DIFFERENCES BETWEEN IN-VITRO AND IN-VIVO POTENCIES OF CORTICOTROPHINS: AN INTERPRETATION IN TERMS OF METABOLIC STABILITY

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SUMMARY

Relative activities of a series of corticotrophin analogues have been measured by means of five different bioassays using the rat.

Similarities in the relative potencies of various ACTH analogues determined using lipolysis or steroidogenesis *in vivo* and for the lipolytic and steroidogenic responses of fat pads and adrenal slices *in vitro* emerged and support the concept of a close structural relationship between the ACTH receptors in adipose and adrenal tissues in the rat.

Potencies based on the steroidogenic response of isolated adrenal cells, adrenal slices or in-vivo experiments differed markedly from each other. Inactivation of peptides did not occur in the isolated cell assay, so it is likely that this assay estimates potency at the receptor level. A number of arguments suggest that the difference between the isolated cell assay and the other steroidogenic assays lies solely in the effects of peptide inactivation in the latter, and this allows the relative metabolic stabilities for the peptide analogues in these assays to be calculated. In this way it can be shown that:

(1) Replacement of L-Ser by D-Ser in amino acid position 1 markedly increases the metabolic stability of the peptide and has only a slight effect on receptor properties.

(2) Shortening at the NH_2-terminus reduces the activity of peptides at the receptor level by several orders of magnitude, but increases their relative metabolic stability.

(3) Introduction of amide groups at the CO_2H-terminus markedly increases receptor potency of (1–16), (1–17) and (1–18) ACTH without affecting their metabolic stability *in vivo*. However, amidation of the CO_2H-terminus does have a large effect on metabolic stability in the adrenal slice assay.

(4) Replacement of Arg by Lys in positions 17 and 18 of (1–18) ACTH increases potency at the receptor level (adrenal cells) but has little effect on metabolic stability.

The comparison of potencies obtained in the various assays, therefore, throws light on the significance of each assay. In addition, the effects of structural modification of analogues can be separately evaluated with respect to the metabolic stability of a peptide and its potency at the receptor level.

INTRODUCTION

The structure–activity relationships of adrenocorticotrophic hormone (ACTH) have been extensively discussed (e.g. Lebovitz & Engel, 1964; Ramachandran, Chung & Li, 1965; Tanaka, 1971; Schwyzer, 1972; Hofmann, 1974). As pointed out by Hofmann (1974) direct comparison of the biological activities of different ACTH analogues can be questionable, since several assay systems have been used for this purpose: ascorbic acid depletion (Sayers, Sayers & Woodbury, 1948), the steroidogenic response of adrenals *in vivo* (Lipscomb & Nelson, 1962; Barthe & Desaulles, 1971), in-vitro assays based on the use of whole adrenal tissue (Saffran & Schally, 1955; Daly, Loveridge, Bitensky & Chayen, 1972), isolated adrenal cells (Sayers, Swallow & Giordano, 1971; Lowry, McMartin & Peters, 1973) or isolated adipocytes (Rodbell, 1964). Widely discrepant activities have been reported for certain ACTH-analogues with different test systems. These discrepancies probably arise because the potency of a peptide in a given assay does not simply depend on its ability to stimulate the target tissue. Processes of transport and inactivation which determine the amount of active peptide reaching the tissue will also have a critical influence on potency and these processes must differ appreciably from one assay to another.

In the course of a study of the inactivation of ACTH (Bennett, Bullock, Lowry, McMartin & Peters, 1974), it was found that the test system using isolated adrenal cells could be improved so that factors leading to degradation of the peptide during the performance of the test were practically eliminated. It was shown that under these conditions the isolated adrenal cell system could be used to determine the biological activity of ACTH peptides at the cellular or receptor level.

In the present study, potencies of different ACTH analogues obtained in the adrenal cell system were compared with potencies obtained in four other in-vivo and in-vitro systems. Synthetic peptides representing significant changes at the NH_2 and CO_2H-termini of the ACTH sequence ranging from (1–13) to (1–24) were chosen.

The results obtained were then used to compare the assays, and to show how each assay can be interpreted in terms of two properties of the peptide, namely its potency at the receptor and its resistance to inactivation.

MATERIALS AND METHODS

The peptides used in this study are listed below. The shortened designation shown in brackets after each peptide is used for reference in the text.

The sequence of corticotrophin-(1–24)-tetracosapeptide is Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro.

