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Fate of Corticotrophins in an Isolated Adrenal-Cell Bioassay and Decrease of Peptide Breakdown by Cell Purification

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1. The fate of corticotrophins in a trypsin-dispersed rat adrenal-cell assay system was investigated with a view to establishing whether differences in the rate of inactivation might contribute to potency differences observed between analogues. 2. Corticotrophin-(1–24)-tetracosapeptide and to a lesser extent synthetic 1–39 corticotrophins were found to be inactivated during incubation with cell suspension. 3. Peptide fragments were isolated by using [[$^3H_2$]Tyr^{23}]corticotrophin-(1–24)-tetracosapeptide as a marker. The fragments indicate a peptidase with a predominantly tryptic specificity. 4. The peptidase is present in the extracellular fluid and is released from cells when they are damaged. 5. Cells were fractionated on an albumin gradient. Cells from the zona fasciculata and the zona intermedia or reticularis were present in fractions which produced fluorogenic steroids in response to corticotrophin. 6. Purification of the cells by centrifugation through albumin decreased degradation by peptidases, so that if the assay is carried out with a dilute suspension of purified cells peptide breakdown should not affect the observed potencies of adrenocorticotrophin analogues. 7. No binding of [[$^3H_2$]Tyr^{23}]corticotrophin-(1–24)-tetracosapeptide to cells could be detected at low concentrations of the peptide. This indicated that less than 120 receptors/cell are occupied during stimulation by a dose that would elicit approx. 80% of the maximal response.

The steroidogenic response of isolated adrenal-cell suspensions can provide a sensitive, accurate bioassay for corticotrophins (Sayers *et al.*, 1971; Lowry *et al.*, 1973). However, to be able to interpret potency differences obtained from any bioassay of peptide-hormone analogues it is necessary to understand their fate in the assay system. It is quite possible for instance that a peptide might show an increased potency not because of an altered interaction with receptors but simply because it is degraded more slowly. In fact this is the explanation proposed by Seelig & Sayers (1971*a*,*b*) for their finding that *N*-terminal replacement of L-Ser^1 by D-Ser^1 increases the potency of corticotrophin analogues. Lowry *et al.* (1973), however, did not find these analogues to be more potent and also consistently found the peptide containing only amino acid residues 1–24 to be five to seven times more potent than the natural peptide, which consists of 39 amino acids. This latter finding differs from results obtained *in vivo* by Schwyzer & Kappeler (1963) and *in vitro* by Sayers *et al.* (1971) and Kitabchi & Sharma (1971), where the peptides in question have been shown to be approximately equipotent.

In order to establish the part that metabolism of the peptides plays in determining their potency we have investigated the fate of corticotrophin-(1–24)-tetracosapeptide in the isolated adrenal-cell assay system in detail and have shown that a simple procedure for cell purification can decrease inactivation of corticotrophins.

Materials and Methods

Materials

[[$^3H_2$]Tyr^{23}]Corticotrophin-(1–24)-tetracosapeptide (46 Ci/mmol) was synthesized in these laboratories (Brundish & Wade, 1973). This peptide has full biological activity. The following synthetic peptides were kindly supplied by Dr. W. Rittel and Dr. B. Riniker, CIBA–GEIGY, Basle, Switzerland: porcine corticotrophin (unrevised sequence, Shepherd *et al.*, 1956), human corticotrophin (revised sequence, Riniker *et al.*, 1972), corticotrophin-(1–24)-tetracosapeptide (SYNACTHEN) and [D-Ser^1,Lys^{17,18}]corticotrophin-(1–18)-octadecapeptide amide. Trasylol (proteinase inhibitor) was obtained from Farben Fabriken Bayer A.G., Leverkusen, Germany. Bacitracin and bovine serum albumin (crystallized) were from Sigma (London) Chemical Co., London S.W.6, U.K., and lima-bean trypsin inhibitor was from Worthington Biochemical Corp., Freehold, N.J. U.S.A.

CM-cellulose (CM 32) was obtained from Whatman Biochemicals Ltd., Maidstone, Kent, U.K.,

and trimethylamine from BDH Laboratories, Poole, Dorset, U.K. Trimethylamine was distilled at 4°C from a solution containing 10% (v/v) acetic anhydride and stored at 4°C as an aqueous solution (approx. 3M).

Methods

Adrenal cells were prepared and the bioassay was carried out as described by Lowry *et al.* (1973). Briefly, the cells were dispersed by stirring adrenal quarters with trypsin and were then harvested from the supernatant. The cells were pelleted by gentle centrifugation (100*g* for 30min at 15°C) and resuspended in Krebs–Ringer bicarbonate buffer (Umbreit *et al.*, 1964) containing 0.1% glucose, 0.5% albumin and 0.05% lima-bean trypsin inhibitor (KRBAI). This suspension was then centrifuged to give a pellet, which was taken up in KRBAI buffer and filtered through 100μm nylon gauze. After incubation of portions of cell suspension with corticotrophins, fluorogenic 11-hydroxy corticosteroids were extracted and assayed by using a H_2SO_4–ethanol procedure.

Procedure for cell fractionation. Cells resuspended in 2ml of medium and filtered as above were layered on top of a linear albumin gradient (2–10%) in the barrel of a 20ml disposable syringe. The outlet of the syringe barrel was cut off and the bottom was sealed by inserting the rubber part of the plunger of another syringe. The gradient volume was 16ml and had a depth of 5cm. After centrifugation at 10*g* for 5min fractions were aspirated by inserting a plunger into the edge of which a polythene conduit (1.5mm internal diam.) had been introduced. The barrel was tilted and the plunger slowly pushed in with the outlet uppermost to aspirate fractions from the top of the gradient through the polythene tubing.

