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EVIDENCE THAT PROTEASE INHIBITORS REDUCE THE DEGRADATION OF PARATHYROID HORMONE AND CALCITONIN INJECTED SUBCUTANEOUSLY

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1 Agents known to delay absorption from a subcutaneous site were tested in chicks for their ability to prolong the hypercalcaemic response to parathyroid hormone (PTH).

2 Polyvinylpyrrolidone was found to enhance the response but gelatine greatly reduced the 2 h hypercalcaemia.

3 The reduction by gelatine was reversed when the protease inhibitor aprotinin was added to the injection vehicle, and hypercalcaemia then persisted for more than 8 h.

4 Of other protease inhibitors studied, ϵ-aminocaproic acid was also found to enhance the hypercalcaemic response to subcutaneous PTH and its fragments but, unlike aprotinin, it was ineffective in the presence of gelatine.

5 By radioimmunoassay and bioassay respectively, it was confirmed that aprotinin raised circulating levels of PTH and also of another peptide hormone, calcitonin, injected subcutaneously.

6 Addition of calcium to the solutions injected subcutaneously abolished the hypercalcaemic response to PTH while injection of calcium and PTH simultaneously but at separate sites left the response unaltered.

7 The two protease inhibitors, ϵ-aminocaproic acid and aprotinin, each restored the response to subcutaneous PTH despite the presence of calcium at the injection site.

8 It was concluded that protease inhibitors injected subcutaneously with PTH and calcitonin in the chick reduced the rate of degradation of these hormones and that the proteases responsible for hormone degradation at the subcutaneous injection site may be released or activated by calcium ions.

Introduction

The growing list of peptide hormones now administered for diagnostic or therapeutic purposes includes insulin, corticotrophin, oxytocin, growth hormone, calcitonin, parathyroid hormone, vasopressin and pentagastrin. Except for buccal and insufflative preparations (of oxytocin and vasopressin respectively), all these hormones are routinely administered by subcutaneous or intramuscular injection. Yet the kinetics of entry of these peptides to the circulation from a subcutaneous site and the extent to which they are metabolically altered before absorption have been little studied except in the case of insulin, and even then, only by isotopic or immunoassay methods which do not permit confident estimation of bioactive levels (Joiner, 1959; Binder, 1969). During experiments to develop a long-acting form of parathyroid hormone for subcutaneous injection in a clinical trial (Reeve, Hesp, Williams, Hulme, Klenerman, Zanelli, Darby, Tregear & Parsons, 1976), we observed that addition of enzyme inhibitors to the injected dose significantly increased the hypercalcaemic response in chicks. In the present paper a number of known enzyme inhibitors and related compounds have been compared in respect of their effect on biological responses and plasma levels following subcutaneous injection to chicks of parathyroid hormone and another hormone peptide, calcitonin.

A preliminary account of these results has been published (Parsons, Rafferty, Stevenson & Zanelli, 1977).

Methods

The substances tested in this study are listed in Table 1. The hormone preparations used were

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Sephadex-purified bovine PTH (prepared at NIMR and NIBSC by the method of Aurbach (1959), further purified in collaboration with Dr J.L.H. O'Riordan of the Middlesex Hospital and freeze-dried in ampoules coded 72/286), and two synthetic fragments representing the amino-terminal 1-34 residues of the bovine (bPTH 1-34) and human (hPTH 1-34) sequences (Potts, Tregear, Keutmann, Niall, Sauer, Deftos, Dawson, Hogan & Aurbach, 1971; Niall, Sauer, Jacobs, Keutmann, Segre, O'Riordan, Aurbach & Potts, 1974). Porcine calcitonin, (batch number VH 1711) was kindly donated by the Armour Pharmaceutical Company Ltd. Compounds used were ϵ-aminocaproic acid, 6-aminohexanoic acid and N,N-dibenzyl-ethylenediamine diacetate (Benzathine) (Koch-Light Laboratories Ltd), benzamidine hydrochloride (Ralph N. Emmanuel Ltd), (−)-noradrenaline tartrate (Levophed) and aprotinin (Trasylol), (Bayer Products Company), phenylmethylsulphonyl fluoride and soya bean trypsin inhibitor (Sigma Chemical Company), gelatine (Difco Laboratories, Detroit), protamine sulphate (International Reference Preparation), lysine vasopressin (Sandoz), isoprenaline sulphate (Burroughs Wellcome), aluminium hydroxide gel (Armour), and 5-aminopentanoic acid and 7-amino heptanoic acid, both kindly synthesized by Dr E.W. Gill, University Department of Pharmacology, Oxford. All other compounds used were supplied by BDH Chemicals Limited.