1. Corticotrophin-(1–24)-tetracosapeptide [(1–24)-OH].
2. Corticotrophin-(1–24)-tetracosapeptide amide [(1–24)-NH_2].
3. Corticotrophin-(1–18)-octadecapeptide [(1–18)-OH].
4. Corticotrophin-(1–18)-octadecapeptide amide [(1–18)-NH_2].
5. Corticotrophin-(1–17)-heptadecapeptide [(1–17)-OH].
6. Corticotrophin-(1–17)-heptadecapeptide amide [(1–17)-NH_2].
7. Corticotrophin-(1–16)-hexadecapeptide [(1–16)-OH].
8. Corticotrophin-(1–16)-hexadecapeptide amide [(1–16)-NH_2].
9. Corticotrophin-(1–13)-tridecapeptide amide [(1–13)-NH_2].
10. [D-Ser^1]-corticotrophin-(1–24)-tetracosapeptide [[D-Ser^1]-(1–24)-OH].
11. [D-Ser^1]-corticotrophin-(1–18)-octadecapeptide amide [[D-Ser^1]-(1–18)-NH_2].
12. [D-Ser^1,Lys^{17,18}]-corticotrophin-(1–18)-octadecapeptide amide [[D-Ser^1,Lys^{17,18}]-(1–18)-NH_2].

13. [$Lys^{17,18}$]-corticotrophin-(1–18)-octadecapeptide amide [[$Lys^{17,18}$]-(1–18)-NH_2].
14. [$Lys^{17,18}$]-corticotrophin-(3–18)-hexadecapeptide amide [[$Lys^{17,18}$]-(3–18)-NH_2].
15. [$Lys^{17,18}$]-corticotrophin-(4–18)-pentadecapeptide amide [[$Lys^{17,18}$]-(4–18)-NH_2].
16. [$Lys^{17,18}$]-corticotrophin-(5–18)-tetradecapeptide amide [[$Lys^{17,18}$]-(5–18)-NH_2].

The peptides (1–24)-OH (Synacthen) and [D-Ser^1,$Lys^{17,18}$]-(1–18)-NH_2 were synthesized by the fragment condensation method as described by Kappeler & Schwyzer (1961) and Riniker & Rittel (1970) respectively. The other corticotrophins used in this investigation were synthesized by similar conventional procedures.

The following ACTH fragments have been described previously: (1–13)-NH_2 (Hofmann & Yajima, 1961); (1–16)-NH_2 (Bajusz & Medzihradszky, 1967); (1–16)-OH (Hofmann, Yanaihara, Lande & Yajima, 1962); (1–17)-OH (Li, Chung, Ramachandran & Gorup, 1962); (1–17)-NH_2 and (1–18)-NH_2 (Ramachandran *et al.* 1965); (1–18)-OH (Otsuka, Inouye, Shinozaki & Kanamaya, 1965).

Steroidogenic assay in vivo

Male rats were hypophysectomized and 24 h later injected intravenously with a range of doses of each peptide. Groups of six rats were killed 0·5, 1, 1·5, 2, 4 or 8 h after injection and plasma corticosteroids measured (Barthe & Desaulles, 1971). Plasma corticosteroid concentration was plotted against time over the 8 h period and an integrated response was assessed by measuring the area under this curve. The potency ratio of analogues was estimated as the ratio of doses producing integrated responses of similar magnitudes.

Assay using rat adrenal slices in vitro

Adrenal slices were incubated with suitable concentrations of ACTH analogues for 2 h and the steroid content of the medium was measured (Saffran & Schally, 1955). Potencies were calculated from a four-point assay.

Assay using isolated rat adrenal cells

Eight twofold dilutions of suitable starting concentrations of each peptide were incubated in duplicate with portions of a homogeneous cell suspension for 2 h at 37 °C and corticosterone was extracted and measured fluorometrically (Lowry *et al.* 1973). A standard dose–response curve was also prepared each time using (1–24)-OH. The potency of the unknown peptide was then calculated by statistical analysis using from 4 to 6 points from the linear part of each dose–response curve (Bliss, 1952).

Lipolytic assay in vivo

Plasma free fatty acid levels were determined colorimetrically (Duncombe, 1963) 30 min after i.v. administration of a range of doses of peptide to rats.