Simple procedure for cell purification. Cells were purified by layering 2ml of suspension on top of 20ml of 2% (w/v) albumin, centrifuging at 10*g* for 5min and harvesting the cells remaining in the apparatus after the top 10ml had been discarded.

Separation and identification of peptide fragments. The general methods of ion-exchange chromatography including type of column, CM-cellulose and buffer gradients used and u.v.-detection system have been described previously (Bennett *et al.*, 1973).

(*a*) Measurement of extent of breakdown of labelled peptide. Supernatants obtained after incubation were subjected to ion-exchange chromatography on CM-cellulose. The columns (0.6cm internal diam. × 8cm length) were equilibrated and eluted with 0.05M-Tris–HCl (pH 7.4) containing 0.2M-NaCl, as this system has been found to give good separation of corticotrophin-(1–24)-tetracosapeptide and its fragments. Approx. 1mg of unlabelled intact peptide was added as carrier to each 200μl of supernatant sample before loading to eliminate adsorption anomalies and to act as a u.v. marker for unchanged peptide. No pH adjustment was necessary, since the incubation buffer was also at pH 7.4.

(*b*) Identification of degradation products. In the experiments to characterize the breakdown products, gradient elution of the column was required and trimethylamine acetate was used as a volatile buffer. Before loading on the column the sample (0.5ml in incubation medium) was freeze-dried and dissolved in a small volume of starting buffer. The column (again 0.6cm internal diam. × 8cm length) was equilibrated with 10mM-trimethylamine acetate (pH 5.0) and after loading was developed by using a concave gradient similar to that described previously (Bennett *et al.*, 1973) but running from 10mM to 600mM buffer with a total volume of approx. 100ml. Fractions (2ml) were collected every 20min.

Peptides were hydrolysed in 1ml of 6M-HCl containing a small crystal of phenol (to minimize tyrosine breakdown) *in vacuo* at 115°C for 18h. Quantitative amino acid determinations were carried out with a Beckman 120C amino acid analyser.

Radioactive counting. Radioactivity was measured in a Packard TriCarb model 3003 liquid-scintillation counting system. Up to 0.5ml aqueous samples were added to vials containing 15ml of scintillator [containing 30g of 2,5-bis-(5-t-butylbenzoxazol-2-yl) thiophen and 340g of naphthalene in a mixture of 2.5 litres of toluene and 1.7 litres of methylCellosolve]. For efficient reproducible counting of the labelled peptide, it was necessary to include 4% Cab-O-Sil (Packard, Downers Grove, Ill. U.S.A.) in the scintillation fluid.

Results

Inactivation of corticotrophins during incubation with isolated adrenal cells

A suspension of cells (1ml), containing the equivalent of half an adrenal, was incubated at 37°C for 1h with 50ng of corticotrophin-(1–24)-tetracosapeptide, 50ng of synthetic porcine corticotrophin (unrevised sequence) or 50ng human corticotrophin (revised sequence). The cell suspensions were centrifuged for 15min at 200*g* and the resulting supernatants were bioassayed at suitable dilutions with fresh cell suspensions, allowance being made for steroids already present in the sample at the start of bioassay. The extent of degradation was determined by comparison with a standard dose–response curve. Typical curves are shown in Fig. 1.

When the decrease in potency is evaluated by linear-regression analysis (Table 1) it can be seen that corticotrophin-(1–24)-tetracosapeptide was inactivated more extensively than either synthetic porcine corticotrophin or human corticotrophin (revised sequence).

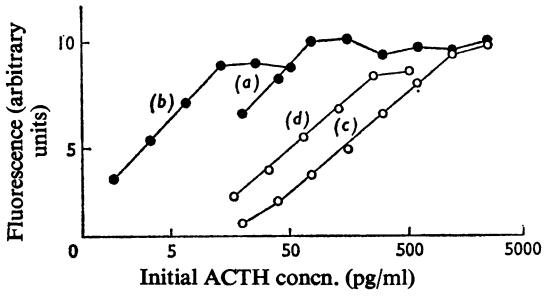


Fig. 1. *Isolated adrenal-cell bioassay of the supernatants of corticotrophins with isolated adrenal-cell suspensions*

Log dose–response curves to (*a*) serial dilutions of supernatant obtained after incubation of corticotrophin-(1–24)-tetracosapeptide; (*b*) corticotrophin-(1–24)-tetracosapeptide standard; (*c*) serial dilutions of supernatant obtained after incubation of synthetic porcine corticotrophin; (*d*) synthetic porcine corticotrophin standard. The supernatant responses are plotted at the peptide concentration that would have been present if no inactivation had taken place. ACTH, adrenocorticotrophin.

Fractionation of cells on albumin gradients

Cells with differing sedimentation velocities were separated by gentle centrifugation after being layered on an albumin gradient as described in the Materials and Methods section. The fractions were examined under the light-microscope (dark-ground illumination) and cell counts were taken. Further portions were incubated with and without corticotrophin-(1–24)-tetracosapeptide to locate the cells that respond to corticotrophin by the production of fluorogenic 11-hydroxy corticosteroids.

The results shown in Table 2 indicated that fluorogenic-steroid production was associated with fractions containing considerable numbers of large, rapidly sedimenting cells. Portions of each fraction were pooled in three groups as shown in Table 2 and these were fixed in suspension by glutaraldehyde and examined under the electron microscope. Typical cells found in the different samples are shown in Plates 1 and 2. The cells were identified by comparison with perfusion-fixed adrenal sections cut to give a

Table 1. *Inactivation of corticotrophins during incubation with isolated adrenal cells*

Experimental conditions were as described in the legend to Fig. 1. The decrease in potency resulting from preliminary incubation of the corticotrophin with isolated adrenal cells was calculated by linear-regression analysis and the 95% confidence limits of each determination were also evaluated.