Insoluble complexes of bPTH 1-84 and hPTH 1-34 were formed with zinc (added in the ratio 5 atoms Zn/molecule PTH), with and without the presence of protamine (40 molecules/1 molecule PTH) by first dissolving the hormone in 0.1 M borate/acetate buffers (pH 5, 7 and 8) and readjusting the pH back to its original value with 1 N NaOH. On addition of 0.01 M $ZnCl_2$ and waiting 30 min to allow flocculation, precipitates were observed at pH 8 and (to a lesser extent) pH 7 but not at pH 5. These precipitates were recovered by centrifugation for 5 min and their hormone content estimated by assaying separately the supernatant fluids and the precipitates redissolved in acid.

A similar method was used to prepare Benzathine/PTH complexes but a precipitate formed only under very acid or very alkaline conditions (pH 0.1 or > 13.0). Even when precipitates formed, all assayable activity was found in the supernatant fluid.

The vehicle used for all injection solutions was sodium acetate trihydrate 10 mg/ml containing crys-

Table 1 Measures applied and substances tested to prolong or enhance the hypercalcaemic effect of parathyroid hormone (PTH) injected subcutaneously

Compounds forming insoluble derivatives	*Doses tested**
Protamine	1, 3 and 30 mg
$Zn(OH)_2$	5 atoms Zn/molecule PTH
Protamine + $Zn(OH)_2$	5 atoms Zn and 40 molecules Protamine/molecule PTH
$Al(OH)_3$ gel	10 atoms Al/molecule PTH
Compounds expected to complex PTH but not forming insoluble derivatives	
Dibenzylethylene diamine (Benzathine)	5 and 45 molecules/molecule PTH
Chondroitin sulphate	1 mg
Polylysine	3 mg
Polyethylene imine	3 and 30 mg
Agents increasing viscosity	
Polyvinylpyrrolidone (PVP)†	3, 10 and 30 mg
Gelatine	15%
Agents with vasoconstrictor activity	
Noradrenaline	2 and 20 μg
Vasopressin (ADH)	10 and 100 mu
Isoprenaline	2 and 20 μg

* By inclusion with the hormone in a subcutaneous injection of 0.4 ml.
† The only compound in this table which enhanced the hypercalcaemic response to parathyroid hormone.

talline bovine serum albumin (Armour) 1 mg/ml, adjusted to pH 7 with dilute HCl and heat-inactivated by placing it in a water bath at 56°C for 1 h.

The duration of action of bPTH 1-84, bPTH 1-34 and hPTH 1-34 and blood levels of calcitonin were investigated in 7 to 11 day old male chicks (Rhode Island Red × Light Sussex cross) weighing 50 to 70 g, fed a powdered balanced diet (MRC 41B). After deprivation of food overnight with access to tap water, the birds were injected subcutaneously, at a site on the lateral thorax, with 0.4 ml of a solution containing the appropriate hormone, using 25-gauge needles on 1 ml syringes. Blood samples were drawn by cardiac puncture with 23-gauge needles into 2 ml heparinised disposable syringes under light halothane anaesthesia (Fluothane, ICI) at predetermined times after injection. Needles were replaced by blind luer-fitting caps, the syringes centrifuged and plasma analysed for calcium (in the case of PTH-treated animals) by measurement of atomic absorption with a Perkin-Elmer spectrophotometer, model 303, using the modified method of Trudeau & Freier (1967), recommended by the manufacturers; or injected into rats (in the case of calcitonin-treated animals) as described below.