Lipolytic assay in vitro

Suitable peptide concentrations were incubated with rat epididymal fat pads as described by Jungas & Ball (1963) but with the addition of 4% bovine albumin to the incubation medium. After a 2 h incubation the glycerol content in the medium was estimated (Wieland, 1957).

Calculation of relative metabolic stability

Comparison of the results obtained in some of the assays has been made by calculating a quantity referred to as the 'relative metabolic stability'. Potency ratios in the isolated

adrenal cell assay are used for reference and the metabolic stability of compound B relative to a reference compound A in a given assay system S is given by:

$$\text{Relative metabolic stability} = \frac{\text{potency of compound B relative to A in assay S}}{\text{potency of compound B relative to A in the isolated adrenal cell assay}}.$$

RESULTS

Potency ratios obtained from five different assay systems

Molar potency ratios, calculated relative to (1–24)-OH, are given in Table 1. For a comparison of the relative sensitivities of the various assay systems, doses of (1–24)-OH producing 50% of the maximal response are listed at the bottom of Table 1. Sensitivity was highest in the adrenal cell assay, being about 10000 times higher than in the adrenal slice or the fat pad assays. The adrenal slice and lipolytic assays *in vitro* had similar sensitivities, whilst *in vivo*, the steroidogenic response was measurable using quantities of peptide which were three to ten times smaller than those required for a lipolytic response. In the two tests *in vivo*, evaluation of the response was, however, different – for the steroidogenic response the evaluation was based on the integrated steroid response (see Methods) whilst lipolysis was only measured 30 min after administration of the peptide.

Table 1. *Molar potency ratios relative to corticotrophin-(1–24)-tetracosapeptides*

(Full peptide structures are given in Materials and Methods.)

Structure	Corticosteroidogenesis			Lipolysis	
	Adrenal cells	Adrenal slices	*In vivo*, i.v.	Fat pads	*In vivo*, i.v.
1. (1–24)-OH	1·0	1·0	1·0	1·0	1·0
2. (1–24)-NH_2	0·4	0·8	1·0	1·0	0·3
3. (1–18)-OH*	$0{\cdot}4\times10^{-2}$	0·2	$0{\cdot}2\times10^{-2}$	$0{\cdot}5\times10^{-1}$	n/a
4. (1–18)-NH_2*	$0{\cdot}3\times10^{-1}$	0·2	$0{\cdot}8\times10^{-1}$	$0{\cdot}6\times10^{-1}$	0·49
5. (1–17)-OH*	$0{\cdot}4\times10^{-3}$	$0{\cdot}8\times10^{-1}$	$0{\cdot}8\times10^{-3}$	$0{\cdot}5\times10^{-1}$	n/a
6. (1–17)-NH_2*	$0{\cdot}2\times10^{-1}$	0·8	$0{\cdot}5\times10^{-1}$	0·6	n/a
7. (1–16)-OH*	$0{\cdot}2\times10^{-4}$	$0{\cdot}2\times10^{-1}$	$0{\cdot}3\times10^{-3}$	$0{\cdot}6\times10^{-2}$	n/a
8. (1–16)-NH_2*	$0{\cdot}3\times10^{-3}$	$0{\cdot}4\times10^{-1}$	$0{\cdot}2\times10^{-2}$	$0{\cdot}3\times10^{-1}$	n/a
9. (1–13)-NH_2	$0{\cdot}3\times10^{-4}$	$0{\cdot}3\times10^{-1}$	$0{\cdot}7\times10^{-3}$	$0{\cdot}3\times10^{-5}$	n/a
10. [D-Ser^1]-(1–24)-OH	0·2	1·0	20·0	1·0	10·0
11. [D-Ser^1]-(1–18)-NH_2	$0{\cdot}5\times10^{-1}$	0·8	9·0	0·8	8·0
12. [D-Ser^1,Lys^{17,18}]-(1–18)-NH_2	$0{\cdot}9\times10^{-1}$	11·0	75·0	2·0	75·0
13. [Lys^{17,18}]-(1–18)-NH_2	0·4	0·8	2·0	2·0	0·8
14. [Lys^{17,18}]-(3–18)-NH_2	$0{\cdot}5\times10^{-3}$	$0{\cdot}4\times10^{-1}$	$0{\cdot}7\times10^{-1}$	$0{\cdot}1\times10^{-1}$	$0{\cdot}2\times10^{-1}$
15. [Lys^{17,18}]-(4–18)-NH_2*	$0{\cdot}4\times10^{-4}$	$0{\cdot}2\times10^{-1}$	$0{\cdot}2\times10^{-1}$	$0{\cdot}1\times10^{-1}$	n/a
16. [Lys^{17,18}]-(5–18)-NH_2	$0{\cdot}2\times10^{-4}$	$0{\cdot}3\times10^{-2}$	$0{\cdot}2\times10^{-1}$	$0{\cdot}1\times10^{-4}$	n/a
Dose of (1–24)-OH giving 50% response	10 pg/ml	100 ng/ml	1 μg/kg†	100 ng/ml	30 μg/kg