	Percentage inactivated	95% confidence limits
Expt. 1		
Corticotrophin-(1–24)-tetracosapeptide	83	82.5–83.5
Synthetic porcine corticotrophin (unrevised sequence)	62	60–65
Expt. 2		
Corticotrophin-(1–24)-tetracosapeptide	88	80–93
Human corticotrophin (revised sequence)	65	40–80

Table 2. *Production of fluorogenic steroids in response to adrenocorticotrophin in cells that have been separated on an albumin gradient*

Cells from 20 adrenals were treated as described in the Materials and Methods section and nine 2ml fractions were collected. The ninth fraction also contained cells resuspended from the bottom of the gradient. Samples of each fraction were diluted to 1ml with KRBAI buffer and incubated with or without 1ng of corticotrophin-(1–24)-tetracosapeptide for 2h. Fluorogenic steroids were then measured and the increment caused by the addition of corticotrophin was calculated.

Fraction no. (numbered from top of gradient)	10^{-4} × Cell count (cells/ml)		Fluorogenic steroid production (ng of corticosterone/ 2h per ml of fraction)	Grouping of fractions for electron microscopy
	5–10 μm	10–20 μm		
1	3		0	A
2	3		10	A
3	6		30	A
4	4	5	50	B
5		9	65	B
6		9	55	B
7		8	50	C
8		4	35	C
9		20	70	C

complete cross-section of the gland. The cells have been named according to the classification of Sabatini & De Robertis (1961) in which the region of cells between the zona glomerulosa and zona fasciculata is called the zona intermedia. These cells have tubulofascicular mitochrondria similar to those of the zona reticularis, but in addition a proportion of the mitochondria contain cristae, which are organized in a parallel array. In the intact adrenals of the rats used in this study hexagonal arrays of microtubules were found in most mitochondria of the zona reticularis and rarely in any other zone. However, these microtubules were never found in isolated adrenal cells. Sample A of the fractionated isolated adrenal cells contained a mixture of damaged cells, necrosing cells, phagocytic cells, small lymphocytes and capillary endothelial cells. Sample B contained cells from the zona fasciculata and either the zona intermedia or the zona reticularis, but it was not possible to distinguish with certainty between the latter two cell types. These cells were rich in lipid droplets and there were many pairs and small clumps. In addition this sample contained some clumps of capillary endothelial cells.

Fraction C contained the same cell types as sample B with the exception of capillary endothelial cells. The ratio of zona fasciculata to the other zones was lower and the cells contained fewer lipid drops. Cells from the zona glomerulosa and the medulla were rarely found in any fraction. The difference in the lipid content of the cells found in fractions B and C may well account for their separation.

Chemical characterization of the incubation products of corticotrophin-(1–24)-tetracosapeptide

To obtain amino acid analyses of peptide breakdown products incubation of a large amount of peptide was necessary; therefore 10mg of peptide was incubated for 4h in 1ml of a suspension containing cells from eight adrenals and a supernatant was prepared by centrifuging at 1000*g* for 10min. To demonstrate that the breakdown pattern was similar to that obtained with lower doses, 200μl from an incubation of a low concentration of labelled peptide (fraction M, 30min; see Table 4) was added to one-third of the above material and gradient elution on CM-cellulose was carried out as described in the Materials and Methods section. The u.v. and radioactivity traces shown in Fig. 2 were obtained.

Table 3 shows the peptides found in the fractions beneath the radioactive peaks which have been identified on the basis of amino acid composition after acid hydrolysis (some are mixtures).

Peaks (II), (III) and (IV) were heavily contaminated with protein, probably albumin from the incubation medium. Fig. 2 and Table 3 show that although breakdown of unlabelled material has been less extensive than that of labelled material the peaks containing radioactivity each contain a major peptide fragment which includes Tyr^{23}, and in some cases other peptides were also present. The cleavage pattern found for the unlabelled peptide therefore gives a good indication of the specificity of the enzyme system responsible for degrading the smaller concentrations used in other experiments.

An amino acid analysis of one-third of the supernatant prepared above without acid hydrolysis revealed very small amounts (<1nmol) indicating negligible release of free amino acids. It is unlikely that free amino acids could have been released and taken up so efficiently by cells, since the free amino acid-pool size in the small volume of cells would be a fraction of the extracellular pool size, while incorporation of released amino acids into protein could not occur without other amino acids not present in the corticotrophin becoming rate-limiting.