Assay of calcitonin levels in chick plasma

The blood levels of porcine calcitonin attained in the chick under the influence of protease inhibitors were measured by bioassay in the rat according to the method described by Kumar, Slack, Edwards, Soliman, Baghdiantz, Foster & MacIntyre (1965), with the exception that the unit of replication was a 'chick-rat pair'. Male Sprague-Dawley rats (50 to 60 g), deprived of food overnight but with free access to water, were injected intravenously via the tail vein with 0.5 ml chick plasma previously withdrawn by cardiac puncture as already described and kept at +4°C between centrifuging and the time of injection (usually 15 min after sampling). (It was found impractical to obtain sufficient plasma to inject groups of rats from each bird). Rats were bled by open-chest cardiac puncture (23-gauge needle on 2 ml heparinized syringe) 1 h after injection of the test and standard doses. Calcium levels were estimated by atomic absorption spectrophotometry as already described and plasma calcitonin levels in the original sample deduced by reference to the hormone standard used in the assay.

Table 2 Peptidase inhibitors and some related compounds tested to enhance the 2 h hypercalcaemic response to subcutaneous parathyroid hormone

	*Doses tested**
Marked enhancement	
Aprotinin (Trasylol)	300 and 3000 kiu, approx 40 and 400 μg
6-Amino-hexanoic acid (EACA)	1, 3, 10 and 30 mg
Some enhancement	
Benzamidine	3 and 30 mg
†5-Amino-pentanoic acid	3, 10 and 30 mg
†4-Amino-butanoic acid (GABA)	3 and 30 mg
†2-Amino-hexanoic acid (Nor-leucine)	3 and 30 mg
p-Amino-hippuric acid	3 and 30 mg
No enhancement	
†7-Amino-heptanoic acid	10 and 30 mg
†2-Amino-pentanoic acid (Nor-valine)	3 and 30 mg
†2-Amino-butanoic acid	3 and 30 mg
p-Amino-benzoic acid	3 and 30 mg
p-Tosylyl-arginine methylester (TAME)	0.3 and 3 mg
N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)	0.3 and 3 mg
Soya bean trypsin inhibitor	1 and 5 mg
Phenylmethylsulphonyl fluoride (PMSF)	0.4 and 4 mg

* By inclusion with the hormone in a subcutaneous injection of 0.4 ml.
† Indicates those compounds which were tested because of their structural relationship with EACA.

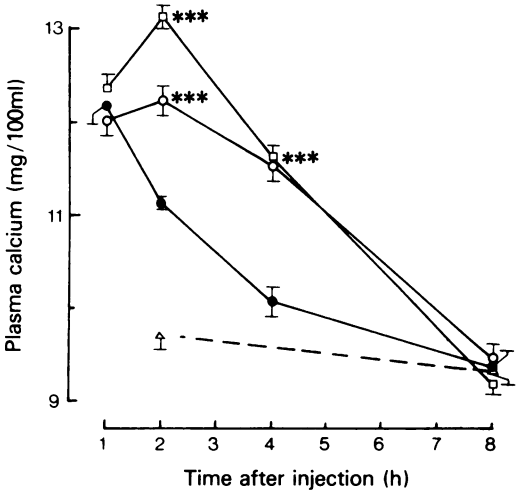

Figure 1 Effect of adding ϵ-amino caproic acid, 1 mg (○) or 10 mg (□), on the mean plasma calcium levels produced by subcutaneous injection of 4.5 μg hPTH 1-34 (●) to chicks. The effect of vehicle plus 10 mg ϵ-amino caproic acid (△) is represented by the broken line. Each point is the mean of six determinations and is shown with its standard error. $***P < 0.001$.

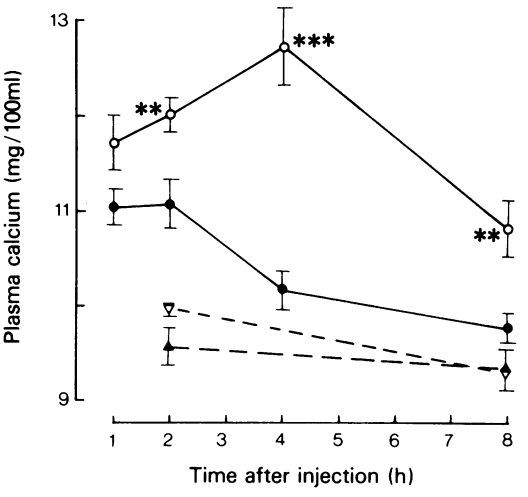

Figure 2 Effect of adding 300 kiu aprotinin (○) on the mean plasma calcium levels produced by subcutaneous injection to chicks of 4.5 μg hPTH 1-34 in 15% warmed gelatine (●). The effects of vehicle plus gelatine (▲) and vehicle plus gelatine plus aprotinin (▽) are represented by the broken lines. Each point is the mean of six determinations and is shown with its standard error. $**P < 0.01$; $***P < 0.001$.

