Metabolism of Two Adrenocorticotrophin Analogues in the Intestine of the Rat

By P. J. LOWRY and C. McMARTIN

Research Division, CIBA Laboratories, Horsham, Sussex RH12 4AB, U.K.

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1. At 30 min after oral administration unchanged Synacthen [corticotrophin-(1–24)-tetracosapeptide] was found in the stomach but could not be detected in the lumen of the small intestine of the rat. 2. Synacthen and 41795-Ba {[D-Ser¹, Lys¹⁷, Lys¹⁸]corticotrophin-(1–18)-octadecapeptide amide} were rapidly metabolized *in vitro* by both intestinal juice and everted pieces of small intestine. Peptide products were not found either in the intestinal tissue or in the fluid bathing the serosal tissue. 3. Glucose but not O₂ was necessary for the breakdown of the two adrenocorticotrophin analogues by everted tissue. 4. When the products obtained after partial digestion were chromatographically separated and identified, a pattern of breakdown emerged. The *N*-terminus of Synacthen and the Phe(7)–Arg(8) bond in both analogues were particularly labile. The D-serine *N*-terminal residue of 41795-Ba conferred a marked protection to aminopeptidase action. 5. The relative susceptibilities of peptide bonds would have been difficult to predict on the basis of existing knowledge of the properties of enzymes of the small intestine.

It is known that ACTH* analogues given orally to rats in large quantities cause steroidogenesis (P. A. Desaulles, CIBA–Geigy, Basle, Switzerland, unpublished work). Thus it appears that these peptides are absorbed from the gut, but only in small amounts. The poor absorption might be due either to extremely rapid breakdown of the peptide in the lumen of the gut or at the gut wall, or to slow transference of intact peptide through the epithelial cells. Probably a combination of these processes limits the absorption.

To find out more about the fate of ACTH analogues when given orally, we have investigated their breakdown in the rat intestine *in vivo* and *in vitro*.

Materials and Methods

Materials

CM-cellulose (CM-32) was obtained from Whatman Biochemicals Ltd., Maidstone, Kent, U.K. AG-50 (X4; less than 400 mesh) and Bio-Gel P-6 (200–400 mesh) were obtained from Bio-Rad Laboratories, Richmond, Calif., U.S.A. Trimethylamine and triethylamine were obtained from BDH Chemicals, Poole, Dorset, U.K.

Where possible all other reagents were A.R. grade.

Synacthen [corticotrophin-(1–24)-tetracosapeptide] and 41795-Ba {[D-Ser¹, Lys¹⁷, Lys¹⁸]corticotrophin-(1–18)-octadecapeptide amide} were kindly supplied by Dr. W. Rittel, CIBA–Geigy, Basle, Switzerland.

* Abbreviation: ACTH, adrenocorticotrophin.

Purification of reagents

Trimethylamine was distilled at 4° from anhydrous liquid trimethylamine containing 10% (v/v) acetic anhydride and stored at 4°C as a concentrated aqueous solution (approx. 3M). Triethylamine was distilled over ninhydrin (10g/l) and the fraction boiling at 88–90°C was collected.

Ninhydrin reagent, as used in the Technicon Automatic Amino Acid Analyser, was used to monitor column effluents.

Separation and purification of peptides

Peptides were separated by column chromatography. All columns were run by means of constant-flow pumps, Technicon multichannel peristaltic pumps with the Bio-Gel and CM-cellulose columns and a Milton–Roy pump with sulphonated polystyrene resin columns. Portions (one-fifth to one-tenth) of the column effluents were continuously monitored by the ninhydrin reaction and the major portion was collected as 2ml fractions. Typical flow rates into the analyser were 0.05ml of sample/min, 0.16ml of ninhydrin reagent/min and 0.10ml of N₂/min. The heating coil was a 2-metre length of Teflon tubing (0.75mm internal diam.) sufficient to give a time-delay of about 10min. This coil was heated in refluxing dibromomethane (b.p. 95–100°C) or the Technicon oil bath, through which N₂ was continuously bubbled.