Localization of the enzyme system that inactivates corticotrophins

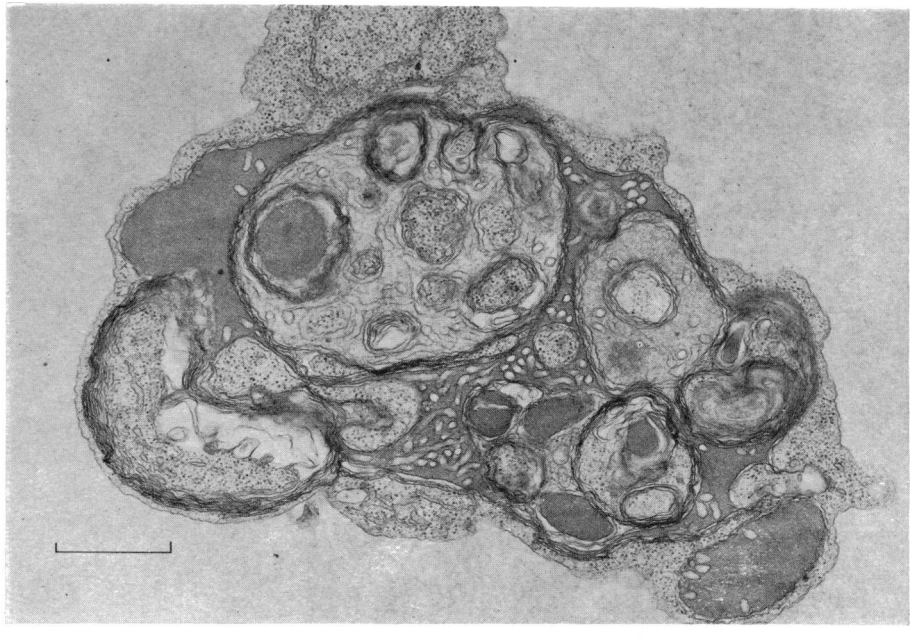
Breakdown in cellular fractions. A freshly prepared suspension of adrenal cells (from ten adrenals in 2ml) was divided into three parts. One part (200μl) was diluted 40 times (with Krebs–Ringer solution+albumin) and called fraction D. Portions (1ml) of fraction D were incubated for 1 and 2h with 50ng of [[^{3}H]Tyr^{23}]corticotrophin-(1–24)-tetracosapeptide. The second part (400μl) was diluted ten times and called fraction M. Portions (1ml) of this were incubated for 5min, 30min and 1h with 50ng of [[^{3}H]Tyr^{23}]corticotrophin-(1–24)-tetracosapeptide. The third part (1.0ml) was layered undiluted on an albumin gradient. The gradient was centrifuged as described in the Materials and Methods section and

EXPLANATION OF PLATES 1 AND 2

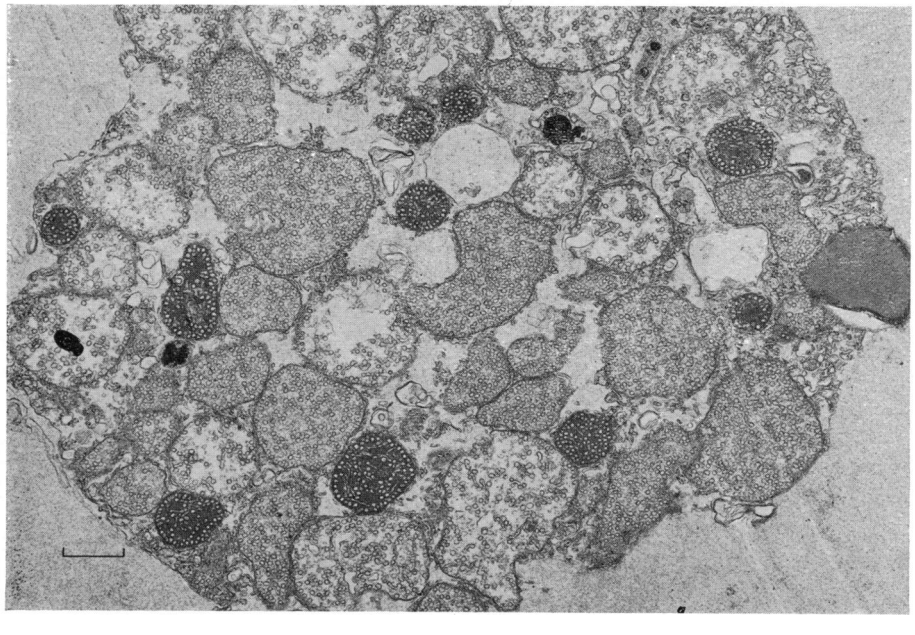
Illustrations of some of the cellular components found in the different fractions of the albumin gradient

1(*a*) A broken cell from the top fraction A. As can be seen from its dimensions, it appears to be much larger than the undamaged cell seen in Plate 2(*a*). The mitochondrial structure indicates that it came from the zona fasciculata. 1(*b*) A necrotic cell from the top fraction A. Most of the cytoplasm and organelles are surrounded by membranes suggestive of developing autophagic vacuoles. 2(*a*) A typical zona fasciculata cell found in fraction C from the gradient. The vesicular mitochondria are the main identifying features. A few lipid drops were found in each cell. 2(*b*) A cell type frequently found in fractions B and C. The tubulo-vesicular mitochondria would indicate either zona intermedia or zona reticularis, but the presence of lysosomes (L) and abnormal mitochondria (AM) would be more representative of the zona reticularis. Each bar represents 1μm.

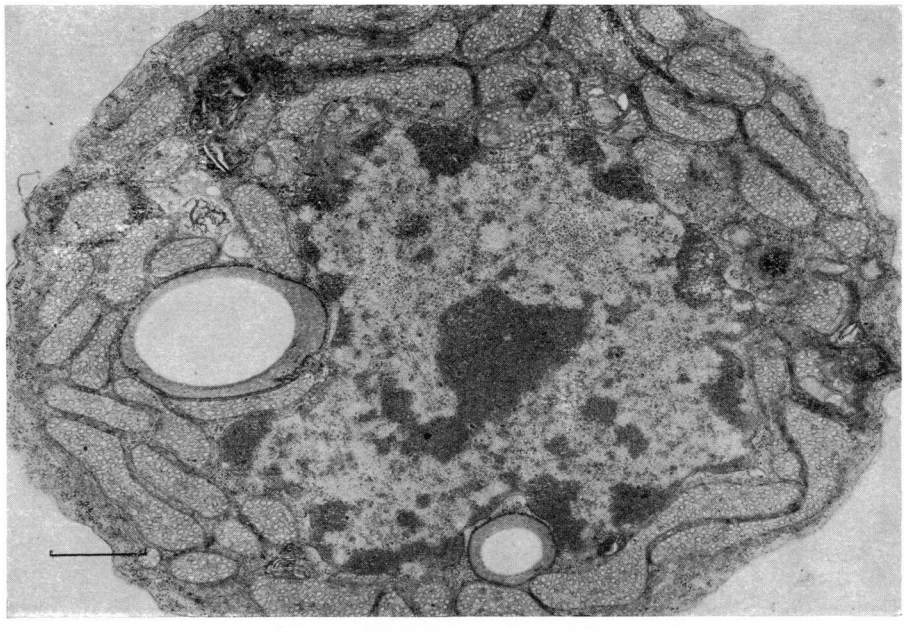
(*a*)