* Tested using purified cells (see Results).
† Half-maximal increase of plasma steroids 30 min after injection.
n/a = not active.

Degree of peptide inactivation occurring in the isolated adrenal cell assay

To simplify interpretation of the results it was necessary to eliminate any possibility of peptide inactivation occurring in the isolated adrenal cell assay. For this reason a number of peptides, chosen to represent the various types of groups which might be exposed to degradative processes, were tested directly for inactivation as described by Bennett *et al.* (1974), i.e. peptide was incubated for 1 h with a portion of dilute cell suspension and the

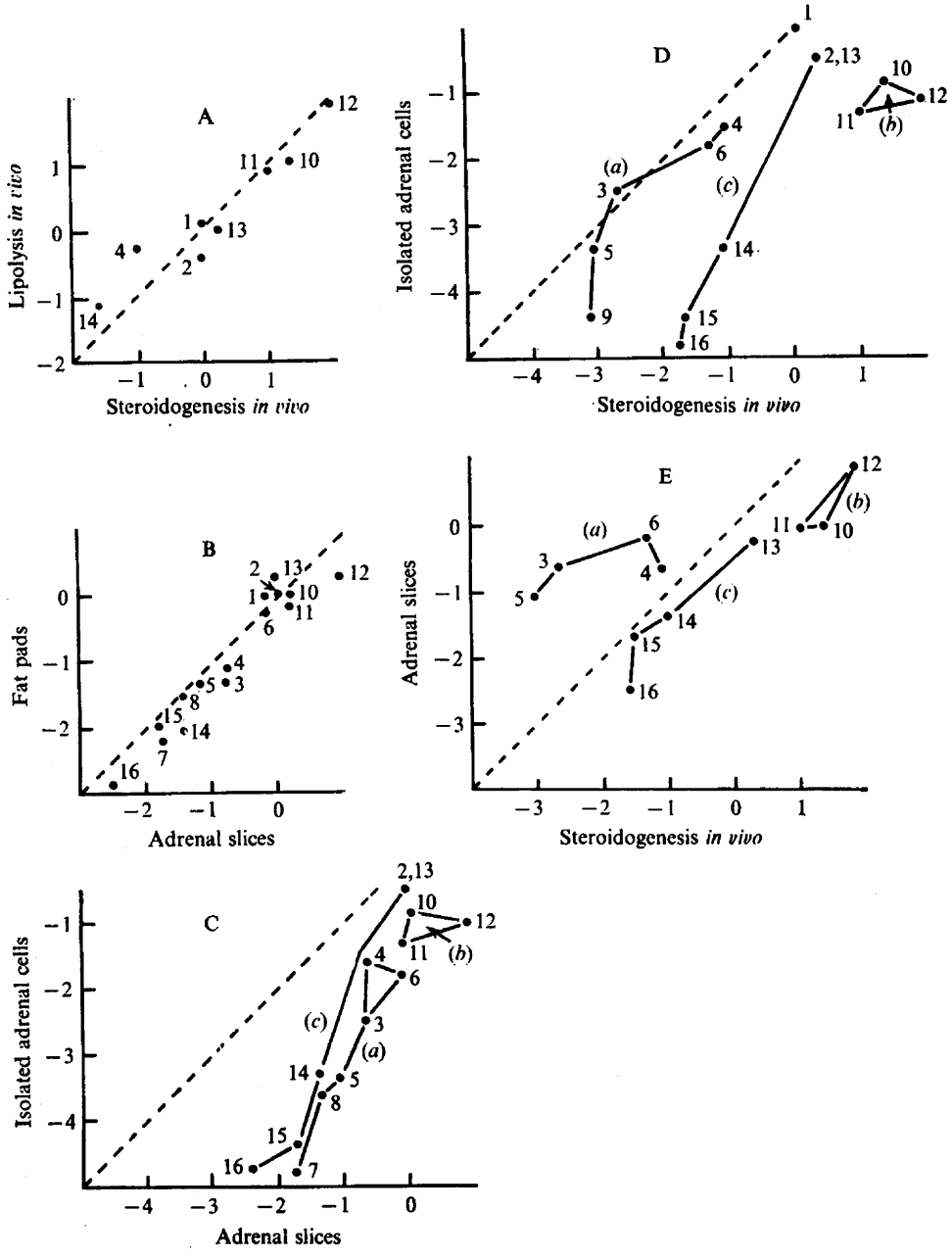

Fig. 1. Comparison of molar potencies relative to (1-24)-OH obtained in five different assays. The logarithm of potency is plotted on each axis. A pair of assays is compared in each graph. The dotted line represents points where the potency relative to corticotrophin-(1–24)-tetracosapeptide is identical in both assays. Numbers refer to the peptides as listed in Materials and Methods. Groups of related peptides in graphs C, D and E are joined by lines. (*a*) Peptides modified at the CO_2H-terminus by deamidation or removal of amino acids. (*b*) Peptides with D-Ser[1] at the NH_2-terminus. (*c*) Peptides shortened at the NH_2-terminus.

supernatant assayed with fresh portions of cell suspension. The following analogues were tested in this way: (1–24)-OH; [D-Ser^1,Lys^{17,18}]-(1–18)-NH_2; (1–18)-NH_2; (1–18)-OH; (1–16)-NH_2; (1–16)-OH; [Lys^{17,18}]-(3–18)-NH_2 and [Lys^{17,18}]-(5–18)-NH_2. Only the (1–18)-NH_2 and (1–16)-NH_2 showed substantial inactivation. A similar experimental procedure showed that inactivation of the last two peptides did not occur when cells were purified as described by Bennett *et al.* (1974); that is, by layering the cell suspension on 2% albumin solution, sedimenting the cells by centrifugation and carefully discarding the supernatant. When cells purified in this way were used to assay (1–18)-NH_2, (1–17)-NH_2 and (1–16)-NH_2, consistent potency ratios were obtained, thus eliminating variations observed with (1–18)-NH_2 when unpurified cells were used.

Comparison between results obtained in the various assays