The proportion of eluate that was not used for the ninhydrin assay passed through a 2.5cm-path-length

cell in a Uvichord II spectrophotometer before fraction collection. The cell was similar in construction to that described by McMartin *et al.* (1969), but it had silica windows (Jencons; H52/2; 20mm), and these were sealed by means of neoprene 'O' rings which fitted into circular grooves cut in the ends of the Teflon body.

The molarities of buffers refer to the concentration of base, acid being added to obtain the desired pH. All buffers contained 0.03% toluene.

Peptides were obtained by freeze-drying the appropriate fractions, and their amino acid composition was determined, after acid hydrolysis (6M-HCl, 115°C, 16h *in vacuo*), by using the Beckman 120C amino acid analyser. Before acid hydrolysis the extinction at 280.9 and 289.0nm was measured for the calculation of tryptophan content (Edelhoch, 1967). In most peaks a single peptide fragment could be identified on the basis of its amino acid composition. Where mixtures of peptides occurred the components were assumed to be those that fitted the analyses and were compatible with the tendency of peptides to be eluted in the order of their basic character.

(1) *Bio-Gel P6 columns*. A column (50cm × 1.2cm) was packed in 1M-acetic acid. The column was eluted with 1M-acetic acid at a flow rate of 0.1 ml/min, since this solvent had been shown to be suitable for the fractionation and purification of small amounts of peptides by Lowry & Chadwick (1970).

(2) *CM-cellulose column*. Fractionated microgranular CM-cellulose (Whatman CM-32) was prepared and packed in glass columns (McMartin & Vinter, 1969).

(a) *High-resolution preparative column*. A column (30cm × 1.2cm) was packed with CM-cellulose in 0.15M-triethylamine-acetate buffer, pH8.5. When this buffer was used the high packing flow rates used with inorganic buffers (McMartin & Vinter, 1969) were found to be unnecessary. The column was water-jacketed and kept at 52°C throughout the run. A two-chamber gradient device was used. The mixing chamber was a 250ml cylindrical Dreschel bottle (5cm diam. × 14cm tall) containing initially 250ml of 0.15M-triethylamine-acetate buffer, pH8.5, and the final buffer chamber was a 500ml Technico volumetric flask filled to the same level as the Dreschel bottle (approx. 500ml) with 0.57M-triethylamine-acetate buffer, pH10.6. The top half of the neck of the volumetric flask was cut off so that the chambers could be connected by a siphon in the shape of an inverted U. After leaving the gradient device the buffer was degassed as it passed through a length of Teflon tubing and a glass trap which were heated in refluxing chloroform. The column was run at a flow rate of 0.23 ml/min. After regeneration with starting buffer the column could be used repeatedly, samples being applied by simply pumping them on to the column.

(b) *Analytical determination of Synacthen*. Small CM-cellulose columns (5cm × 0.6cm) were used for the semiquantitative determination of Synacthen and its breakdown products. Sodium phosphate buffer, pH7.4 (0.05M), containing 0.2M-NaCl, at a flow rate of 0.1 ml/min eluted Synacthen from the column after approx. four bed volumes.

(3) *Sulphonated polystyrene columns*. For the separation of acidic and neutral peptides AG-50W (X4; less than 400 mesh) was fractionated in 0.5M-NaHCO₃ by the method of Hamilton (1958) to give a bead diameter of 20 ± 5 μm. The column (46cm × 0.6cm) was run at 52°C. The gradient was prepared by a two-chamber device. The mixing vessel was a Portland Plastics Polyacrylic beaker, 105cm tall with a 55cm internal-diameter circular base and a 73cm internal-diameter circular top and straight sides. Initially this chamber contained 300ml of pH2.85 buffer (60mm-trimethylamine, 0.17M-acetic acid, 0.4M-formic acid). The final buffer chamber was a 250ml conical flask (Davisil, narrow-necked) containing 250ml of 0.4M-trimethylamine-acetate buffer, pH7.0. The chambers were connected by a siphon and the column was eluted at a flow rate of 26 ml/h.