(*b*)



(a)



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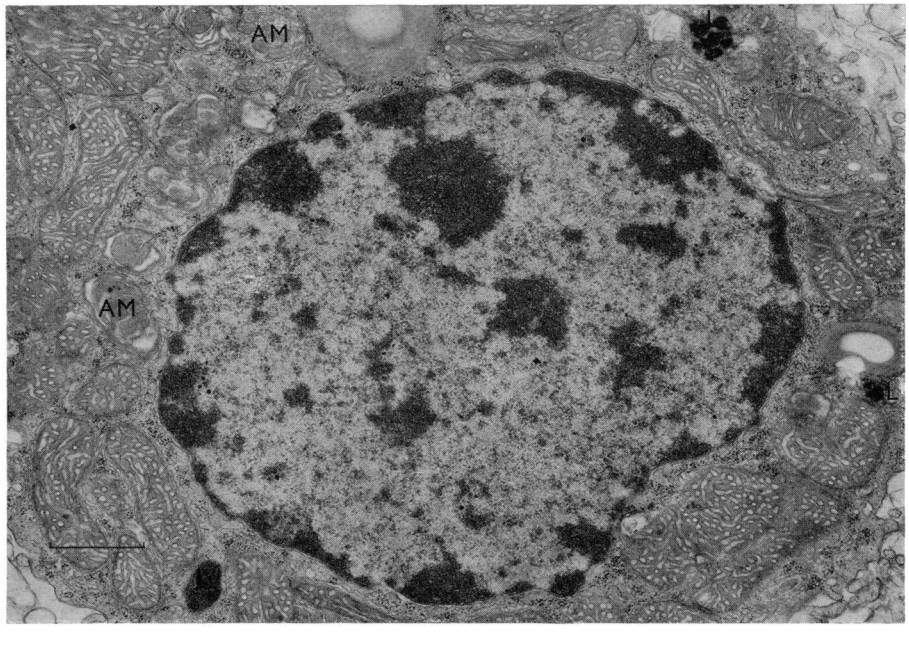

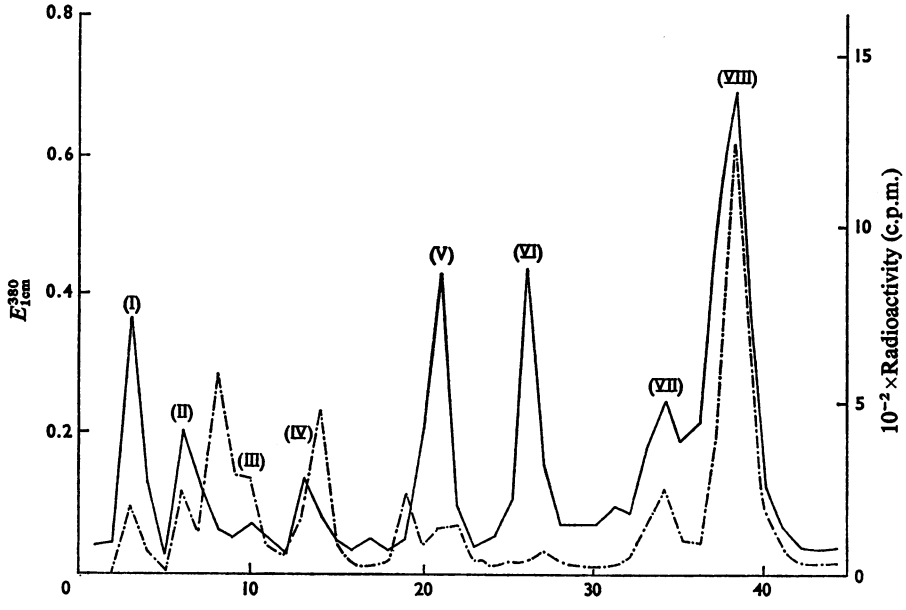

Fig. 2. *U.v.-absorption and radioactivity traces of the gradient elution on CM-cellulose of the combined supernatants of incubations of high (unlabelled) and low (labelled) concentrations of corticotrophin-(1–24)-tetracosapeptide with isolated adrenal cells*

For details see the text. Peaks of radioactivity are numbered. Peaks of u.v.-absorbing material that do not correspond to radioactive peaks may represent peptide fragments that do not contain [^{3}H]Tyr or may be unlabelled products in the medium, which contains albumin and trypsin inhibitor. - · - ·, u.v. absorption; ——, radioactivity.

Table 3. *Peptides found in peaks (I), (V), (VI), (VII) and (VIII) of Fig. 2*

Peak no.	Peptide fragment composition	Total Quantity of peptide (nmol)
(I)	Corticotrophin-(22–24)-tripeptide	20
	Corticotrophin-(1–7)-heptapeptide	10
(V)	Corticotrophin-(9–16)-octapeptide	10
	Corticotrophin-(17–24)-octapeptide	30
(VI)	Mainly corticotrophin-(16–24)-nonapeptide	70
(VII)	Mainly corticotrophin-(9–24)-hexadecapeptide	45
(VIII)	Mainly corticotrophin-(1–24)-tetracosapeptide, i.e. Ser^{1}-Tyr-Ser-Met^{4}-Glu-His-Phe-Arg^{8}-Trp-Gly-Lys-Pro^{12}-Val^{13}-Gly-Lys-Lys^{16}-Arg-Arg-Pro-Val^{20}-Lys-Val-Tyr-Pro^{24}	350

separated into equal top and bottom 9ml fractions. The cells in both fractions were sedimented by centrifugation at 200*g* for 5min. The supernatant from the top fraction was called fraction A. Small cells from the top fraction were resuspended in 9ml of KRBAI buffer and termed fraction B, and large cells from the bottom fraction were resuspended in an equal volume and called fraction C.

