-3β-ol had been prepared as described previously (Arthur *et al.*, 1976*b*). All other chemicals were of analytical grade and were supplied by BDH Chemicals, Poole, Dorset, U.K. or Koch–Light Laboratories, Colnbrook, Bucks., U.K.

Pretreatment of animals

Female rats (150–200g) of the Wistar strain were obtained from the University of Edinburgh Small Animal Breeding Station, Roslin, Midlothian, U.K. Normally the rats, six per experimental group, received one of the following treatments: (*a*) diethyl ether anaesthesia for 10min to raise their blood concentrations of corticotropin, before killing by cervical dislocation, (*b*) intraperitoneal injection with 10mg of cycloheximide in 0.5ml of 0.9% (w/v) NaCl 10min before killing by cervical dislocation or (*c*) animals were kept as quiescent as possible before stunning and cervical dislocation. In one experiment, aminoglutethimide phosphate [15mg in 0.15ml of 0.9% (w/v) NaCl] was injected subcutaneously, followed 45min later by a subcutaneous injection of corticotropin (10 units; a unit is 10μg) and followed in certain experiments a further 20min later by an intraperitoneal injection of cycloheximide [10mg in 0.5ml of 0.9% (w/v) NaCl]. After a further 10min, these animals were killed by cervical dislocation.

Preparation of adrenal mitochondria

Adrenal glands were removed rapidly from the animals and trimmed free of fat. The glands were homogenized in 0.25M-sucrose (usually 12 glands in 10ml of medium) with a Teflon/glass homogenizer. After sedimentation of the nuclei, cell debris etc., at 600$g_{av.}$ for 10min, the mitochondrial fraction was then sedimented at 8500$g_{av.}$ for 15min. The mitochondrial fraction was washed by resuspension in 15ml of 0.25M-sucrose and resedimented as above. The final pellet was taken up in 0.25M-sucrose to give a protein concentration of about 5mg/ml. These manipulations were conducted at 4°C. Protein concentrations were determined by the method of Lowry *et al.* (1951), with bovine serum albumin (type F) as standard.

Mitochondrial sterol side-chain cleavage activity

This was assayed in a volume of 1ml at 37°C in a medium consisting of 250mM-sucrose, 20mM-KCl, 15mM-triethanolamine hydrochloride, 10mM-potassium phosphate, 5mM-$MgCl_2$, 0.2mM-$NADP^+$, 0.1mM-EDTA and 0.1% (w/v) bovine serum albumin, pH7.4, at a mitochondrial protein concentration of about 1mg/ml. Cyanoketone (2α-cyano-4,4,17α-trimethyl-17β-hydroxyandrost-5-en-3-one), 6μM, was included in incubations to prevent further metabolism of pregnenolone. The sterol side-chain cleavage reaction was initiated with 10mM-DL-isocitrate, after the mitochondria were incubated at 37°C for various times with sterol (final concentration, 100μM), normally added in 10μl of ethanol. Portions (0.2ml) of the reaction mixture were transferred into 4ml of methanol to stop the reaction at 2, 5 and 10min intervals after the addition of isocitrate (zero time). Chloroform (5ml) and water (2ml) were added to the extract and the organic layer was assayed for pregnenolone by using a radioimmunoassay technique. This was performed as described by Abraham *et al.* (1973), except that antisera had been raised in New Zealand White rabbits to pregnenolone 20–albumin conjugate (Dighe & Hunter, 1974). The coefficient of variation of this assay in the range used was ±11%. The [7α-^{3}H]pregnenolone was used also to monitor recovery of pregnenolone through the extraction procedure.

Spectral studies

These were carried out in an Aminco–Chance split-beam spectrophotometer fitted with a temperature-regulated cell-holder.