Methods for separating peptides

Since the procedure for fractionating peptides played a large part in the subsequent experiments some important aspects of the method are described below.

Satisfactory separation of the peptide fragments formed from Synacthen and 41795-Ba was accomplished by using two types of column in succession. A CM-cellulose column was used for the basic peptides, and the acidic and neutral peptides which were rapidly eluted from this column as an unresolved band were then applied to a sulphonic acid resin column.

Elution was carried out by using trimethylamine and triethylamine volatile buffers. The use of these bases in buffers has been described by Porath (1955). These buffers offer advantages over pyridine, since they do not oxidize peptides (Lowry & Chadwick, 1970) or absorb at 280nm, and unlike NH₃, when purified do not react with ninhydrin. In addition they are very volatile.

Simple distillation did not completely eliminate ninhydrin-positive material, but treatment of trimethylamine with acetic anhydride to acetylate primary and secondary amines before distillation was effective in eliminating ninhydrin-reactive material. With triethylamine, addition of ninhydrin before and during distillation resulted in a distillate free of ninhydrin-positive compounds.

Although CM-cellulose columns when tested at room temperature in a constant buffer had efficiencies of around 100 theoretical plates/cm for amino acids, the efficiency for Synacthen was never more than

20 theoretical plates/cm even at very low flow rates (3ml/h per cm²). Raising the temperature to 52°C resulted in higher efficiencies for Synacthen (50 theoretical plates/cm at 15ml/h per cm²), although this change did not increase the efficiency towards amino acids.

At the elevated temperature bubbles of air began to form after a few hours in the columns that were pumped at low pressures. To remove dissolved gases the buffer was passed through Teflon tubing and a glass trap heated in refluxing chloroform. The trap consisted of an elongated glass bulb with a capacity of approx. 3ml with Teflon tubing sealed into the upper and lower extremities. The upper and lower halves of the bulb were made separately and the tubing was drawn into tight-fitting holes before the halves were fused together. After a couple of hours of use no more gas would appear in the trap and it seemed most likely that the dissolved gas diffused through the Teflon tubing, since the surrounding atmosphere would be pure chloroform vapour. This explanation is confirmed by the fact that when the ninhydrin reaction was carried out in a Teflon coil heated in an oil bath the colour yield was decreased by a factor of three unless N₂ was flushed through the oil. Presumably O₂ diffused through the Teflon coil and destroyed the coloured ninhydrin complex.

Procedures for separating neutral peptides in the presence of amino acids must have high resolution and the use of small-bead-size 4% cross-linked resin and the trimethylamine-formate-acetate buffer appears to satisfy these requirements. The very sharp peaks obtained even with the *N*-terminal heptapeptide of ACTH may be a result of the use of this organic buffer.

The recovery of peptides from both the sulphonic acid and CM-cellulose columns was quantitative provided that polystyrene fraction-collector tubes were used, and down to 10nmol of peptide could be detected with ease by the ninhydrin monitoring system.

Animal experiments

In vivo. Male Wistar rats (100–200g) were lightly anaesthetized with ether. Synacthen (20mg) with Phenol Red (1mg) as a marker substance in 1ml of water was introduced into the stomach of each rat by means of a fine polyvinyl tube (1.6mm internal diam.) connected to a syringe. After 30min the animals were killed by decapitation. The stomach and small intestine were separately tied off, excised quickly and the contents were washed out with 10ml of 0.1M-acetic acid. Samples (0.3ml) from each were added to 2.7ml of 0.1M-NaOH and the amount of Phenol Red was determined by measuring the extinction at 530nm. The remainder of the samples were freeze-dried. Samples were analysed on a column (1.2cm × 50cm)

of Bio-Gel P-6 equilibrated with 1M-acetic acid. The eluate was monitored in the normal way by the ninhydrin reaction.