Radioimmunoassay of parathyroid hormone levels in chick plasma

The amounts of bPTH 1-84 and hPTH 1-34 reaching the circulation were determined by non-equilibrium radioimmunoassay, essentially by the method of Segre, Tregear & Potts (1975). Iodination of the hormone preparations was carried out with slight modifications of the chloramine T method (Hunter & Greenwood, 1962), tracers being purified on biogel P100 (bPTH 1-84) or biogel P10 (hPTH 1-34) immediately before use. Guinea-pig anti-PTH serum 211/32 was used, raised against partially purified bPTH 1-84 and prepared under a collaborative arrangement between Burroughs Wellcome and the Medical Research Council. This antiserum has been shown to recognize determinants both within the hPTH 1-34 fragment (the region of the molecule required for biological activity) and in the carboxy-terminal two-thirds of the molecule. Serum from vehicle-injected chicks was included in the assay to allow appropriate correction for incubation damage or non-specific binding.

Studies to exclude complexing of calcium by aprotinin

Determination of the extent of possible sequestration of calcium by aprotinin at the site of injection was investigated by the method of Hummel & Dreyer (1962). A column of Sephadex G-50 was equilibrated with Tris buffer (pH 7.4) containing 1.25 mM $CaCl_2$. Aprotinin (purified by gel filtration on a Sephadex G-50 column and lyophilised) was dissolved in the buffer at a concentration of 6.25 mM and added to the column. Its elution was detected by u.v. spectrophotometry (Pye Unicam SP500) at 280 nm and compared with serial measurements of the calcium concentration of the eluate, determined by atomic absorption spectrophotometry as previously described.

Results

Preliminary investigations are summarized in Table 1 which lists the measures tested to delay absorption of injected PTH and its fragments, either by formation of an insoluble complex or by utilising the retarding effect of viscosity on absorption. As shown, a variety of substances with local vasoconstrictor activity were also tested.

The only agent among those listed in Table 1 which proved to prolong the hypercalcaemic action of bPTH 1-84 and hPTH 1-34 was polyvinylpyrrolidone. The fact that gelatine caused no prolongation left it uncertain whether the effect of polyvinylpyrrolidone was indeed due to greater viscosity of the injection

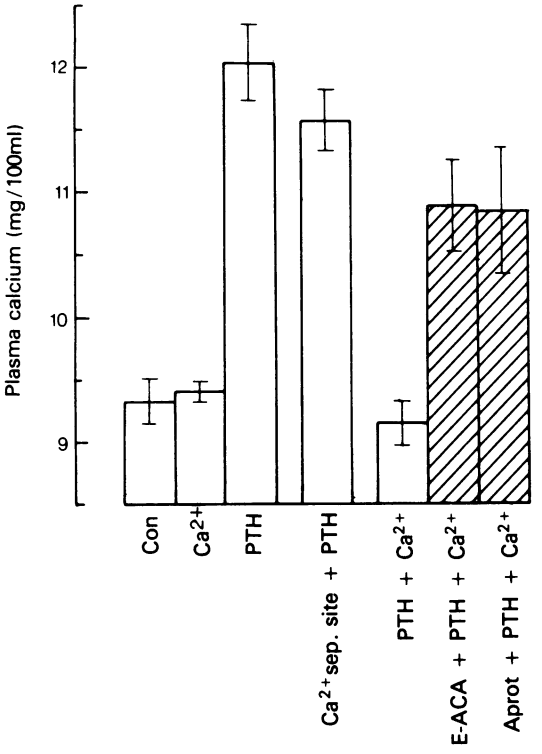

Figure 3 Mean plasma calcium levels measured 1 h after subcutaneous injection of vehicle alone (Con); vehicle plus 50 μmol calcium (Ca^{2+}); 4.5 μg hPTH 1-34 (PTH); PTH plus calcium injected at separate sites; PTH plus calcium combined; PTH plus calcium plus 10 mg ϵ-amino caproic acid (E-ACA) combined; and PTH plus calcium plus 300 kiu aprotinin. Each column is the mean of six determinations and is shown with its standard error.