Results and Discussion

Pregnenolone formation from endogenous precursors in adrenal mitochondria obtained from stressed, cycloheximide-treated and quiescent rats

In Fig. 1, the formation of pregnenolone from endogenous precursors was compared in adrenal mitochondria from three experimental groups of rats, (*a*) stressed, (*b*) cycloheximide-treated and (*c*) quiescent animals. The derivation of these three groups is described in the Materials and Methods section. Pregnenolone synthesis was measured in the mitochondrial preparations as described in the Materials and Methods section, except that no additional cholesterol was added to the assay system. The results shown are similar to those obtained by Simpson *et al.* (1972). Adrenal mitochondria from stressed animals produced more pregnenolone than those from the cycloheximide-treated or quiescent groups. The adrenal mitochondria from cycloheximide-treated animals were slightly less active

than those from quiescent animals when pregnenolone synthesis was monitored. However, this latter result depended much on the quiescence of this control group. The difficulty of obtaining groups of strictly quiescent animals regularly was such that as a routine we compared two experimental groups of animals, (*a*) stressed and (*b*) those pretreated with cycloheximide as described in the Materials and Methods section. A conclusion from this experiment was that the pool of available steroidogenic cholesterol was greater in the adrenal mitochondria from stressed animals than those of the quiescent or cycloheximide-treated groups, even though the adrenal mitochondria from the cycloheximide-treated animals contained more non-esterified cholesterol than those from the stressed group (Mahaffee *et al.*, 1974; Arthur *et al.*, 1976*a*). Jefcoate *et al.* (1974) showed that disruption of the integrity of the mitochondria from cycloheximide-treated animals resulted in a release of the suppressed rate of steroidogenesis. This implies an increased

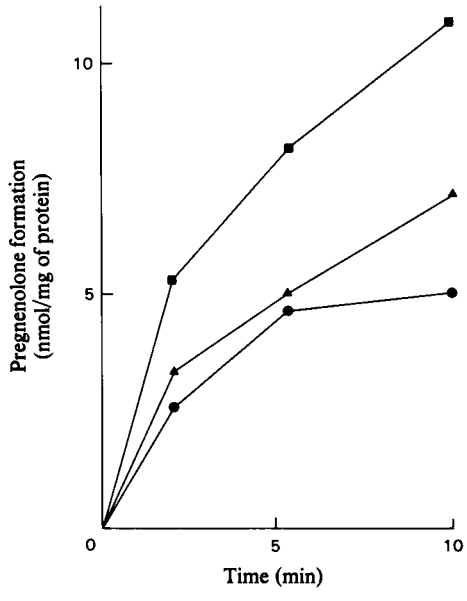

Fig. 1. *Pregnenolone formation from endogenous precursors in adrenal mitochondria obtained from diethyl ether-stressed, quiescent or cycloheximide-treated rats*

Adrenal mitochondria were prepared from rats that had been subjected to a diethyl ether stress (■), cycloheximide therapy (●) or quiescence (▲) as described in the Materials and Methods section. Cholesterol desmolase assays utilizing endogenous substrate precursors were performed at 37°C as described in the text. Each point represents the mean of triplicate determinations on two independent preparations.

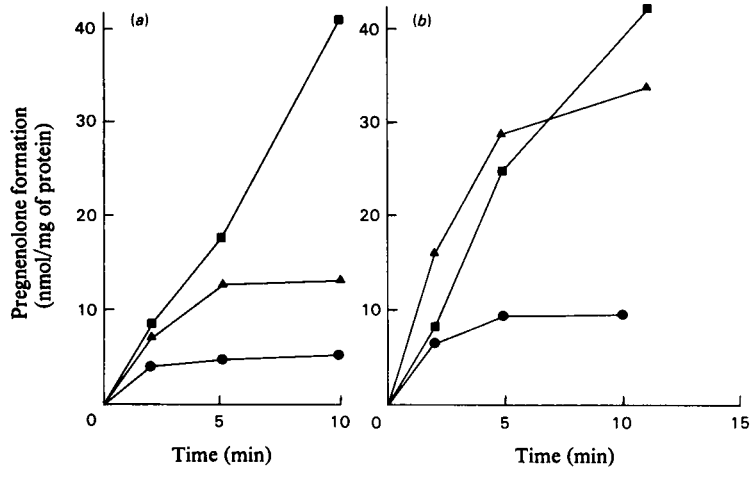

Fig. 2. *Pregnenolone formation on addition of exogenous sterol to adrenal mitochondria obtained from rats subjected either to diethyl ether stress or to cycloheximide treatment*