In vitro. Male Wistar rats were killed by decapitation and the small intestine was quickly excised. Each intestine was washed out with 10ml of water and the suspension was centrifuged for 10min at 600g. The supernatant was used as the concentrated contents of the small intestine. A tenfold dilution of this solution was used to obtain slow digestion of added peptides.

Peptides were also incubated with 2.5cm segments of everted small intestine. Fresh everted tissue was washed with Krebs-Ringer bicarbonate buffer (Laser, 1961) containing 0.1% glucose. It was then incubated, with shaking, in 2ml of Krebs-Ringer bicarbonate buffer containing 0.1% glucose and 20mg of peptide for 30min at 37°C.

In the experiments *in vitro* digestion was stopped by the addition of the sample to 2ml of ice-cold 10% (w/v) trichloroacetic acid to give a final concentration of 5% trichloroacetic acid and after centrifuging the supernatant was applied directly to chromatographic columns. Some experiments were carried out to determine the uptake and transport of peptides and amino acids by the everted sac of the small intestine. A piece (2.5cm) of everted tissue containing 0.2ml of Krebs-Ringer bicarbonate buffer was tied at both ends and placed in 2ml of buffer containing 20mg of Synacthen and incubated as described above. The intact sac was thoroughly washed in buffer and then the serosal contents were collected and treated with trichloroacetic acid as described above. The tissue was washed again and homogenized in ice-cold 5% (w/v) trichloroacetic acid with an ultra-Turrax homogenizer (Janke und Kunkel, Staufen i. Br., W. Germany), and the homogenate was centrifuged at 600g for 10min and the supernatant was collected. Peptides in these two extracts were investigated by using the small CM-cellulose columns as described under 2(b) above.

Recoveries of peptides added to samples immediately before trichloroacetic acid precipitation were around 50%.

Results

Experiments in vivo

Fig. 1 shows the analyses on Bio-Gel P-6 of standards and typical samples of the contents of rat stomach and small intestine collected 30min after the administration of Synacthen and Phenol Red by stomach tube. The chromatograph of stomach contents, which contain 50% of the administered Phenol Red, showed a peptide peak of similar molecular weight to Synacthen, judged by its elution volume, the area of which would correspond to 50% of the administered peptide. Amino acid analysis of this

Fig. 1. Amounts of Synacthen in rat stomach and intestine 30 min after oral administration *in vivo*

(a) Standard Bio-Gel P-6 chromatograph showing the retention times and peak sizes of Synacthen (Syn) (1 mg) and glycine (Gly) (20 μ g). (b) Bio-Gel P-6 chromatograph of one-tenth of the stomach contents of a rat 30 min after oral administration of 20 mg of Synacthen. (c) Bio-Gel P-6 chromatograph of one-tenth of the stomach contents of an untreated rat. (d) Bio-Gel P-6 chromatograph of one-tenth of the contents of the small intestine of a rat 30 min after oral administration of 20 mg of Synacthen.

Fig. 2. Separation of peptide fragments formed from Synacthen during incubation with intestinal juice

(a) CM-cellulose chromatograph (see the Materials and Methods section under 'High resolution preparative column'). (b) Sulphonic resin chromatograph of the products in bands X and Y of Fig. 2 (a). —, Band X; ----, band Y. Peptides found in peaks A–J are indicated in Fig. 4.

peptide showed that it contained amino acids corresponding to the full 1–24 sequence. Although 25% of the Phenol Red was present in the sample from the small intestine, neither Synacthen nor an appreciable increase in amino acids and small peptides could be detected.