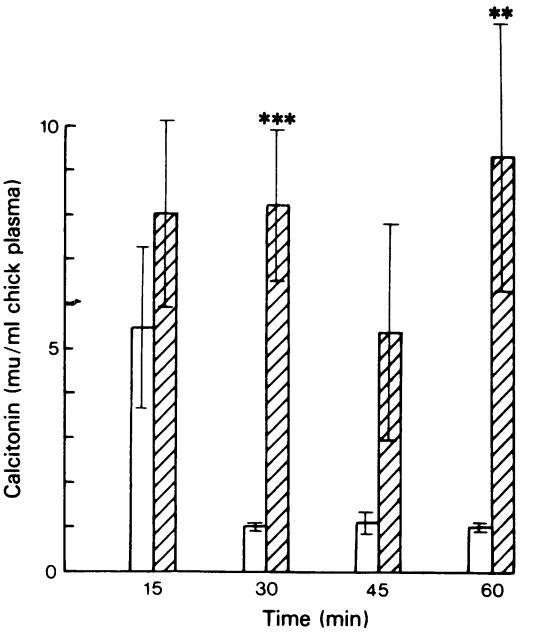

Figure 4 Effect of 300 kiu aprotinin on the bioactive calcitonin levels achieved in chick plasma at various times after subcutaneous injection of 30 iu calcitonin. Open columns, calcitonin alone; hatched columns, calcitonin plus aprotinin. Each column is the mean of six determinations and is shown with its standard error. The concentrations of calcitonin in chick plasma samples were measured by the rat intravenous hypocalcaemia bioassay (see text). $^{**}P < 0.01$; $^{***}P < 0.001$.

medium or reflected some quite different property of the molecule.

Since polyvinylpyrrolidone might be reducing enzymatic destruction of PTH by tissue proteases at the site of injection, we tested a variety of known enzyme inhibitors and related compounds as listed in Table 2. The plasmin inhibitor ϵ-amino caproic acid markedly potentiated the hypercalcaemic response to PTH and its fragments, measured at 2 h ($P < 0.001$) and 4 h ($P < 0.001$) after injection (Figure 1). A significant increase was also seen at 4 h with the kallikrein inhibitor aprotinin ($P < 0.001$). When ϵ-amino caproic acid or aprotinin alone was added to the injection vehicle there was no change in calcium from control values. Lesser, but nonetheless statistically significant enhancement of the response was also caused by the higher doses of 5-aminopentanoic acid, 4-aminobutanoic acid, *p*-aminohippuric acid, 2-aminohexanoic acid and Benzamidine at 2 and 4 h (Table 2).

Although when added alone, gelatine reduced the response to hPTH 1-34 at 2 and 4 h after injection, when administered in conjunction with aprotinin it enhanced the effect at 4 h ($P < 0.001$) and prolonged it to 8 h ($P < 0.01$), by which time the effect of PTH alone had disappeared (Figure 2). No such enhancement of the response to PTH and gelatine was seen when ϵ-amino caproic acid was added to the injection medium ($P > 0.5$). When $CaCl_2$ (50 μmol) was added to the injection medium (without gelatine), even the 1 h hypercalcaemic response to hPTH was almost abolished (Figure 3). An identical dose of $CaCl_2$ injected subcutaneously at a separate site left the hypercalcaemic response unaffected. The inhibitory effect of subcutaneous calcium was overcome by addition of aprotinin or ϵ-aminocaproic acid to the solution injected (Figure 3). When aprotinin (purified by gel filtration) was eluted from a column of Sephadex G-50, previously equilibrated with calcium chloride,