Adrenal mitochondria were prepared from adrenals of rats given either (*a*) 10mg of cycloheximide or (*b*) ether anaesthesia 10min before killing as described in the Materials and Methods section. Pregnenolone formation was monitored in such mitochondria on addition of 10mM-isocitrate after equilibration of the mitochondria with either 100μM-cholesterol (▲) or 100μM-26-hydroxycholesterol (■) for 15min at 37°C, or in the absence of exogenous sterol (●). The assays were performed as described in the text. Each point represents the mean of triplicate determinations on two independent preparations.

pool size of steroidogenic cholesterol, produced by the disruption rather than by a direct effect of the cycloheximide-sensitive factor on the desmolase. Such observations initiated our present investigations into the fate of exogenous sterol, whether it be cholesterol or some alternative substrate, in adrenal mitochondria from stressed and cycloheximide-treated animals.

Pregnenolone formation in adrenal mitochondria obtained from stressed or cycloheximide-treated rats in the presence of added cholesterol or 26-hydroxycholesterol

In Fig. 2 pregnenolone formation was monitored in adrenal mitochondria when either cholesterol or 26-hydroxycholesterol was used as the extramitochondrial source of sterol. Mitochondria were prepared from adrenals of animals either diethyl ether-stressed or treated with cycloheximide as described in the Materials and Methods section. The sterols, at a final concentration of 100 μM, were added in a small volume of ethanol (10 μl) and equilibrated with the mitochondria for 15 min before addition of the source of reducing equivalents, isocitrate (designated zero time). 26-Hydroxycholesterol was efficiently converted into pregnenolone in both groups of mitochondria, whereas cholesterol was metabolized much less efficiently in the mitochondria from the cycloheximide-treated animals. Thus the cycloheximide-sensitive factor would appear to regulate the fate of exogenous cholesterol, but not of the 26-hydroxycholesterol. Since sterols are rapidly taken up by adrenal mitochondria (Boyd *et al.*, 1975), it would appear likely that the cholesterol was taken up into at least two distinct compartments with one of these readily accessible to the enzyme, whereas the hydroxysterol entered a single pool which was accessible to the enzyme. The cycloheximide-sensitive factor appeared to control the rate or the extent of cholesterol translocation between its compartments.

Sterol binding to adrenal mitochondrial cytochrome P-450

Mason *et al.* (1978) concluded that the intramitochondrial translocation of cholesterol to the steroidogenic sites was slow and was probably a rate-limiting process. This conclusion was based both on optical difference spectroscopic observations as well as on equilibration studies of adrenal mitochondria with exogenous cholesterol. In Fig. 3 the binding of cholesterol and of 24-hydroxycholesterol to cytochrome *P*-450, the terminal oxidase and substrate-binding site of the sterol desmolase, was studied. In this experiment, either cholesterol or 24-hydroxycholesterol was added to rat adrenal mitochondria in 30 μl of ethanol to a final concentration of 100 μM, an equal amount of solvent being added to the reference cuvette. The temperature was maintained at 37°C. The optical difference spectrum in the 350–500 nm region was recorded at various times after addition of the sterols. A so-called 'type I' spectrum, typical of the interaction of cytochrome *P*-450 and substrate (Schenkman *et al.*, 1967), was obtained with a maximum at 385 nm and a minimum at about 422 nm. Maximum binding of the hydroxysterol occurred within 6 min and maximum cholesterol interaction was not observed until at least 1 h after the addition of cholesterol to the adrenal mitochondria. Thus the exogenous 24-hydroxycholesterol equilibrated rapidly with the desmolase, whereas the exogenous cholesterol entered the steroidogenic pool slowly.