Experiments *in vitro*

(a) *Digestion with intestinal fluid.* The rate of disappearance of intact Synacthen was measured by using the analytical CM-cellulose column system. Incubation of 10 mg of Synacthen at 37°C with 1 ml of

a 1:10 dilution of a suspension of the contents of the lumen of the small intestine in 10ml of water resulted in the loss of 50% of the peptide in 30min.

These conditions were used for the hydrolysis of 20mg each of Synacthen and 41795-Ba, portions of

the same sample of intestinal juice being used. Chromatograms of the peptides formed are shown in Figs. 2 and 3. Chromatograms of intestinal juice incubated without added peptide showed small amounts of amino acids and negligible quantities of

Fig. 3. Separation of peptide fragments formed from 41795-Ba during incubation with intestinal juice

(a) CM-cellulose chromatograph (see the Materials and Methods section under 'High resolution preparative column'). (b) Sulphonic resin chromatograph of the products in bands X and Y of Fig. 3(a). —, Band X; ----, band Y. Peptides found in peaks A-U are indicated in Fig. 5.

Fig. 4. Diagram showing peptides and amino acids formed from Synacthen by intestinal juice

Double horizontal lines show major products, single horizontal solid lines products found in intermediate amounts and dashed horizontal lines minor products as judged by the peak area on the chromatographs. The letter above each horizontal line indicates the peak in Fig. 2 in which the peptide was found. The histogram shows free amino acids corrected for blank values obtained when intestinal juice was incubated without added peptide. Where an amino acid occurs in several places the measured quantity has been distributed so as to be consistent with adjacent amino acid contents and the peptide pattern.

Fig. 5. Diagram showing peptides and amino acids formed from 41795-Ba by intestinal juice

The letter above each horizontal line indicates the peak in Fig. 3 in which the peptide was found and the histogram represents free amino acids which have been allocated, as described in the legend of Fig. 4.

Fig. 6. Separation of peptide fragments formed from Synacthen during incubation with everted small intestine

(a) CM-cellulose chromatograph (see the Materials and Methods section under 'High resolution preparative column').
(b) Sulphonic resin chromatograph of the products in band X (Fig. 6a). Peptides found in peaks A-J are indicated in Fig. 8.

peptides. Figs. 4 and 5 show the amino acids and peptides released from Synacthen and 41795-Ba in the intestinal juice.

(b) *Digestion with everted sac.* Measurement of the amount of unchanged Synacthen in solution showed that incubation of everted sac at 37°C resulted in the loss of 50% of the 20mg of added Synacthen in 30 min.

Some noteworthy observations were made about the conditions of incubation. It was found that the presence of a small amount of glucose was essential for hydrolysis to occur, although O₂ was not required, since breakdown proceeded as rapidly when the incubation was carried out under N₂. Measurement of peptides and amino acids in the serosal fluid, the

tissue and the mucosal fluid revealed that all the increase in peptide products and amino acids occurred in the mucosal fluid.

Chromatograms of the products formed from Synacthen and 41795-Ba on incubation with adjacent everted segments taken from the middle of the small

intestine are shown in Figs. 6 and 7. Blank chromatograms of everted tissue incubated without added peptide showed small amounts of amino acids, but negligible quantities of peptides. Figs. 8 and 9 show the amino acids and peptides released from Synacthen and 41795-Ba on incubation with everted sacs.

Fig. 7. Separation of peptide fragments formed from 41795-Ba during incubation with everted small intestine (a) CM-cellulose chromatograph (see the Materials and Methods section under 'High resolution preparative column'). (b) Sulphonic resin chromatograph of band X (Fig. 7a). Peptides found in peaks A-S are indicated in Fig. 9.

Fig. 8. Diagram showing peptides and amino acids formed from Synacthen by everted small intestine The letter above each horizontal line indicates the peak in Fig. 6 in which the peptide was found and the histogram represents free amino acids which have been allocated, as described in the legend of Fig. 4.