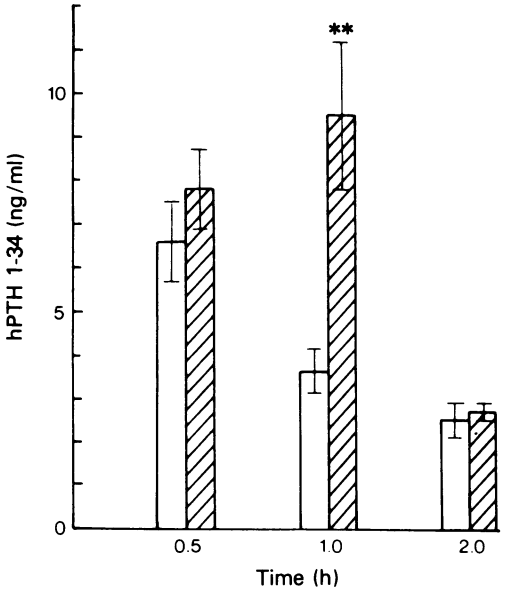

Figure 5 The effect of 300 kiu aprotinin on the mean plasma levels of immunoreactive hPTH 1-34 achieved at various times after subcutaneous injection of 4.5 μg hPTH 1–34. Open columns, hPTH 1-34 alone; hatched columns, hPTH 1-34 plus aprotinin. Each column is the mean of six determinations and is shown with its standard error. $^{**}P < 0.01$.

the concentration of calcium in the eluate remained unaltered in the fractions where the aprotinin was detected by its ultraviolet absorption.

Circulating levels of hormone measured at various intervals after injection in two types of experiment

(1) The concentration of calcitonin in the plasma of chicks was determined at various time intervals after subcutaneous injection of the hormone with and without addition of aprotinin. As shown in Figure 4, the plasma concentrations of calcitonin 30 and 60 min after the injection were significantly ($P < 0.001$ and $P < 0.01$ respectively) higher in birds which received calcitonin plus aprotinin than in those which received calcitonin alone.

(2) In birds which received subcutaneous injections of the amino-terminal synthetic fragment hPTH 1-34, a homologous radioimmunoassay showed higher blood levels 1 h post-injection when aprotinin was added than in birds which received no inhibitor. The effect (Figure 5) was highly significant ($P < 0.01$). A similar increase in the mean hormone level after injection was seen with ϵ-amino caproic acid, but the values were more scattered and the difference did not reach statistical significance ($P > 0.2$). Immunological and biological activity are closely correlated in this short fragment of the PTH molecule, which exhibits all the known actions of the hormone (Parsons, Rafferty, Gray, Reit, Zanelli, Keutmann, Tregear, Callahan & Potts, 1975), and this was reflected in the greater hypercalcaemic responses seen with both inhibitor-treated groups.

Measurement of blood levels by a radioimmunoassay having carboxyterminal as well as amino-terminal specificity showed increased circulating amounts of immunoreactivity 1 h after injection in birds which received bPTH 1-84 in the presence of aprotinin or ϵ-amino caproic acid. However in these experiments with the native hormone, lack of parallelism between the dilution curves and the standard curve indicated immunochemical heterogeneity in the plasma samples, and it was concluded that metabolic alteration (presumably cleavage) of the molecule had occurred (Segre, Niall, Habener & Potts, 1974).

Discussion

Subcutaneous injection is by far the commonest method of administering peptide hormones, most of which are poorly absorbed across epithelial cells. The unusual pharmacokinetic properties of peptides suggest that there are two major respects in which present techniques can be improved. Firstly, there are reasons for believing that a large proportion of the injected dose is often denatured at the injection site and that it may be possible to prevent such losses. Secondly, controlled release preparations may have a value in minimizing unwanted effects of a hormone which is just as important as their use to prolong therapeutically effective blood levels.

One reason for suspecting considerable local inactivation of a peptide at subcutaneous injection sites can be found by comparing subcutaneous and intravenous dose requirments in the light of the different rates of entry to the circulation associated with these two routes. Neither the therapeutically useful actions of hormones nor the responses measured in *in vivo* bioassays develop instantaneously; they typically increase for tens or hundreds of minutes after an effective blood level is reached. When this time of onset substantially exceeds the circulating half-life (as is true for most peptide hormones), a dose infused into a vein will produce a greater biological response than if it is injected as a single intravenous bolus. This proposition, which seems self-evident, has been confirmed in our own laboratory in the case of insulin-induced hypoglycaemia (Stevenson, Tsakok and Parsons, unpublished observations) and it makes little difference whether the response is scored as the measured change at a fixed time or as the area under a time-curve. The reason is presumably that drug

wastage resulting from concentration-dependent rates of metabolism and excretion at peak blood levels far above the maximal effective range is minimized by controlling the rate of entry to the circulation.