Samples (1ml) of fractions A, B and C were incubated for 5, 30 and 60min with 50ng of [[^{3}H]-Tyr^{23}]corticotrophin-(1–24)-tetracosapeptide. Blank samples were prepared by incubating labelled peptide with KRBAI buffer for these times and also for 2h. After incubation 100μg of unlabelled corticotrophin-(1–24)-tetracosapeptide was added as carrier and the samples were cooled and centrifuged at 200*g*

for 5min, and portions of the supernatant were subjected to chromatography by using constant molarity as described in the Materials and Methods section. A typical result is shown in Fig. 3. About 10% of the total radioactivity was present in peak III in all the samples, including the blanks, and this peak is therefore irrelevant to the assessment of breakdown. A small amount of breakdown was observed in the blank incubations (less than 20% in 2h) and this was presumably caused by peptidases in the albumin which are resistant to the trypsin inhibitor added. The results shown in Table 4 have been corrected for this breakdown and therefore show the contribution of the cell fractions to degradation.

Table 4 shows that corticotrophin-(1–24)-tetracosapeptide is broken down to a much greater extent by the supernatant than by either of the cell fractions. This shows the presence of an extracellular proteolytic enzyme which might be released from broken cells. It can also be seen that there is more rapid degradation in a concentrated cell suspension than in a dilute one. The possibility that the trypsin used for dispersing the cells might contain proteolytic enzymes capable of degrading the peptides in the presence of trypsin inhibitor was tested by adding 50μl of the trypsin solution used for cell dispersal (0.25mg of trypsin/ml) to 0.95ml of KRBAI buffer and incubating at 37°C for 1h with 1ng of corticotrophin-(1–24)-tetracosapeptide/ml. The sample showed no loss of biological activity compared with a control incubation in KRBAI buffer alone.

Effect of cellular disruption. Freshly prepared adrenal cells were 'purified' as described under 'Methods'. The resulting cell suspension was divided into two batches. One was disrupted by agitation on a Whirlimixer (Fisons Scientific Apparatus Ltd., Loughborough, Leics., U.K.) for 10min (D), and the other was a control (C). Portions of batches D and C (1ml) were incubated with 50ng of labelled peptide for 5 and 30min. In addition 1ml of diluent (containing albumin) was incubated with 50ng of labelled peptide for 5 and 30min. The results are shown in Table 5. There is greater degradation with the disrupted cell preparation. This again suggests release of a proteolytic enzyme from damaged cells.

Effect of cell purification and of peptidase inhibitors on the sensitivity of the assay

Cells which had been purified by gentle centrifugation through 2% albumin (see the Materials and Methods section) were slightly more sensitive to corticotrophin-(1–24)-tetracosapeptide. A typical result is shown in Fig. 4. The effect of peptidase inhibitors was tested on unpurified cell suspensions, but as shown in Fig. 5 bacitracin had no effect and similar results were obtained for trasylol at 100μg/ml. Bacitracin was tested because, although it is not a recognized peptidase inhibitor, it has been suggested that it can inhibit the breakdown of glucagon by cell-membrane preparations (Illiano & Cuatrecasas, 1972). The results suggest that peptidases responsible

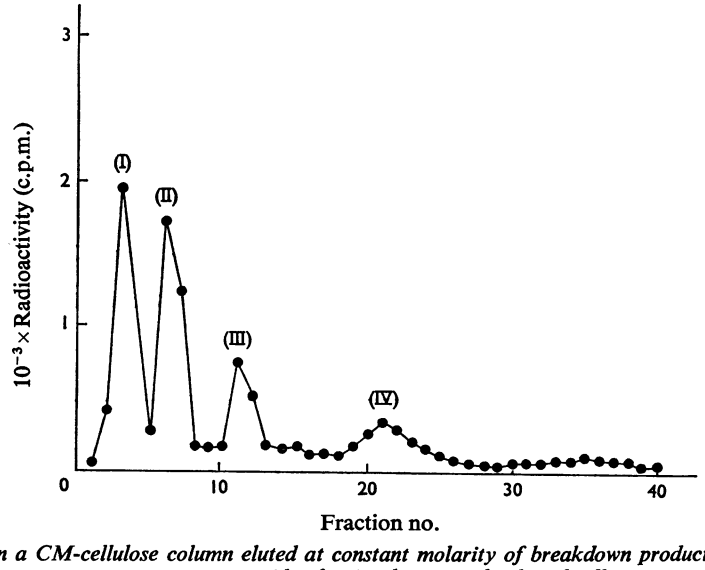

Fig. 3. *Separation on a CM-cellulose column eluted at constant molarity of breakdown products of corticotrophin-(1–24)-tetracosapeptide after incubation with adrenal cells*

A CM-cellulose column was eluted at constant molarity as described in the Materials and Methods section. Peak (IV) represents unchanged corticotrophin-(1–24)-tetracosapeptide.

Table 4. *Percentage of chromatographically separated breakdown products of corticotrophin-(1–24)-tetracosapeptide after incubation with adrenal cells*

A typical separation is shown in Fig. 3 in which the labelling of the peaks is indicated. Peak (IV) represents unchanged corticotrophin-(1–24)-tetracosapeptide.