Relationships between potency ratios obtained from the five assay systems are shown in Fig. 1 A–E, log potencies from one assay being plotted against those from another. A good correlation existed between results obtained from the steroidogenic response of adrenal slices *in vitro* versus the lipolytic response of fat pads *in vitro* (Fig. 1 B) and also between those representing steroidogenesis versus lipolysis in the intact animal (Fig. 1 A) although not all compounds were active in the latter assay. Comparison of the results of the three steroidogenic assays, on the other hand, failed to reveal any equivalent relationship (Fig. 1 C and D). Although potencies obtained from isolated adrenal cells and adrenal slices showed a degree of correlation, the changes in potency were much more pronounced in the adrenal cell assay (Fig. 1 C). In Fig. 1 C, D and E compounds which have structural similarities have been linked (*a*) for analogues which were shortened at the CO_2H terminal end, (*b*) for D-Ser^1 analogues and (*c*) for analogues shortened at the NH_2-terminal end. There was a tendency for groups of related compounds to lie in separate regions of the graph.

Relative metabolic stability

As explained further in the Discussion, the lack of equivalence between the three steroidogenic assay systems can be attributed to inherent differences in peptide metabolism. Consequently, it was possible to use the results to obtain information about the relative metabolic stabilities of peptides in certain assays.

The isolated adrenal cell assay has been shown to be free of metabolic interference. It, therefore, reflects the steroidogenic activity of a peptide at the receptor level. By comparing potencies obtained in this assay with those obtained *in vivo* it is thus possible to assess the effects of structural modification of a peptide on its metabolism in the intact animal. This interpretation can be placed on a quantitative basis, and a relative metabolic stability calculated as described in Materials and Methods. Each of the Tables 2–5 lists relative metabolic stabilities for a group of related analogues calculated taking the first member of each group as a reference compound. A high value indicates a decreased susceptibility to breakdown in the whole animal in comparison to the reference compound.

Relative metabolic stabilities for the adrenal slice assay, similarly calculated, are also shown in these Tables.