Fig. 9. Diagram showing peptides and amino acids formed from 41795-Ba by everted small intestine

The letter above each line indicates the peak in Fig. 7 in which the peptide was found and the histogram represents free amino acids which have been allocated, as described in the legend of Fig. 4.

Discussion

Orally administered Synacthen produces steroidogenesis in the rat equivalent to that obtained with about one-hundredth of the same dose subcutaneously, or one-three-thousandth of the dose intravenously (P. A. Desaulles, CIBA-Geigy, Basle, Switzerland, unpublished work). May & Whaler (1958) have shown that the protein *Clostridium botulinum* type A toxin is absorbed from the upper, and to a lesser extent from the lower half of the small intestine of the rabbit, whereas absorption from the stomach only occurred in a few animals. Other investigators have reported that certain small peptides may be transferred intact from the lumen into the mucosal cells of the small intestine (Matthews *et al.*, 1969; Fern *et al.*, 1969). It seems likely therefore that the small intestine is the site of absorption of Synacthen after oral administration.

Our experiments show that breakdown of large doses (20mg) of Synacthen does not occur to an appreciable extent in the stomach. The absence of Synacthen from the small intestine after oral administration *in vivo* and its rapid hydrolysis by gut segments and intestinal juice suggest that Synacthen is rapidly broken down as soon as it leaves the

stomach. Metabolism of the peptide appears to occur on the mucosal side of the small intestine.

The peptide cleavage patterns (Figs. 4, 5, 8 and 9) obtained after very mild digestion with everted sac and intestinal juice show that a few bonds in Synacthen and 41795-Ba are particularly susceptible to hydrolysis. The enzymes in intestinal juice cleave Phe⁷-Arg⁸ and Arg⁸-Trp⁹ bonds extensively in both peptides. Since only a small amount of a single peptide fragment containing intact Phe⁷-Arg⁸ was found (D-Ser¹-Lys¹⁶) it seems likely that Phe⁷-Arg⁸ is the first bond to break and this is followed by cleavage of Arg⁸-Trp⁹ and His⁶-Phe⁷ bonds to release free amino acids. Examination of the peptides found shows that primary sites of hydrolysis also occur at Tyr²-Ser³, Lys¹⁵-Lys¹⁶-Arg¹⁷, Val²⁰-Lys²¹-Val²² in Synacthen and Tyr²-Ser³, Gly¹⁴-Lys¹⁵-Lys¹⁶-Lys¹⁷-Lys¹⁸-NH₂ in 41795-Ba. The extensive breakdown at these and other sites which might be expected from the pancreatic enzymes present in intestinal fluid (trypsin, chymotrypsin, carboxypeptidases) does not occur and the Phe⁷-Arg⁸ bond stands out as being the most susceptible region of the molecule.

Digestion with washed everted small intestine results in a breakdown pattern similar in many respects to that obtained with intestinal fluid. Exten-

sive endopeptidase action is evident at Phe⁷-Arg⁸ site in both peptides and partial cleavage occurs at many of the other sites that were attacked by intestinal juice. The everted sac differs from intestinal juice in releasing more free phenylalanine than arginine and in attacking the *N*-terminus of Synacthen and the Ser³-His⁶ region in 41795-Ba. D-Ser¹ affords remarkable protection of the *N*-terminus of 41795-Ba.

Thus only the everted sac has appreciable aminopeptidase activity, whereas endopeptidases responsible for cleaving the Phe⁷-Arg⁸ bond occur not only in the intestinal fluid but also in the intestinal mucosa.

A peptidase that can be inhibited by di-isopropyl phosphorofluoridate has been detected in the brush border by Woodley & Kenny (1969), and it is possible that this enzyme is responsible for the endopeptidase activity we have observed.

To summarize, our results suggest that the peptide analogues that we have studied are cleaved rapidly in the small intestine at a few specific sites. Peptides lacking such sensitive regions should therefore have an appreciably increased exposure to the absorptive processes of the small intestine.

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