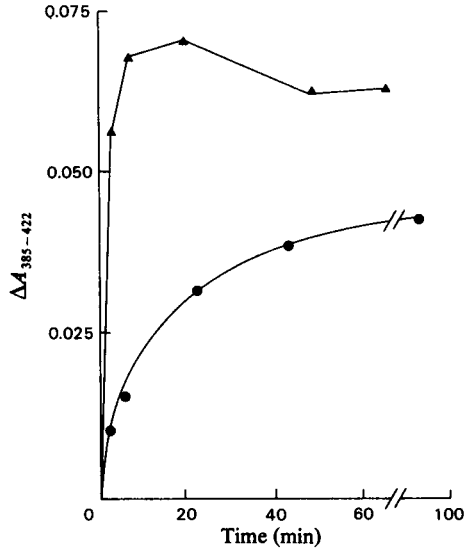

Fig. 3. *Time-dependence of sterol binding to rat cytochrome P-450 in adrenal mitochondria*

Adrenal mitochondria from diethyl ether-stressed rats were suspended in 3 ml of standard buffer medium at a concentration of 0.8 mg of protein/ml in both sample and reference optical cells. A portion of either cholesterol (●) or 24-hydroxycholesterol (▲) (in 30 μl of ethanol; final concentration 100 μM) was added to the sample cell and an equal volume of ethanol was added to the reference cell. The optical difference spectra over the range 360–500 nm were monitored at various times after the addition of sterol. The temperature was maintained at 37°C. The results are the mean of three experiments.

Effect of equilibration time with exogenous sterol on sterol side-chain-cleavage activity of adrenal mitochondria

The temporal difference in the ability of hydroxysterols and cholesterol to interact with the sterol

desmolase was also seen when the rate of conversion of sterol into pregnenolone was determined after various equilibration periods of the sterol with rat adrenal mitochondria. Mitochondria obtained from the adrenals of diethyl ether-stressed rats were incubated at 37°C with either 100 μM-26-hydroxycholesterol or 100 μM-cholesterol for various periods before the initiation of sterol metabolism by the addition of the electron donor isocitrate. Pregnenolone formation was monitored over a further 2 min assay period. The results (Fig. 4) showed that maximum rates of pregnenolone formation from 26-hydroxycholesterol were obtained after the shortest equilibration period (3 min), whereas with cholesterol as substrate such rates required up to 30 min equilibration. This experiment confirmed that the more polar hydroxysterol was directly taken up into a steroidogenic sterol pool, whereas cholesterol had to migrate in a rate-limiting process from a non-steroidogenic into a steroidogenic environment. When mitochondria from the adrenals of rats treated with cycloheximide were used in a similar study, although the final rates of pregnenolone formation from cholesterol were lower, a similar period of equilibration was needed to give maximal rates. Thus the cycloheximide-sensitive process appeared not to be concerned directly with the rate of intramitochondrial cholesterol translocation but with the size of the steroidogenic pool of cholesterol.

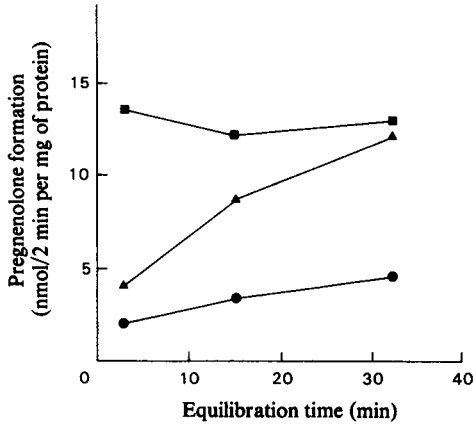

Fig. 4. *Effect of equilibration time with exogenous sterol desmolase activity in adrenal mitochondria*

Either 100 μM-cholesterol (▲) or 100 μM-26-hydroxycholesterol (■) was equilibrated with rat adrenal mitochondria obtained from diethyl ether-stressed rats for various times before addition of 10 mM-isocitrate to initiate pregnenolone production. This was determined as described in the Materials and Methods section. The experiment was repeated with 100 μM-cholesterol added to mitochondria from adrenals of rats treated with 10 mg of cycloheximide before killing (●). Each point represents the mean of triplicate determinations on two independent preparations.