ACTH analogues progressively shortened at the NH_2-terminus (Table 2) exhibited a marked loss in potency in the adrenal cell assay, whilst the loss in activity with decreasing chain length was much less pronounced in the adrenal slice assay and in steroidogenesis *in vivo*, i.e. the relative metabolic stability was higher for the NH_2-terminally shortened peptides.

As shown in Table 3, introduction of a CO_2H-terminal amide group increases the potency in the adrenal cell assay and to a lesser extent in steroidogenesis *in vivo;* therefore the relative metabolic stability is slightly lower for amides.

Table 2. *Effect of shortening the NH_2-terminus of [$Lys^{17,18}$]-corticotrophin-(1–18)-octadecapeptide amide on potency and metabolic stability in steroidogenic assays*

(Values are calculated relative to [$Lys^{17,18}$]-(1–18)-NH_2. For full structures see Materials and Methods.)

	Isolated adrenal cells	*In vivo*, i.v.		Adrenal slices	
	(Relative potency *in vitro*)	Relative potency	Relative metabolic stability	Relative potency	Relative metabolic stability
13. [$Lys^{17,18}$]-(1–18)-NH_2	1·0	1·0	1·0	1·0	1·0
14. [$Lys^{17,18}$]-(3–18)-NH_2	$1·0\times10^{-3}$	$4·0\times10^{-2}$	40	$5·0\times10^{-2}$	50
15. [$Lys^{17,18}$]-(4–18)-NH_2	$1·0\times10^{-4}$	$1·0\times10^{-2}$	100	$3·0\times10^{-2}$	300
16. [$Lys^{17,18}$]-(5–18)-NH_2	$5·0\times10^{-5}$	$1·0\times10^{-2}$	200	$4·0\times10^{-3}$	80

Table 3. *Effect of deamidation and shortening of the CO_2H terminus of corticotrophin-(1–18)-octadecapeptide amide [(1–18)-NH_2] on potency and metabolic stability in steroidogenic assays*

(Values are calculated relative to (1–18)-NH_2. Full structures are given in Materials and Methods.)

	Isolated adrenal cells	*In vivo*, i.v.		Adrenal slices	
	(Relative potency *in vitro*)	Relative potency	Relative metabolic stability	Relative potency	Relative metabolic stability
4. (1–18)-NH_2	1·0	1·0	1·0	1·0	1·0
3. (1–18)-OH	$1·0\times10^{-1}$	$3·0\times10^{-2}$	0·3	1·0	10
6. (1–17)-NH_2	$7·0\times10^{-1}$	$6·0\times10^{-1}$	0·9	4·0	6
5. (1–17)-OH	$1·0\times10^{-2}$	$1·0\times10^{-2}$	1·0	$4·0\times10^{-1}$	40
7. (1–16)-NH_2	$1·0\times10^{-2}$	$3·0\times10^{-2}$	3·0	$2·0\times10^{-1}$	20
8. (1–16)-OH	$7·0\times10^{-4}$	$4·0\times10^{-3}$	6·0	$1·0\times10^{-1}$	143
9. (1–13)-NH_2	$1·0\times10^{-3}$	$9·0\times10^{-3}$	9·0	$2·0\times10^{-1}$	200

Table 4. *Effect of* D-*Ser^1 replacement on potency and metabolic stability of corticotrophin-(1–24)-tetracosapeptide, corticotrophin-(1–18)-octadecapeptide amide and [$Lys^{17,18}$]-corticotrophin-(1–18)-octadecapeptide amide in steroidogenic assays*

(Values are calculated relative to the L-Ser^1 analogue in each case. Full peptide structures are given in Materials and Methods.)

	Isolated adrenal cells	*In vivo*, i.v.		Adrenal slices	
	(Relative potency *in vitro*)	Relative potency	Relative metabolic stability	Relative potency	Relative metabolic stability
1. (1–24)-OH	1·0	1·0	1·0	1·0	1·0
10. [D-Ser^1]-(1–24)-OH	$2·0\times10^{-1}$	20·0	100·0	1·0	5·0
4. (1–18)-NH_2	1·0	1·0	1·0	1·0	1·0
11. [D-Ser^1]-(1–18)-NH_2	2·0	113·0	57·0	4·0	2·0
13. [$Lys^{17,18}$]-(1–18)-NH_2	1·0	1·0	1·0	1·0	1·0
12. [D-Ser^1,$Lys^{17,18}$]-(1–18)-NH_2	$2·0\times10^{-1}$	38·0	190	14·0	70

In the adrenal slice assay, the differences in potency between free acids and amides are much smaller. Whether amides or free acids are considered (Table 3), shortening at the CO_2H-terminal results in a pronounced loss in potency in the adrenal cell assay and in steroidogenesis *in vivo*. In the adrenal slice assay the decrease in activity with decreasing chain length was less pronounced. Correspondingly the relative metabolic stability was higher in the adrenal slice assay than *in vivo*.