		Percentage of breakdown products of corticotrophin-(1–24)-tetracosapeptide			
Time of incubation at 37°C		5min	30min	1h	2h
Treatment of cells	Peak				
A (supernatant)	(I)	12	29	39	
	(II)	11	29	26	
	(IV)	53	25	8	
B (small cells)	(I)	8	18	20	
	(II)	4	11	9	
	(IV)	85	62	53	
C (large cells)	(I)	0	7	8	
	(II)	2	8	7	
	(IV)	90	83	82	
M (unfractionated cells diluted 1:10)	(I)	13	37	50	
	(II)	14	19	13	
	(IV)	52	25	15	
D (unfractionated cells diluted 1:40)	(I)			19	30
	(II)			10	12
	(IV)			61	39

Table 5. *Percentage breakdown products of corticotrophin-(1–24)-tetracosapeptide after incubation with control and disrupted adrenal cells*

Peak (IV) represents unchanged corticotrophin-(1–24)-tetracosapeptide.

		Percentage of breakdown products of corticotrophin-(1–24)-tetracosapeptide	
Time of incubation at 37°C		5 min	30 min
Treatment of cells	Peak		
Control	(I)	1	10
	(II)	6	19
	(IV)	88	57
Disrupted	(I)	3	20
	(II)	17	32
	(IV)	79	33

for degrading corticotrophins in the cell suspension are not inhibited by trasylol or bacitracin and must in addition be resistant to the lima-bean trypsin inhibitor, which is always present in the incubation medium.

Binding of radioactivity to cells

Cells were purified as described in the Materials and Methods section and a concentrated suspension (containing cells derived from eight adrenals/ml) was incubated for 2min at 37°C with 10ng of [[$^3H_2$]-Tyr^{23}]corticotrophin-(1–24)-tetracosapeptide/ml. A portion of the whole suspension was counted and the

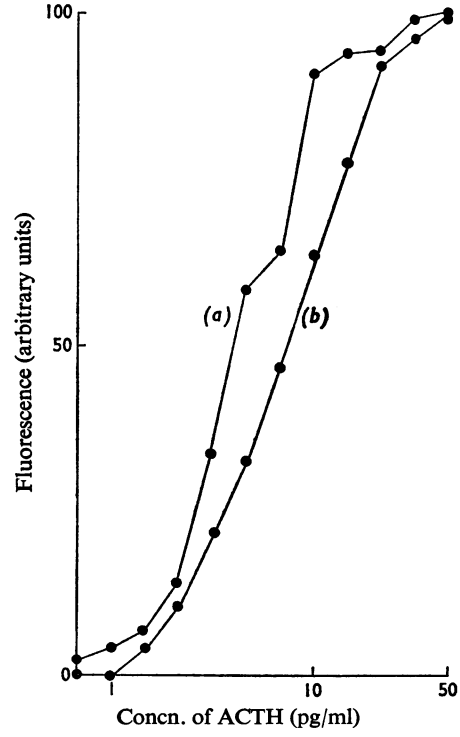

Fig. 4. *Log dose–response curves*

(*a*) Cells purified as described under 'Methods'; (*b*) unpurified cells. The cells were incubated with serial dilutions of corticotrophin-(1–24)-tetracosapeptide and assayed for steroids as described in the Materials and Methods section.

cells were then immediately centrifuged at 200*g* for 5min at room temperature and a portion of the cell-free supernatant was counted for radioactivity. The results are shown in Table 6. Although the radioactivity in the supernatant is apparently slightly less than that in the whole cell suspension, in view of the low values of the radioactive counts the difference is probably not significant. The safest conclusion to draw from these experiments would therefore be that no more than 10% of the peptide was bound to the cells.

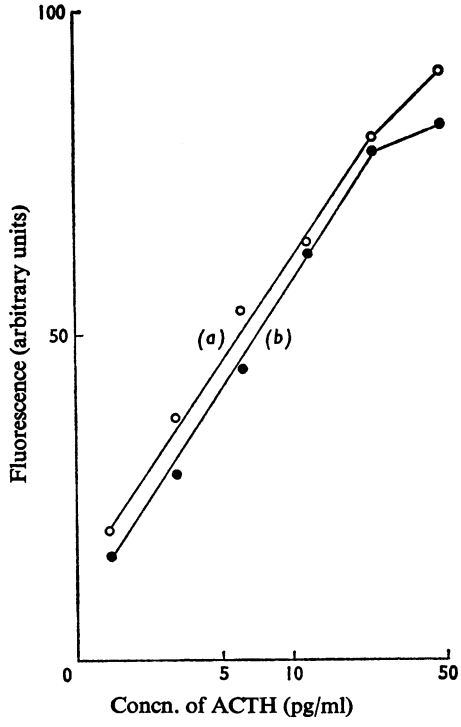

Fig. 5. *Effect of bacitracin on a log dose–response curve to corticotrophin-(1–24)-tetracosapeptide*

(*a*), Control without bacitracin; (*b*) cell suspension containing 0.5mg of bacitracin/ml.

Table 6. *Binding of* [[^3H]*Tyr*^{23}]*corticotrophin-(1–24)-tetracosapeptide to adrenal cells*

The peptide was briefly incubated at a concn. of 10ng/ml with purified isolated adrenal cells as described in the Results section and the radioactivity in the extracellular fluid (supernatant) was compared with that in the whole cell suspension. Results from duplicate incubations are shown. Each sample was counted for 100min to accumulate at least 3000 counts. The results are corrected for background.