Sterol side-chain cleavage activities in adrenal mitochondria obtained from ether-stressed and cycloheximide-treated rats

Arthur *et al.* (1976*b*) reported that the adrenal mitochondrial desmolase showed a broad specificity with regard to the nature of the side chain of the sterol nucleus, since the enzyme could cleave a variety of polar and non-polar side chains. Since adrenal mitochondrial metabolism of 26-hydroxycholesterol (see Fig. 2) and of 25-hydroxycholesterol (Jefcoate *et al.*, 1974) was insensitive to cycloheximide, the metabolism of a wider range of cholesterol analogues in mitochondria from adrenals

Table 1. *Rate of pregnenolone formation from various sterols in adrenal mitochondria obtained from rats either ether-stressed or treated with cycloheximide*

Adrenal mitochondria either from ether-stressed or from cycloheximide-treated rats (see the Materials and Methods section) were equilibrated with 100 μM-sterol for 15 min at 37°C. The rate of pregnenolone formation over the initial 2 min of the reaction after addition of 10 mM-isocitrate was determined. Triplicate determinations were carried out on duplicate samples from two separate experiments. The results are expressed as the means ± S.E.M. and the significance is given by using a paired Student's *t* test. Abbreviation: N.S., not significant.

Added sterol	Pregnenolone formed (nmol/min per mg of protein)		
	Ether-stressed	Cycloheximide-treated	
Endogenous	2.1 ± 0.3	0.4 ± 0.1	$P < 0.005$
Cholesterol	3.9 ± 0.3	2.1 ± 0.3	$P < 0.005$
26-Norcholest-5-en-3β-ol	5.5 ± 0.4	2.5 ± 0.2	$P < 0.005$
Desmosterol	6.3 ± 0.3	3.4 ± 0.3	$P < 0.005$
5-Cholen-3β-ol	4.0 ± 0.3	1.9 ± 0.2	$P < 0.005$
20α-Hydroxycholesterol	6.1 ± 0.2	5.6 ± 0.5	N.S.
24-Hydroxycholesterol	11.7 ± 0.3	11.7 ± 0.4	N.S.
25-Hydroxycholesterol	5.6 ± 0.2	5.5 ± 0.4	N.S.
26-Hydroxycholesterol	7.7 ± 0.4	7.6 ± 0.6	N.S.

of rats either stressed or treated with cycloheximide *in vivo* was investigated. The sterols listed in Table 1 were added separately, at a final concentration of 100 μM, to adrenal mitochondria obtained from either ether-stressed or cycloheximide-treated rats. After equilibration at 37°C for 15min, the rate of pregnenolone formation was determined over a 2min assay period after the addition of isocitrate. The metabolism of 26-norcholest-5-en-3β-ol, desmosterol and 5-cholen-3β-ol, as well as of cholesterol (both exogenous and endogenous), was sensitive to the inhibitor, whereas that of the 20α, 24-, 25- and 26-hydroxycholesterols was not. It was concluded that sterols with non-polar side chains occur in at least two pools within the adrenal mitochondria, one of these being readily accessible to the desmolase and the size of which could be regulated by the cycloheximide-sensitive factor. On the other hand, those sterols with polar (hydroxy) side chains penetrate directly to a sterol pool that is in rapid equilibration with the enzyme. Neither the rate of uptake nor the size of this latter pool appeared to be affected by a cycloheximide-sensitive process. It would seem likely that cholesterol was partly excluded from this steroidogenic polar sterol pool, a feature in agreement with the spectral studies on the different sterol-binding sites in rat adrenal mitochondria (Jefcoate, 1975). However, since exogenous cholesterol metabolism was never completely abolished in adrenal mitochondria from cycloheximide-treated animals, perhaps there was partial penetration of the 'polar' sterol pool by cholesterol.