Table 5. *Effect of replacement of* $Arg^{17}Arg^{18}$ *by* $Lys^{17}Lys^{18}$ *on potency and metabolic stability of corticotrophin-(1–18)-octadecapeptide and* [D-Ser^1]*-corticotrophin-(1–18)-octadecapeptide amide in three steroidogenic assays*

(Values are calculated relative to the $Arg^{17}Arg^{18}$ analogue in each case. Full structures are given in Materials and Methods.)

	Isolated adrenal cells	*In vivo*, i.v.		Adrenal slices	
	(Relative potency *in vitro*)	Relative potency	Relative metabolic stability	Relative potency	Relative metabolic stability
4. (1–18)-NH_2	1·0	1·0	1·0	1·0	1·0
13. [$Lys^{17,18}$]-(1–18)-NH_2	13·0	25·0	2·0	4·0	9·3
11. [D-Ser^1]-(1–18)-NH_2	1·0	1·0	1·0	1·0	1·0
12. [D-Ser^1,$Lys^{17,18}$] -(1–18)-NH_2	2·0	8·0	4·0	14·0	7·0

Replacement of the NH_2-terminal serine by a D-serine (Table 4) had a small effect on potency in the adrenal cell assay, whilst it had a variable effect on potency in the adrenal slice assay and greatly increased potency *in vivo*. Obviously this replacement markedly enhanced metabolic stability *in vivo*.

Replacement of $Arg^{17}Arg^{18}$ by $Lys^{17}Lys^{18}$ in (1–18)-NH_2 (Table 5) increased potency similarly in all three assays, i.e. it enhanced activity at the receptor level without markedly affecting metabolic stability. It is of interest that in the case of [D-Ser^1]-(1–18)-NH_2 this replacement had less effect on potency in adrenal cells but a more pronounced effect on metabolic stability.

DISCUSSION

The results show that modification of the ACTH molecule can have quite different effects on its potency *in vivo* and *in vitro*. To understand these differences it is necessary to consider in more detail the processes which may influence the potency of a peptide in a given assay. These may be divided into: (*a*) aspects of metabolic handling of the peptide, e.g. transport and catabolism which determine the concentration of biologically active peptide in the vicinity of the receptor or molecular site of action, (*b*) the hormone–receptor interaction which determines the relationship between this local concentration and the response.

The isolated adrenal cell assay, where peptide has direct access to the cell membrane and peptide inactivation is minimal, should reflect the effect of changes in peptide structure on potency at the receptor. Differences between relative potencies obtained in this assay and those from another steroidogenic assay, e.g. *in vivo*, should therefore give an estimate of relative susceptibility to inactivation in that assay. This way of interpreting results depends, however, on the assumption that the specificity of the receptor has not been modified during isolation of adrenal cells. The integrity of receptors in isolated cells is demonstrated by the sensitivity of their response to physiological levels of ACTH and a high degree of

structural specificity for different ACTH analogues. In addition, differences between the potencies of three ACTH analogues determined *in vivo* and determined using the isolated cell assay can be completely accounted for by experimentally observed differences in their blood levels *in vivo* (Table 6).

Modification at the NH_2-terminus

Modification at the NH_2-terminus (Table 2) has a pronounced effect on potency in the isolated cell assay and removal of the two terminal amino acids causes a 1000-fold reduction in potency. In contrast, the effects of removal of NH_2-terminal amino acids are less marked on potency *in vivo*.

Table 6. *Comparison of relative metabolic stabilities estimated from potencies by the method proposed in this paper with those obtained directly from blood concentration measurements*

Relative metabolic stability was calculated from potencies as described in Materials and Methods and was also estimated directly as the ratio of blood concentrations of peptide obtained 40 min after the start of a 20 min infusion of equal amounts of the three peptides (McMartin & Peters, 1975).