Sample	Radioactivity of sample (c.p.m./0.5ml)
Cell suspension	35
	34
Supernatant	31
	33

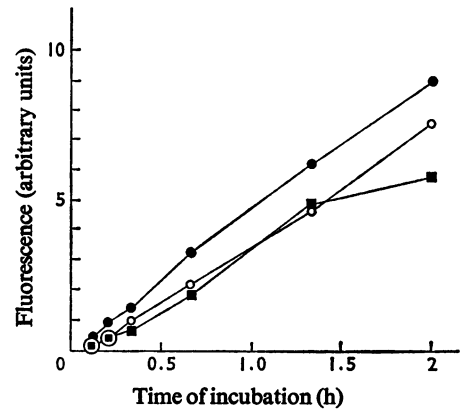

Fig. 6. *Graph showing time-course of fluorogenic corticosteroid production in isolated adrenal cell suspensions*

Corticosteroid production in response to (●), 1ng of corticotrophin-(1–24)-tetracosapeptide/ml, (○), 10pg of corticotrophin-(1–24)-tetracosapeptide/ml, (■), 30pg of [D-Ser^1, Lys^{17},^{18}]corticotrophin-(1–18)-octadecapeptide amide/ml.

Time-course of steroid production

Fig. 6 shows the accumulation of fluorogenic steroids during a 2h period after addition of corticotrophins to a dilute cell suspension. The results show that the rate of formation of steroid in response to submaximal doses of corticotrophin-(1–24)-tetracosapeptide and [D-Ser^1, Lys^{17}, Lys^{18}]corticotrophin-(1–18)-octadecapeptide amide is approximately constant over the 2h period. This result is similar to that obtained by Swallow & Sayers (1969) using 1–39 natural corticotrophin.

Discussion

The results show that, even in the presence of lima-bean trypsin inhibitor, isolated adrenal-cell suspensions can rapidly degrade corticotrophins. The rate of breakdown can be decreased by using dilute cell suspensions (Table 4) and this is presumably the explanation of the findings of Lowry *et al.* (1973) that the assay was more sensitive in dilute cell suspensions. The decreased rate of degradation at low cell concentrations is also consistent with the approximately linear time-courses of steroid production found in

Fig. 6. The present results show that the rate of breakdown can also be greatly diminished by purification of the cells by centrifugation through albumin and that a purification step of this sort will also increase the sensitivity of the response to corticotrophin-(1–24)-tetracosapeptide. When cell purification is combined with the use of a dilute cell suspension one can expect the effects of degradation on the response of the assay to be minimal.

Comparison of the inactivation of synthetic 1–39 corticotrophin and corticotrophin-(1–24)-tetracosapeptide (Table 1) shows that the latter is more susceptible to proteolytic attack.

Differences in the stability of corticotrophins have also been observed in plasma by Imura *et al.* (1967), where the shorter-chain analogues also proved to be more labile. These findings suggest that the natural hormone is stabilized by the *C*-terminal peptide sequence, possibly as the result of the formation of a certain amount of secondary or tertiary structure.

One of the reasons for carrying out the preset investigation was to try to understand the greater potency of the tetracosapeptide compared with the natural sequence which was observed in the isolated cell bioassay by using dilute cell suspensions (Lowry *et al.*, 1973). The shorter peptide was consistently five to seven times more potent, a result which did not agree with findings *in vivo* (Schuler *et al.*, 1963) or *in vitro* (Sayers *et al.*, 1971; Kitabchi & Sharma, 1971), where the peptides were virtually equipotent.

In view of the fairly low rate of degradation of the tetracosapeptide in dilute cell suspensions and the even lower rate of inactivation of the 1–39 sequence it seems certain that the shorter peptide is a more potent stimulator of the receptors in the isolated adrenal cells used. It is possible that in other assays the shorter peptide is more extensively degraded and the difference in its stability compared with the natural peptide results in an apparent decrease in its relative potency.

The peptide fragments formed during incubation show that the enzymes involved have a predominantly tryptic specificity, i.e. cleaving Arg^{8}–Trp^{9}, Lys^{16}–Lys^{17} and Lys^{21}–Val^{22} bonds. The fact that no free amino acids were released demonstrates that aminopeptidases are not present and that carboxypeptidases capable of liberating *C*-terminal arginine or proline must also be absent.

The results showed that the enzymic activity was present in the extracellular fluid but could be released from disrupted cells (Tables 4 and 5). Matsuyama (1969*a*,*b*) found that corticotrophin was mainly inactivated by a microsomal fraction from adrenal homogenates, and although our experiments differ because a trypsin inhibitor is present it is likely that the microsomal peptidase he observed is the same as the tryptic-like peptidase found in the present experiments. As the breakdown of corticotrophin is greatly decreased when purified cells are used it is unlikely that the enzyme plays a role in the catabolism of corticotrophins under normal physiological conditions.

The observations on the binding of tritiated corticotrophin-(1–24)-tetracosapeptide to cells (Table 6) show that at a physiological concentration the proportion of the peptide bound must be less than 10% (1 pg/ml). The cell suspension contains at least 100000 cells/adrenal (Lowry *et al.*, 1973) and as cells from eight adrenals were present in each ml this means that less than 1 pg was bound to more than 1.6×10^{6} cells. The molecular weight of the peptide is about 3000, so that 1 pg consists of

$$\frac{6 \times 10^{23} \times 10^{-12}}{3 \times 10^{3}} = 2 \times 10^{8} \text{ molecules.}$$

It would appear therefore that on average fewer than 120 receptors are occupied in each cell under conditions where an approximately 80% response was induced. This value may, however, give a false impression of the total number of receptors per cell, since only a fraction of the receptors may need to be occupied to produce a maximal response (the spare receptor concept, see Stephenson, 1956) and in addition some of the cells may not respond to corticotrophins and may not have receptors.

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