Effect of cycloheximide treatment on cholesterol side-chain cleavage activity of adrenal mitochondria from aminoglutethimide-treated rats

The complexity of the cycloheximide-sensitive process can be illustrated by the studies on adrenal mitochondria obtained from animals treated *in vivo* with aminoglutethimide, a specific inhibitor of cholesterol desmolase, before stress or cycloheximide treatment. As shown by Mahaffee *et al.* (1974), such adrenal mitochondria accumulate large amounts of non-esterified cholesterol during the blockade *in vivo*. Preparation of adrenal mitochondria from aminoglutethimide-treated rats resulted in removal of aminoglutethimide from the mitochondria and subsequently high desmolase activities were observed. Mason *et al.* (1978) have demonstrated that the addition of exogenous cholesterol to such adrenal mitochondria resulted in no further stimulation of pregnenolone formation. To explain the high rates of pregnenolone production observed, it was suggested that the cholesterol accumulated in a compartment that was readily accessible to the mitochondrial desmolase. The results (Fig. 5) illustrate the rate of pregnenolone formation in adrenal mitochondria from rats that, subsequent to aminoglutethimide and corticotropin treatment, received an injection of cycloheximide 10min before killing. These mitochondria produced much lower amounts of pregnenolone than did mitochondria from animals that had solely the aminoglutethimide block, where the accumulated cholesterol could be rapidly metabolized on removal of aminoglutethimide.

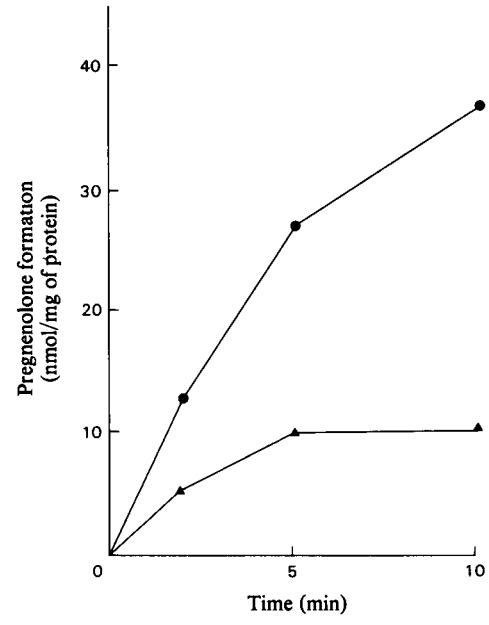

Fig. 5. *Effect of cycloheximide treatment on pregnenolone formation in adrenal mitochondria from rats given an aminoglutethimide blockade*

Pregnenolone production was monitored as described in the Materials and Methods section in adrenal mitochondria obtained from rats given either corticotropin (10 units) and aminoglutethimide phosphate (15mg) therapy (●) or corticotropin and aminoglutethimide phosphate plus cycloheximide (10mg) treatment (▲) before killing. The detailed treatment regimens are given in the text. Each point represents the mean of triplicate determinations on two independent preparations.

Although the results in Fig. 5 do not rule out a direct effect of a cycloheximide-sensitive factor on the enzyme, they would appear also not to favour this factor or process being concerned with a cholesterol-transporting protein acting intramitochondrially (Simpson *et al.*, 1972; Paul *et al.*, 1976; Williams-Smith *et al.*, 1976). It is proposed that the cycloheximide-sensitive factor regulates the size of the steroidogenic cholesterol compartment. Mason *et al.* (1978) have proposed that Ca^{2+} ions and polylysine

stimulate steroidogenesis by promoting the displacement of cholesterol in the mitochondrial membrane produced by phase separation of some form, the cholesterol being extruded into a steroidogenic compartment. The cycloheximide-sensitive factor might, therefore, act in a similar manner to polylysine, i.e. performing as an extrinsic protein on the mitochondrial membrane structure, and producing changes in the bulk membrane properties, especially in the distribution of lipid. The cycloheximide-sensitive factor, as well as Ca^{2+} and polylysine (J. I. Mason, J. R. Arthur & G. S. Boyd, unpublished work), had no effect on the adrenal mitochondrial metabolism of the hydroxysterols. This is consistent with the idea that these polar sterols presumably enter a separate compartment to cholesterol with easy access to side-chain cleavage cytochrome *P*-450. The region of the membrane accommodating the polar sterols would be distinct and not subject to displacement forces produced by such extrinsic proteins. Further study on insertion of sterols into mitochondrial membranes and the role of extrinsic proteins on the membrane character would seem useful in the study of sterol metabolic regulation.

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