	Potency		Relative metabolic stability	
Analogue	Isolated adrenal cells *in vitro*	*In vivo*	From potency	From blood levels of peptide
(1–24)	1·0	1·0	1·0	1·0
Human (1–39)	0·14*	1·5†	11	22
[D-Ser^1,Lys^{17,18}]-(1–18)-NH_2	0·1	75	750	700

* Lowry, McMartin & Peters (1973).
† Barthe, Desaulles, Schär & Staehelin (1964).

These differences between the assays are indicative of a high relative metabolic stability for the shortened molecule. This suggests that the (1–18) analogue is cleaved readily at the NH_2-terminus *in vivo* and that the (3–18), (4–18) and (5–18) analogues are less susceptible. This concept finds support in the fact that (as shown in Table 4) replacement of L-Ser^1 by D-Ser^1 only affects potency slightly in the cell assay but greatly enhances it *in vivo*. Thus, the D residue appears to stabilize the molecule. It is, of course, possible that the metabolic stability might not be a simple reflection of breakdown since other processes, e.g. uptake into tissues like liver and kidneys, may also regulate blood concentrations. However, studies with tritium-labelled corticotrophins have suggested that extremely rapid extracellular cleavage of (1–24)-OH takes place in the first few circulations after injection and that [D-Ser^1,Lys^{17,18}]-(1–18)-NH_2 is substantially more resistant to this process (Baker, Bennett, Hudson, McMartin & Purdon, 1976). In this investigation it was demonstrated that rapid cleavage of (1–24)-OH occurred at both the NH_2- and CO_2H-termini. Thus, the interpretation of the present findings in terms of peptidases attacking the NH_2-terminus is fully consonant with direct observations of the fate of corticotrophins. The metabolic stabilities calculated for the adrenal slice assay for this series of compounds indicate that a similar type of metabolic process is operating.

Progressive shortening at the CO_2H-terminus

Progressive shortening at the CO_2H-terminus resulted in a marked loss of activity at the receptor level. This loss was less pronounced in the analogues with a CO_2H-terminal amide. Activity at the receptor level seems to be enhanced by factors which increase the charge at the CO_2H-terminus. A similar pattern of relative activities was observed for

in-vivo steroidogenesis, and this implies that changes in the CO_2H-terminal region have no marked effects on relative metabolic stability.

In the adrenal slice assay the effects of modification at the CO_2H-terminus on metabolic stability were much more pronounced. In this system the effects of the metabolic degradation were especially marked for the longer peptides and it appeared that the specificity of peptidases active in the adrenal slices differed from those *in vivo* in that they were directed more towards the CO_2H-terminus. Table 7 summarizes some of the conclusions with respect to the effect of modification of the structure of $Lys^{17,18}$-(1–18)-NH_2. This table illustrates how metabolic stability *in vivo* and receptor potency vary quite independently of each other.

Table 7. *Effects of changes in the structure of* [$Lys^{17,18}$]-*corticotrophin-(1–18)-octadecapeptide amide on receptor potency (isolated adrenal cells) and apparent metabolic stability* in vivo

NH_2 Ser^1-Tyr Ser Met ---------- $Lys^{17}Lys^{18}H_2$

	Replacement by D-Ser^1	Removal of Ser^1-Met^4	Removal of $Lys^{17}Lys^{18}NH_2$ to give (1–16)-OH	Replacement by $Arg^{17}Arg^{18}$
Receptor activity	0·3-0·03	< 0·03	< 0·03	0·3-0·03
Metabolic stability	> 30	> 30	30-3	3-0·3

So far no reference has been made to the significance of the lipolytic assays. The similarity of lipolytic and steroidogenic assays *in vivo* suggests that, as proposed by Lebovitz & Engel (1964), ACTH receptors in the fat cells and target adrenal cells must be closely related if not identical. The close resemblance of potencies obtained using fat pads and adrenal slices also supports this conclusion and leads us to the further supposition that the metabolic processes limiting the activity in these two assays must be remarkably similar.

In conclusion we have tried to show how a comparison of the results obtained with different assay methods for a series of analogues can provide a better understanding of the significance of each assay and also provide information about fundamental properties of the peptides. Thus, the in-vivo assay yields essential information for selection of potentially useful analogues and in conjunction with the isolated adrenal cell assay indicates how modification of the peptide sequence can independently affect its properties at the receptor and its metabolic stability *in vivo*. The lipolytic assays compared with the in-vivo and adrenal slice steroidogenic assays reveal close similarities in the peptide receptor interactions responsible for these different types of response.

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