In the absence of local inactivation, these considerations would predict that smaller doses should be required to elicit a measured biological response, because absorption from a subcutaneous site involves relatively slow permeation through connective tissue and vascular membranes before entry to the capillary bloodstream (Schou, 1961). This is exactly the opposite of what is found on comparing typical subcutaneous and intravenous dose requirements for bioassay of a variety of peptide hormones (Table 3). Significant local subcutaneous inactivation of the peptides concerned seems by far the most likely explanation.

It seemed possible to evaluate the extent of subcutaneous inactivation by infusing a hormone intravenously (thus ensuring complete entry to the bloodstream) while reducing the rate of entry according to a predetermined programme to imitate the time-course of blood levels and responses normally associated with the subcutaneous route. Preliminary experiments of this type with insulin have confirmed that in the rat, not more than 50% of a subcutaneously injected dose normally enters the bloodstream in bioactive form (Stevenson, Tsakok & Parsons, 1978).

The present paper describes the addition of a range of protease inhibitors to injection solutions in an attempt to decrease the extent of local inactivation of the amino-terminal fragment of parathyroid hormone, injected subcutaneously to chicks. Two of those tested (ε-aminocaproic acid and aprotinin) were found to enhance significantly the hypercalcaemic response to subcutaneously injected hPTH 1-34. In confirmation of the postulated mechanism, aprotinin was shown to increase the circulating level of immunoreactive hPTH 1-34 significantly 1 h after injection and the level of bioactive calcitonin 30 and 60 min after injection.

None of the measures tested in this study gave very effective control of the rate of absorption of PTH. The fact that injection of the zinc-protamine complexes gave rise to hypercalcaemia the time course of which could not be distinguished from that due to a dose of uncomplexed PTH, is presumably explained by the observation that although the complexes were stable at pH 8 they began to dissociate at pH 7. This is in contrast with the stability of zinc-protamine insulin complexes, which dissociate only at pH 5 and below (Schlichtkrull, Pingell, Heding, Brange & Jorgensen, 1975).

It seems worth recalling why PTH is among those hormones for which there are the strongest theoretical reasons to expect a therapeutic benefit from controlled administration. Prolonged exposure to PTH blood levels in the upper part of the normal range increases calcium absorption from the intestine and retention by the kidney (Parsons & Reit, 1974) and low doses also appear to favour anabolic actions on the skeleton which may be useful in the treatment of osteoporosis (Reeve *et al.*, 1976). However, even transient exposure to a high blood level induces osteolytic effects which long outlast the elevated PTH blood level (for review, see Parsons, 1976). Thus PTH offers one of the best examples for the generalization that, because hormones have multiple dose-related actions, the patterns of response seen in normal physiology depend on precisely regulated circulating levels as much as on selectivity of receptors.

Practical application of these principles requires controlled release, since when systemic metabolic destruction of a hormone remains unchanged, the rate of its entry to the circulation determines the blood concentration. The fact that adding calcium to the subcutaneous injection solutions abolished hypercalcaemia unless protease inhibitors were added is probably due either to calcium activation of proteases (Hino & Nagatsu, 1975) or release of cellular proteases at the injection site (Ignarro & George, 1974). The finding increases the likelihood that subcutaneous inactivation of PTH may prove a significant practical problem. Other factors that release or activate lysosomal enzymes may also increase local hor-

Table 3 Typical dose requirements for bioassay of various peptide hormones given subcutaneously, expressed as multiples of the corresponding intravenous doses

Hormone	*i.v. dose*	*s.c. dose**
Insulin	1	2
Calcitonin	1	2
Parathyroid Hormone	1	3
Corticotrophin	1	10

* These figures are based on experience in our own laboratories, with the exception of that for corticotrophin, for which we thank Dr P. L. Storring, N.I.B.S.C.

mone destruction, and the chronic presence of any subcutaneous depot or implant might be expected to have just such an effect. Thus this paper provides several lines of evidence that development of an effective delayed-release dose form of PTH may depend on success in controlling its degradation.

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